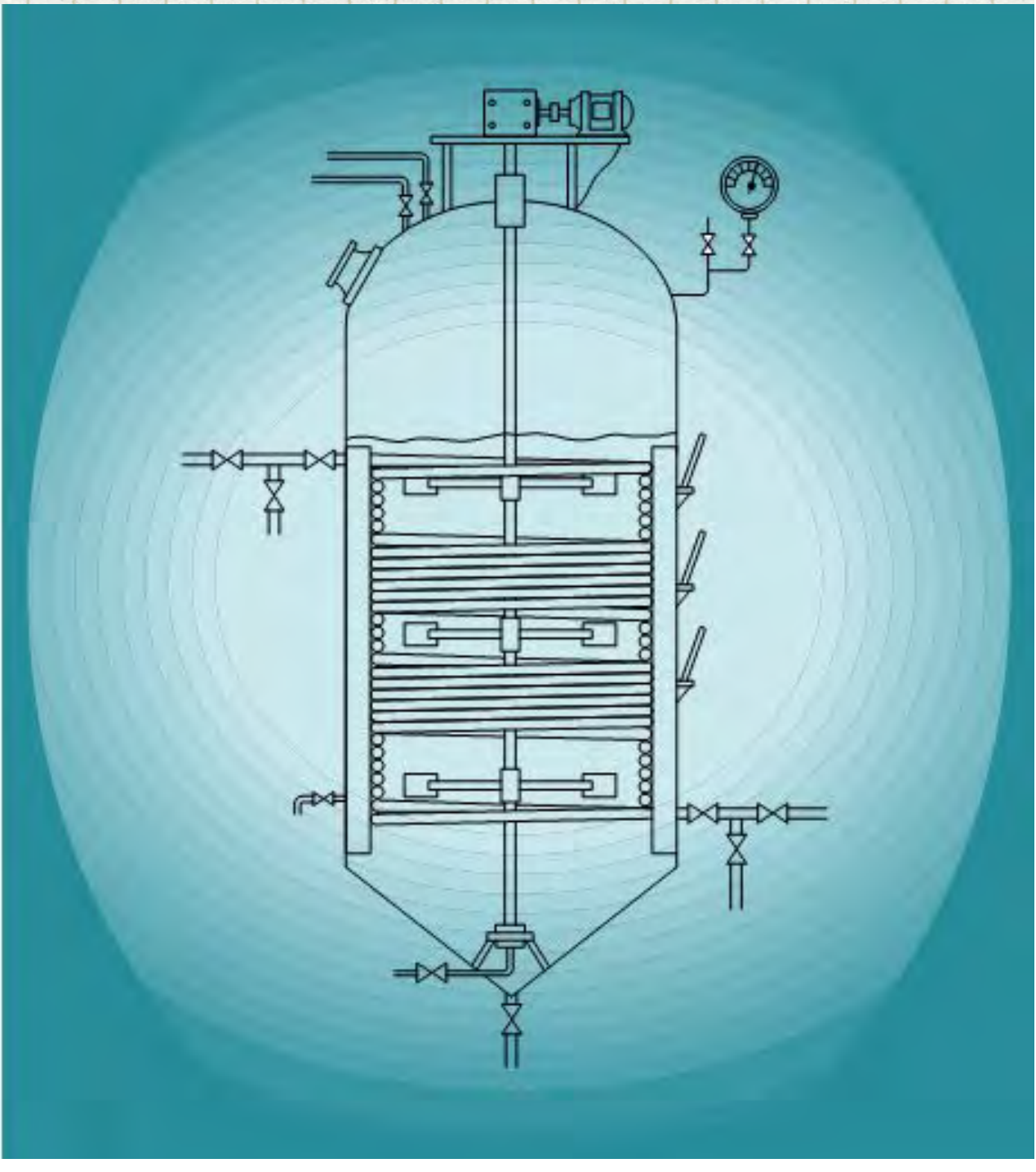
Essentials of

Industrial Microbiology

Basanta Rai



ESSENTIALS OF

INDUSTRIAL MICROBIOLOGY

Basanta K. Rai

PREFACE

The present book is basically a compilation of a series of lectures on INDUSTRIAL MICROBIOLOGY and MICROBIAL BIOCHEMISTRY I have delivered over the years to B. Tech (Food Technology) and B. Sc. (Microbiology) respectively at Central Campus of Technology, Dharan, Nepal.

The chapters included herein more than cover the current syllabus of Industrial Microbiology for B. Tech (III year). Within the scope and limitation of the syllabus, I have tried to put together information as meticulously as possible. Some descriptions have become outdated, genetic engineering in particular. However, the basic concept is still useful. The book contains a large number of cross-referenced diagrams, tables and index to assist the students/readers.

Thanks are due to those authors whose books I have freely consulted. As an acknowledgement, I have appended a short bibliography, which I hope will be helpful to the students in finding out additional reading materials.

I am very much thankful to NAAST College, Dharan-16, Nepal for providing the much-needed computer facility during the early stages of the work (1998!).

I am very much hopeful that the book will fulfill its intended purpose. Any criticism for the improvement (updating) of the work will be thankfully received.

Dharan, Mar 2012 Basanta Kumar Rai

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CHAPTER 1

THE SCOPE OF INDUSTRIAL MICROBIOLOGY

1.1 INTRODUCTION

Industrial microbiology is one of the most important areas of applied microbiology. Basically, it deals with screening, improvement, management, and exploitation of microorganisms for the production of various useful end products in large quantities (commercial scale).

From industrial microbiology standpoint, microorganisms can be considered *chemical factories in miniature* because they have immense capability to transform an array of raw materials into diverse end products. The overall reaction characterizing the industrial application of microorganisms can be summarized as:

$$\text{Substrate (raw materials)} \xrightarrow{\text{microorganisms}} \text{End products and/or Services}$$

Microorganisms are involved in the above reaction in ways more than one. The products and services these microorganisms are capable of generating can be limited only by imagination. Some of the more important categories of products/services presently available through the use of microorganisms are:

1. Microbial cell (biomass)
    - Live (bakers yeast, test-cultures for microbiological assay, etc.)
    - Dead or processed (yeast autolysates, single cell protein, etc.)
2. Enzymes (invertase, lipase, pectin esterase, rennin, etc.)
3. Metabolites
    - Primary metabolites (metabolites needed for the growth of the organisms themselves, e.g., ethanol, vitamins, amino acids, etc.)
    - Secondary metabolites (metabolites not essential for growth but are produced as a survival tool in response to environmental conditions, e.g., antibiotics, polysaccharides, etc.)
4. Transformed products
    - Semi-synthetic penicillins, etc.
5. Biofertilizers
    - Microbial inoculants such as *Rhizobium, Mycorrhiza*, etc.
6. Biopesticides
    - *Bacillus thuringiensis* against lepidoptereans
7. Waste degradation
    - Sewage treatment by fermentation/digestion

1.2 INDUSTRIALLY IMPORTANT GROUPS OF MICROORGANISMS

The following is a brief treatment of some of the important groups of industrially important microorganisms. Mushroom has not been dealt with for obvious reasons.

1.2.1 BACTERIA

They are unicellular prokaryotes that reproduce predominantly by binary fission. Typically, the size is around 1-2 μm. The basic shapes are described as spherical, rod-shaped, and helical (see Fig. 1.1a). Some bacteria of industrial importance and their associated uses are:

- *Streptomyces* sp.— for streptomycin production
- *Bacillus subtilis*— for fermented soy food, protease, antibiotics
- *Corynebacterium* sp.— for amino acid production
- *Lactobacillus* sp. — for fermented dairy products, fermented vegetables
- *Bacillus thuringiensis* — for biopesticides
- *Xanthomonas campestris* —for xanthan gum
- *Acetobacter* sp. — for vinegar production

1.2.2 MOLDS

Molds are multicellular eukaryotes. They lack chlorophyll. They have mycelial structure, which gives the impression of a fluffy/cottony colony (see Fig. 1.1c). Some of the industrially important molds and their uses are:

- *Penicillium chrysogenum* — for penicillins
- *Aspergillus oryzae* — for amylase, oriental alcoholic beverages, etc.
- *Mucor miehi* — for fungal rennet
- *Rhizopus oligosporus* —for tempeh
- *Aspergillus niger* — for citric acid and fumaric acid

1.2.3 YEASTS

They lack chlorophyll and are therefore either saprophytes or parasites. They are unicellular and non-motile. They are bigger than bacteria (5-20 μm) and generally reproduce by budding (see Fig. 1.1b). Some of them produce *pseudo*mycelia (e.g., *Candida* sp). Some of the industrially important yeasts are:

- *Saccharomyces* sp. — for alcoholic fermentation, bakers yeast
- *Candida utilis* — for feed yeast
- *Cryptococcus* — for feed yeast
- *Ashbya gossypyii* — for riboflavin (vitamin B_2) production

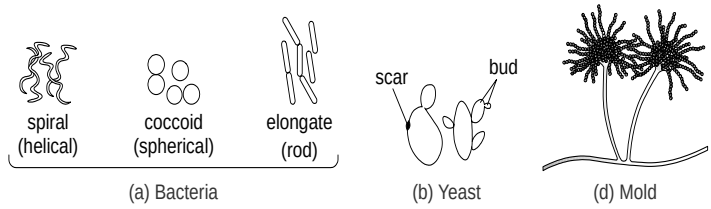


Fig. 1.1 Typical morphology of bacteria, yeast, and mold

1.3 DESIRABLE PROPERTIES OF INDUSTRIAL MICROORGANISMS

It is difficult to generalize but the most important desirable properties an industrially important microorganism must possess are:

(i) Rapid growth in relatively cheap and readily available substrate
(ii) Production of the desired metabolite in large quantities
(iii) Non-pathogenicity
(iv) Genetic stability
(v) Temperature tolerance
(vi) Short fermentation time
(vii) Ease of removal of cells from the fermentation broth
(viii) Amenable to genetic manipulation.

In practice, however, no single microorganism has all the above-listed properties. The choice is therefore a compromise.

CHAPTER 2

SCREENING OF MICROORGANISMS

2.1 INTRODUCTION

A set of highly selective procedures that allow the detection and isolation of microorganisms producing the desired metabolite (having desired properties) constitutes *Primary Screening*. Ideally, primary screening should be rapid, inexpensive, predictive, specific but effective for a broad range of compounds, and applicable on a large scale. Primary screening is time-consuming and labor-intensive since a large number of isolates have to be screened to identify a few potential ones.

However, this possibly is the most critical step since it eliminates the large bulk of unwanted and useless isolates, which are either non-producers or *producers of known compounds*. The need for the latter would become obvious in the light of the fact that till 1987 more than 3000 different metabolites were well characterized, and every year about 100 new ones are added to this list. Therefore, the rapid and accurate determination of new metabolites is necessary to *avoid a wasteful duplication of effort*. Some simplified examples of screening approaches are given in Table 2.1.

Table 2.1 Simplified examples of screening of microorganisms

Product	*Screen*
Amylase	Starch-Agar method
Protease	Precipitation Test
Antibiotics	*Primary Screening*: Crowded-Plate Technique *Secondary Screening*: Specialized Tests
Amino acids	Auxanography

Rapid and effective screening techniques have been devised for a variety of microbial products, which utilize either a property of the product or that of its biosynthetic pathway for the detection of desirable isolates. The initial screening is done (ordinarily) in plates using agar media. The microorganisms thus selected are subjected to *Secondary Screening*. This screening differs from the primary screening both with respect to objective and the level of sophistication. A vast amount of information regarding the organism as well as the metabolite is obtained here. Several trials are done to optimize the cultural condition for maximizing the product yield. Some of the tests done are:

1. For Microorganisms

- Classification and identification
- Cultural requirements
- Pathogenicity
- Genetic stability
- Scope for improvement, e.g., by mutation, genetic manipulation

2. For Product Yield

- Comparison with yield from known commercial strains

3. For Metabolite

- Identification of the compound
- Immediate or potential use
- Toxicity to animals
- Novelty (newness)

4. For Process

- Shake-flask culture (behavior)
- Pilot-scale culture (inoculum build-up)
- Large-scale culture (main fermentation)

Indeed, a host of techniques must be used to gather all of the above-mentioned information. The final isolates are quite often further tested *vis-à-vis* strain improvement. This final level of selection is termed *Rational Screening* or *Selective Screening*.

2.2 EXAMPLES OF SCREENING

2.2.1 SCREENING OF AMINO ACID PRODUCERS

The most widely used technique in this case is *auxanography*. Auxanogram is a plate culture in which diffusion gradients have been produced in the medium involving one or more substances that affect the growth of the microorganisms. The test primarily measures the ability of an isolate (the test organism) to interact with test compounds, e.g., ability of the microorganism to metabolize different sugars, the antibiotic activity of a test compound, etc. Auxanogram was originally developed by Biejerinck in 1889 to determine the ability of yeasts to utilize different sugar substrates. The method is now widely used to identify yeasts at the level of species.

STEPS

Preparation of first plate

1. A filter strip (1.5×12 cm) is put across the bottom of a Petri dish in such a way that the two ends pass over the edge of the dish (see Fig. 2.1).
2. A paper disc of the size of the Petri dish is placed over the paper strip on the bottom of the dish
3. Molten nutrient agar is poured on the paper disc in the dish and allowed to solidify
4. Microbial source material, such as soil, is subjected to dilution such that aliquots on plating will produce well-isolated colonies
5. Aliquots are plated out

Preparation of second plate

1. A minimal medium lacking the amino acid, say lysine, under consideration is seeded with a special mutant (test organism) that cannot itself synthesize lysine. Because of this inability, the test organism, ideally, cannot grow in this minimal medium.
2. The seeded medium is poured on to a fresh Petri dish.
3. The plate is allowed to set.

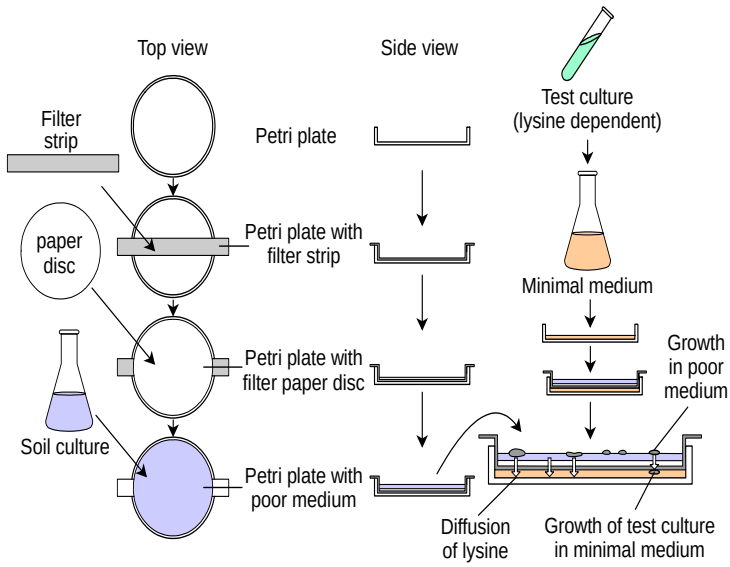


Fig. 2.1 Auxanographic screening of lysine producers

4. Now, the agar in the first plate (prepared is step 1) is carefully and aseptically lifted out with the help of a pair of tweezers and spatula and placed on the surface of the second plate. Without inverting, the plate is incubated at a suitable temperature.

The lysine produced by the colonies present in the upper layer can diffuse into the lower layer of agar containing the test organism. Any growth observed in the lower layer can now be regarded as growth stimulated by lysine diffused from the colony just above in the first agar. The corresponding colony in first plate can now be subcultured for further assay. Obviously, the entire work must be done aseptically.

2.2.2 SCREENING OF ANTIBIOTIC PRODUCERS FROM SOIL

A typical laboratory method for the isolation and testing of antibiotic producers from soil sample is given in Fig. 2.2. The method is normally called *Crowded Plate* method. The overall screening protocol is outlined in Fig. 2.3.

PROCEDURE

1. Take 10 g soil and prepare 10^{-1} dilution
2. Transfer about 2 loopfuls of sample suspension on the agar plate labeled '1'
3. Spread the suspension over the entire surface with a dally rod
4. Use the residue of the dally rod as an inoculum for the plate labeled '2'
5. Spread thoroughly as in step 3
6. Carry out the sequential transfers to 3 other properly labeled plates. Use the methods given in steps 1 to 5
7. Incubate (inverted) all the plates at 25°C for 5-7 days. Observe for the zones of inhibition in between
8. Subculture the selected colonies from the crowded plate

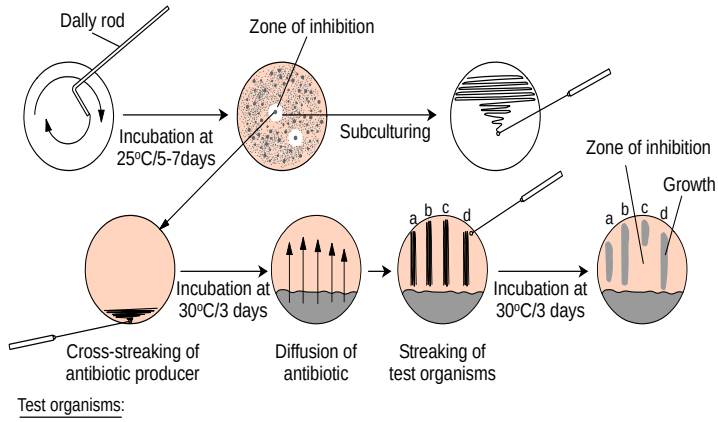


Fig. 2.2 Simplified protocol for the isolation of antibiotic producers from soil

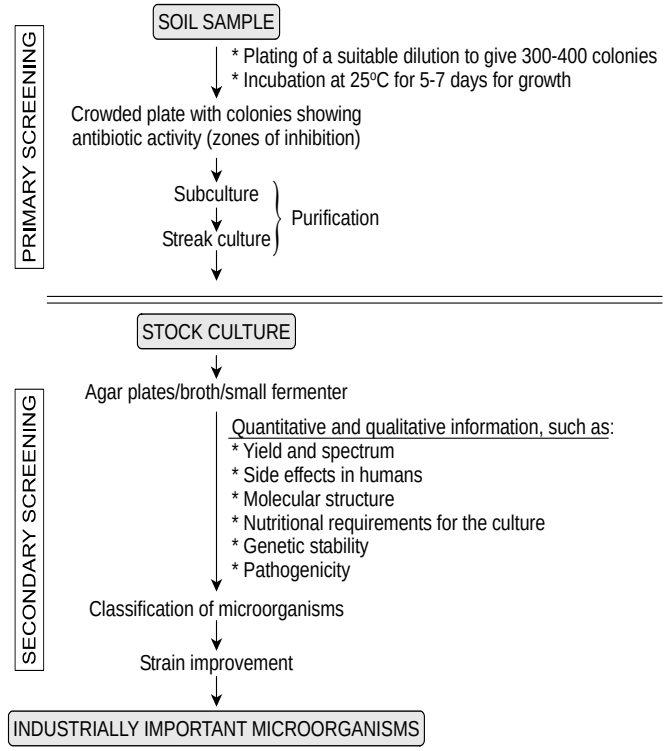


Fig. 2.3 Screening of antibiotic producer from soil

CHAPTER 3

GENERAL TECHNIQUES OF SELECTION OF MICROORGANISMS

3.1 INTRODUCTION

The microbial profile of our environment is as diverse as can be, both with respect to type and number. Several selection techniques are available at present for isolating microorganisms of our interest from any environment. Basically, such methods function by facilitating the growth of the desired species so that the subsequent isolation becomes easier. There are three main groups of selection methods: (i) Chemical, (ii) Physical, and (iii) Biological. The basic strategy in all of the above methods is to create environment conducive to physical segregation or even encouragement of the growth of the desired species while discouraging or even inhibiting the rest.

3.1.1 CHEMICAL METHODS

- Use of special nitrogen or carbon source (for example, cellulose medium for isolating cellulolytic organisms)
- Use of dilute media (this has limited use in industrial microbiology)
- Use of toxic or inhibitory substances (for example media amended with antibiotics, dyes, bile salts, etc.)

3.1.2 PHYSICAL METHODS

- *Heat treatment:* spore-formers can be selected by heating the sample to 80°C for 10 min and culturing.
- *Incubation temperature:* selection of psychrophiles, thermophiles, etc., is possible by this method.
- *pH of the medium:* this is especially useful for the isolation of yeasts/molds and archeans.
- *Cell size and motility*: microorganisms can be selected based on size by using filters of varying pore sizes.

3.1.3 BIOLOGICAL METHOD

Nature also exerts a selective force on microorganisms. Sometimes, animals can serve as a reservoir of a given species of microorganism. For example, when sputum organisms from a patient suffering from streptococcal pneumonia are injected into laboratory mice, all organisms but *Streptococcus pneumoniae* are killed by the defense mechanism of the mouse. The mouse thus becomes, in a sense, a biological reservoir of pneumococci!

3.2 PURE CULTURE TECHNIQUES

Selective methods can be employed to obtain a large proportion of the microorganisms we are interested at. It now becomes easier to carry out further isolation works. A variety of techniques can be employed for the isolation. The descendant of a single isolation in pure culture constitutes a *strain.* A strain is usually made up of succession of cultures and is often derived from a single colony. If a strain is derived from a single parental cell, it is termed a *clone.*

3.2.1 GENERAL METHODS OF ISOLATION

- Streak-plate technique (radiant, continuous, discontinuous, etc.)
- Pour-plate technique
- Spread-plate technique
- Hypheal tip technique
- Micromanipulator technique

3.2.1.1 Streak-plate technique

It is a very simple and rapid technique of isolation. In it, the sample broth is streaked onto a dry agar surface in a series of non-overlapping streaks (see Fig. 3.1). The process thins out the cells and at some point the cells are separated sufficiently apart to give rise to discrete colonies.

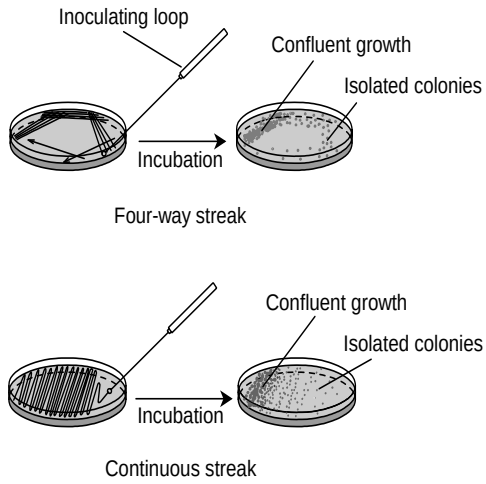


Fig. 3.1 Streak-plate isolation

3.2.1.2 Pour-plate technique

It entails mixing of sample broth in a melted agar medium and plating out a suitable dilution. The method has some limitations in that psychrophiles or organisms that cannot withstand a temporary shock of 45-50°C cannot survive. Besides, the isolated

colonies remain embedded in the medium and subculturing them entails digging through the agar. The single most important advantage in it is that it can be used for quantitative (enumeration) purpose also. A variation of this method is to transfer 0.1ml of the pre-diluted sample broth and pour 15-20 ml liquid agar medium over it. The plate is rotated gently in the shape of **'8'** to affect uniform mixing.

3.2.1.3 Spread-plate technique

It involves spreading onto the solid agar surface about 0.1 ml of the culture. Turntables can be used for the spreading. In manual method, a loopful of culture is transferred to the agar surface and spread uniformly with a bent glass rod (called *dally rod*). To affect finer isolation, the residue in the rod is used to spread-plate yet another fresh plate (see Fig. 3.2). In this way, 4-6 plates can be used for the spreading. As the spreading progresses, at some point the cells will be sufficiently apart to the affect isolation.

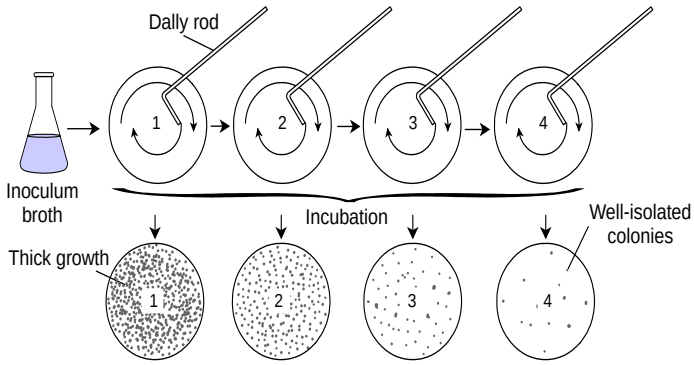


Fig. 3.2 Spread-plate isolation

3.2.1.4 Hypheal tip method

This method is useful particularly for the isolation of molds. A small segment of mycelia that radiate outward can be aseptically cut out and placed on a fresh medium for growth.

3.2.1.5 Micromanipulator technique

It is used only when clones are required. With this method, an unequivocal selection of a single cell is possible. The method uses an instrument called *micromanipulator* (a high resolution microscope fitted with manipulating ancillary) and needs considerable expertise. During manipulation, the cell is first identified. Isolation takes place on an agar surface. A sharp needle (provided with the manipulator) is brought close to the cell. The needle is allowed to rest on the agar surface near the cell so that a small dent is formed. Next, the field is shifted away from the needle tip. The dented impression made by the needle during the shifting causes the cell to follow the course of the needle and thus gets separated. The cell is later cut out from the agar surface and subcultured (see Fig. 3.3 for the schematic diagram).

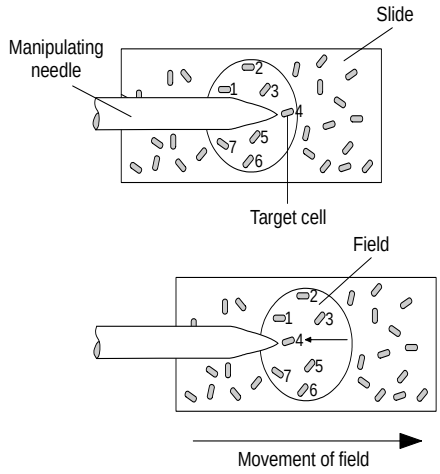


Fig. 3.3 Isolation by micromanipulator

CHAPTER 4

STRAIN IMPROVEMENT

4.1 INTRODUCTION

The initial source of an industrial microorganism is the natural environment, but the original (wild) isolate is greatly modified in the laboratory. As a result of this modification, progressive improvement in the yield can be anticipated. The most dramatic example is that of penicillin produced by *Penicillium chrysogenum*. Over the years, the yield has improved by 50,000 fold from the initial yield of a mere 1-10 μg/ml. The basic objective in improving strain is to make it reliable and efficient so that the microbial process becomes economical.

After an organism is screened, it becomes necessary to increase the product yield from fermentation to minimize production costs. Product yield can be improved by:

- Developing a suitable medium for fermentation
- Refining the fermentation process
- Improving productivity of the strain

Generally, major improvements arise from the last approach; all fermentation enterprises place considerable emphasis on this aspect. The techniques and approaches used to genetically modify strains to increase the production of the desired product, collectively, are called *strain improvement* or *strain development*. Strain improvement is based on following 3 approaches:

1. Mutant selection (induced mutation)
2. Recombination
3. Recombinant DNA Technology (Genetic Engineering).

It is important to note here that productivity is not alone the function of large yield. Productivity is also the function of microbial properties such as resistance to infection, temperature tolerance, resistance to analogs, genetic stability, appropriate flocculation characteristics, etc. Strain improvement programs therefore place due emphasis on these aspects also.

4.2 MUTATION APPROACH

Simply stating, mutation is a stable change in a gene such that the changed condition is stably inherited by offsprings. Mutation can occur either spontaneously or by deliberate induction. The former method cannot be relied on, as it is terribly inefficient. A more direct approach is to use mutagenic agents (*mutagens*). The frequency of mutation can be achieved at very high levels with this method.

4.2.1 THE BASIC PROCEDURE

In this method, the cells or spores are contacted with (exposed to) a given mutagen for a given time period. The exposure may take place on the surface of agar in a Petri dish, in a liquid culture, etc; the process usually kills about 99% of the exposed cells. Of the remaining that is isolated by screening (subculturing), only a small percentage will have actually mutated. Not all the mutants will have the property as desired, though. Those that bear the desired property are identified, selected, and tested further using other special procedures.

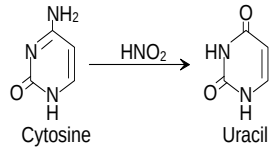
4.3 COMMON MUTAGENS

The most commonly used mutagens for strain development can be classified into two groups, *viz.*, *chemical*, and *radiation*

4.3.1 SOME COMMON EXAMPLES OF CHEMICAL MUTAGENS

- *Nitrous acid (HNO_2)*

Mutation in microorganisms occurs by the removal of amino group from nucleotide base. It is carried out by suspending the cells in an acidic buffer and adding sodium nitrite to it. For example, oxidative deamination of cytosine results in uracil (see Fig. 4.1). This change causes abnormal base pairing and consequent replication errors.



Cytosine Uracil

Fig. 4.1 Mutation by nitrous acid

- *Alkylating agents*

Alkylating agents give relatively high mutant yields. The most common alkylating agents are Ethyl Ethane Sulfonate (EES), Ethyl Methane Sulfonate (EMS), Diethyl Sulfonate (DES), N-Methyl-N'-Nitro-N-Nitrosoguanidine (NTG), etc. Mutation is carried out by suspending the cells in neutral buffer followed by addition of mutagen. The reaction may be stopped by using sodium thiosulfate or by dilution during plating. Alkylating agents add alkyl groups to the nitrogen in the 7th position of the purine. This alkylation creates labile N-glycosidic bond that hydrolyzes to leave depurinated site (see Fig. 4.2). If not repaired immediately, any base may join to fill up the gap. This event leads to unmatched bases. NTG is the most potent of chemical mutagens. It can produce very high mutant yields. It is also an alkylating agent and acts primarily at the replication fork.

Mutation using NTG can be achieved by adding ~ 0.5 g NTG/liter of broth culture and allowing the mixture to stand at 28°C for 30 min. The cells are then recovered

by centrifuging. The recovered cells are washed with suitable buffer and plate-cultured for the regrowth into colonies with diagnostic features.

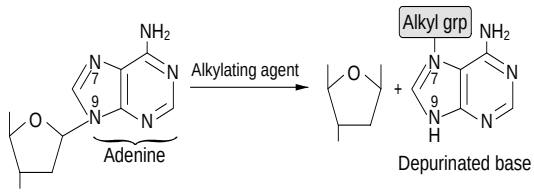


Fig. 4.2 Action of alkylating agent

- *Base analogs*

Compounds such as 5-bromouracil and 2-aminopurine are base analogs of thymine and adenine respectively. By simple analogy, according to the principle of A=T, G≡C, 5-bromouracil should base-pair with adenine. However, due to structural differences, 5-bromouracil pairs with guanine instead. Such a mispairing produces abnormal hydrogen bondings and subsequent replication error. See the structure of the analogs in Fig. 4.3.

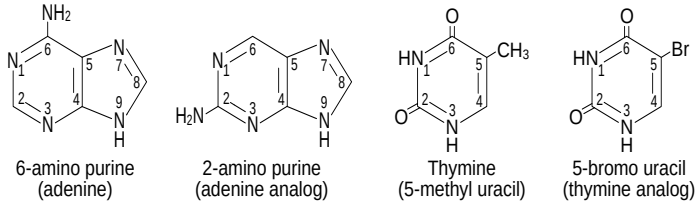


Fig. 4.3 Examples of base analogs

- *Intercalating agents*

These are flat molecules that can slip in (intercalate) between the base pairs in the central stack of DNA helix. This results in the distortion of DNA structure and consequent error in replication of DNA. Some of the common examples of such mutagens are nitrogen mustards, proflavine, acridine orange (Fig. 4.4), etc.

Nitrogen mustards are some poly-(β-chloroethyl) amines with the general formula $RN(CH_2\ CH_2Cl)_2$, where R is an alkyl, alkylamine, or alkyl chloride group.

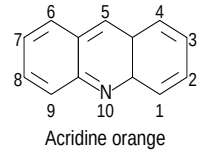


Fig. 4.4 Structure of acridine orange

4.3.2 RADIATION

Several systems of *mutagenesis* have been developed using X-rays, γ-rays, neutrons, UV-rays, etc. Radiation systems are relatively convenient. If handled properly, they may be used to produce high mutant yields. Radiations used for mutation can be conveniently divided into two groups, *ionizing radiations* and *UV-radiations* (non-ionizing).

- *Ionizing radiations*

These include X-rays, γ-rays, neutrons, etc., and are used only when chemical agents or UV-rays are ineffective. Ionization methods work by causing chromosomal breakage, for instance, *translocation* and *transversion.* X-rays are produced by bombarding solids with electrons. The wavelength of the rays is typically 100-150 nm. Gamma rays have wavelength less than 100 nm. The most common source of γ-radiation is cobalt-60.

- *UV-rays*

UV-rays entering around 260-280 nm are strongly absorbed by nucleic acids and the rays are therefore *genotoxic*. The rays work principally by forming dimers between two thymines (see Fig. 4.5).

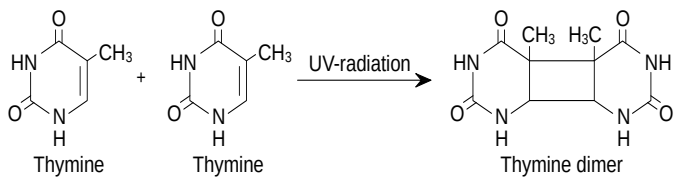


Fig. 4.5 Formation of thymine dimers

Dimer structures bring about replication errors. However, the user must be careful in handling UV-light, as this is mutagenic if exposed to skins and eyes. Care should be taken not to expose the treated cells immediately to the visible light. The exposure leads to *photo-reactivation*, a term referring to restoration to full activity (viability) of the treated cells upon exposure to visible light. The visible light reactivates a special enzyme, called *PRE*, which restores the DNA structure by splitting (unlinking) the dimer. The reactivation rate is about 80%. The organism can have other survival mechanisms also. One system uses a family of genes called *uvr genes*, using which a short fragment of single-stranded DNA (containing the dimer) is cut off and later replaced with a sound one. Similarly, dimers can also be repaired by recombination.

4.4 IMPROVEMENT IN THE YIELD

The ability of mutants to produce large amounts of end product is based on one or more of the following:

- Resistance to infection
- Improved foaming characteristics
- Change in cell wall permeability
- Development of analog resistance
- Mutation to auxotrophy
- Change to constitutivity from inducibility

Brief descriptions of some of the more important points are as follows:

4.4.1 CELL WALL PERMEABILITY

The end products of a microbial process should preferably be *extracellular* so that the recovery becomes easier. Due to *homeostatic* system, microorganisms cannot produce within the cells more metabolites than can be compatible (in quantity, that is). In such cases *leaky* cells have been produced and successfully used. Such cells do not experience any concentration effect within the cell because the product is continuously secreted out in the medium. This leads to overproduction of the desired metabolite.

4.4.2 ANALOG RESISTANCE

Organisms, from simple to developed, balance their metabolic reactions by a mechanism called *feed-back inhibition*. If a specified metabolite crosses a critical limit, the same metabolite will exert an influence on enzyme(s) several steps ahead of the metabolic sequence that led to its formation. One consequence of this is, under normal condition, the product we desire cannot be produced in large amounts. Thus, feed-back inhibition works as an off-on switch of metabolic regulation. Organisms also have another regulatory mechanism known as *repression*. Repression works at the level of transcription and is therefore much slower in action. Organisms use repression mechanism for the fine-tuning of metabolic regulation. One of the various alternatives for overcoming this problem is to *desensitize* the enzyme(s) involved in feedback inhibition. For this, the cells are treated with the analogs of metabolite responsible for repression (for example, deoxyglucose is an analog of glucose). The analogs are generally toxic to the organisms and most of them cannot survive this treatment. The few cells that survive the analog treatment are called *analog resistant* strains. These cells not only become immune to analogs but also develop insensitive feedback enzyme systems. This faulty metabolic regulation leads to uncontrolled production (overproduction) of the desired metabolite (see Fig. 4.6 also).

4.4.3 AUXOTROPHIC MUTANTS

Auxotrophic mutants are strains of microorganisms, derived by mutation, that require one or more *key factors* for growth (not needed by the parent organism). The requirement arises because the organism's ability to synthesize the key metabolite has been lost due to *genetic alteration*. The organism cannot grow unless these metabolites are supplied externally. This loss of ability is termed *auxotrophy*. If the organism is dependent on lysine, for example, it becomes a *lysine auxotroph*. The

position in the pathway where the synthesis of such a compound has been blocked is called *metabolic block* (Fig. 4.6).

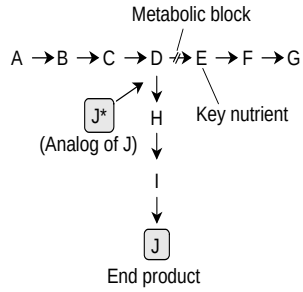


Fig. 4.6 Mechanism of auxotrophy

Since E is the key nutrient, the organism cannot survive without it (and so called *E-dependent* or *E-auxotroph*). If E can be supplied (externally) in regulated amounts the cell can be made to run the pathway as if it were normal. However, since the pathway is blocked at E, the central pathway diverts towards J via H. If J is the product of our interest, overproduction of J through metabolic block between D and E is obvious. However, J cannot accumulate in concentrations more than that is compatible. Slight excess of J causes an inhibition on enzyme between D and H. To overcome this, the organism is again mutated for analog resistance so that the enzyme fails to recognize J* (analog) and therefore J (main product). This event leads to another level of overproduction of J.

Auxotrophic mutants are of immense value in industrial microbiology, particularly in the production of primary metabolites such as amino acids. The cultured organism can be constantly supplied with regulated amounts of the compound just following the metabolic block. The organism carries out normal metabolic reactions but since there is a metabolic block ahead, the compounds just preceding the block will tend to accumulate.

4.4.3.1 Example of strain improvement by analog resistance

The selection of mutants resistant to glucose analog *2-deoxyglucose* (DOG, see Fig. 4.7) has proved extremely valuable when applied to brewing yeasts. In normal fermentations, due to catabolite repression, the yeast takes up maltose only when about half of the glucose present in the *wort* has been metabolized. Mutants resistant to the action of DOG are *derepressed*: they have the ability to utilize maltose and glucose simultaneously, and have improved fermentation characteristics.

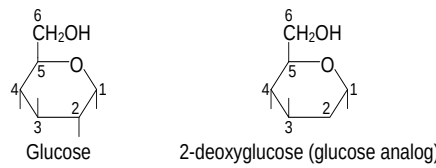


Fig. 4.7 Glucose and glucose analog

4.5 STRAIN IMPROVEMENT BY RECOMBINATION

Recombination can be defined as formation of *new gene combination* among those present in different strains. It is used to combine desirable *alleles* (one of two or more alternative forms of genes) present in two or more strains into one to increase product yield or to generate new products. Recombination may be based on:

- Sexual recombination
- Parasexual cycle
- Protoplast fusion

4.5.1 SEXUAL RECOMBINATION

Conjugation, mediated by *sex factor*, occurs in many bacteria and actinomycetes, including *Streptomyces*. Conjugation leads to formation of, usually, a partial *diploid* in which crossing-over produces recombinant genotypes. Recombinants are recovered and used for genetic studies like *linkage mapping*. Similarly, yeasts have two mating types: the cells of the opposite mating types fuse to form diploid *heterozygous* cells (which are non-mating types). The diploid cells undergo meiosis to produce four haploid spores, which give rise to vegetative cells.

4.5.2 PARASEXUAL CYCLE

Most industrially important fungi are asexual. However, their *haploid* hyphae sometimes fuse to produce *heterokaryons* (cells having two distinct nuclei). Later on the two nuclei of heterokaryons fuse and produce diploid nuclei. Occasionally, mitotic recombination coupled with meiotic reduction yields haploid nuclei from the diploid ones, giving rise to recombinants. In some cases, attempts have been made to use parasexuality for strain improvement, e.g., in *Penicillium chrysogenum*.

4.5.3 PROTOPLAST FUSION

Protoplasts of bacteria, actinomycetes and fungi are isolated by treatment with a variety of enzymes responsible for degrading cell walls, e.g., cellulase, pectinase, etc. An *osmoticum*, usually sorbitol, is necessary for protoplast stability, and fusion is induced by polyethylene glycol (PEG: $HO(CH_2CH_2O)_nH$) treatment. Protoplast fusion has been used to produce *Cephalosporium acremonium* (mold) strains with ability to give significantly higher yields of Cephalosporin C.

Protoplasts can also be prepared by treatment of bacteria (in logarithmic growth phase) with penicillin and then continuing culturing for several generations. The cells are harvested, washed, and suspended in a suitable hypertonic solution until used.

Since protoplasts formed are destroyed under hypotonic conditions, the extent of formation of protoplast is determined indirectly from the number of normal cells surviving under hypotonic conditions.

4.6.3.1 Basic process of PEG-induced protoplast fusion

PEG induces reproducible and high-frequency recombination. It also has low toxicity to most cell types. A protoplast mixture is treated with 28-50% PEG (mol wt. 1500-6000) for 15-30 min followed by gradual washing of the protoplast to remove PEG; protoplast fusion occurs during the washing process. The washing medium may be alkaline (pH 9-10) and may contain a high Ca^{2+} concentration (50 mMol/L); this approach is a combination of PEG and high pH with Ca^{2+} treatment, and is usually more effective than either treatment alone. PEG is negatively charged and binds to cations (e.g., Ca^{2+}) which in turn bind to the negatively charged molecules of plasma membrane (plasmalemma). The PEG can also bind to the cationic molecules of plasma membrane. During the washing process, PEG molecules pull out the plasmalemma components bound to them. This disturbs plasmalemma organization thereby leading to fusion of protoplasts located close to each other (Fig. 4.8).

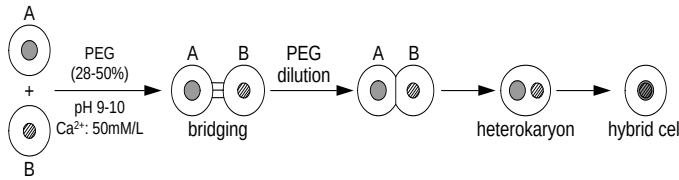


Fig. 4.8 Outline of PEG-induced protoplast fusion

Regeneration of the transformed cells is carried out by spread-plating the treated cells on a suitable hypertonic agar medium (containing sodium succinate and polyvinyl pyrrolidone) and incubating at 30-35°C. The transformants are obtained by selecting cells with some given phenotypic characteristics.

CHAPTER 5

GENETIC ENGINEERING

5.1 INTRODUCTION

An array of sophisticated techniques used to isolate, manipulate, move, propagate and express DNA is called *genetic engineering*. It is also called *recombinant DNA technology* (rDNA technology). The science has two main applications, *viz*., in (i) basic research, and (ii) applied research. Genetic engineering for commercial application is sometimes called *biotechnology*. In a true sense, genetic engineering is one of the many disciplines of biotechnology. The tendency to use the two terms interchangeably is simply an indication of how overwhelmingly biotechnology is dependent on genetic engineering.

5.2 BASIS OF GENE MANIPULATION

The basis of gene manipulation is the *gene cloning*. Since the cloning is carried out at a *molecular level* it is also called *molecular cloning*. The term *cloning* has several contextual meanings. In a biological sense, a clone refers to cells with an identical *genotype*. The cell multiplication is therefore asexual. In microbiology, the descendants of a single cell are called clones. As used in genetic engineering, a clone refers to *identical host cells that carry identical recombinant DNA molecule*. The objective of gene cloning is to isolate and produce specific genes in pure form and large quantities.

5.3 TOOLS NEEDED FOR GENE CLONING

Several tools are required for gene cloning. The tools used in genetic engineering differ from those used in classical engineering in that the former is molecular in nature. Some of the indispensable tools are given in Table 5.1.

5.4 TECHNIQUE OF GENE CLONING

The basic steps are:

- Obtaining the DNA of our interest (*foreign* DNA)
- Insertion of foreign DNA into a suitable cloning vector to form a *recombinant/ hybrid/ chimeric* DNA.
- Introduction of the recombinant vector into the *host* cell (organism).
- Selection of host cells that have been transformed (i.e., selection of clones).
- Amplification of the clones.

Table 5.1 Tools of molecular cloning

	Category	Tools
1	Enzymes	Restriction enzymes DNA polymerase Reverse transcriptase DNA ligase
2	Marker genes/Reporter genes	Lac *z* gene, β-galactosidase gene, etc.
3	Cloning vectors	Modified λ phage, plasmids, phasmids (phagemids), cosmids
4	Advanced vectors	Shuttle vectors, expression vectors
5	Linkers and adaptors	Linkers and adaptors
6	Substrate	Foreign DNA/Passenger DNA/DNA insert

5.4.1 OBTAINING DNA OF INTEREST

The DNA to be inserted into the host cell can be obtained by 3 methods, *viz*., (i) *from the genome*, (ii) *by reverse transcription*, and (iii) *by chemical* or *enzymatic synthesis*. A brief description of the different methods is given in the following paragraphs.

5.4.1.1 Genomic DNA

Isolation of a specific gene is a daunting job. Even in a simple organism like *E. coli*, a given gene accounts for <0.05% of the genome. Sorting out the specific gene is therefore a very complex job. When the genomic DNA is absolutely necessary, one can obtain them from *genomic libraries*. A genomic library is a collection of *plasmid* clones or *phage lysates* containing recombinant DNA molecules so that the sum total *DNA inserts* (noun) in this collection, ideally, represents the entire *genome* of the concerned organism.

The genomic library is constructed by first extracting the total DNA and breaking it into appropriate sizes by mechanical means, sonication, or enzymes. The fragments are separated according to sizes in *agarose gel*. The fragments are collected and inserted in *cloning vectors* (discussed later). This approach is called *shotgun approach* because the whole genome of a cell is cloned in the form of random and unidentified clones. See Fig. 5.1 for the outline of gene library preparation.

5.4.1.2 DNA by reverse transcription

Prokaryotic DNAs are *naked* (they are free from interfering structures, e.g., histones). They also do not contain *introns* (non-coding DNA). The *primary transcript* of *m*RNA in prokaryotes therefore does not undergo processing before being expressed. Human (eukaryotic) genomes present several difficulties in constructing a gene bank. The chromosomes exist as a complex of protein (nucleoprotein), the separation of which is difficult. Besides, they contain introns. Introns are eukaryotic DNA sequences widely dispersed throughout the genome. Introns are transcribed but

never expressed. Using genomic DNA from eukaryotes therefore presents problems in knowing whether the selected fragment of DNA is an intron or an *exon* (coding DNA). After transcription, *m*RNA in eukaryotes undergoes processing so that it becomes free from intron sequences before being expressed. Such pure *m*RNAs can be obtained by running the total RNA extract through *poly-U sepharose* or *poly-T cellulose* columns. Since *m*RNAs have *poly-A* tails, they form hydrogen bond with poly-U sepharose or poly-T cellulose of the support while the rest pass down. The bound *m*RNAs are later eluted from the column.

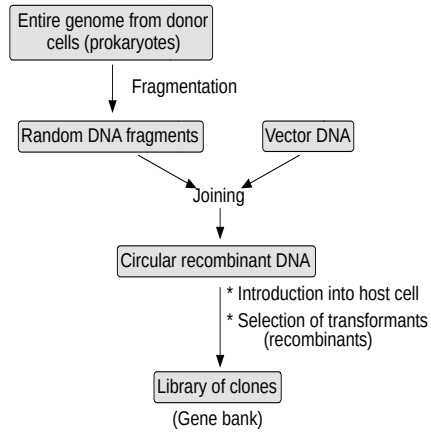


Fig. 5.1 Preparation of gene bank

This processed *m*RNA can now be used to produce exon DNA. Since the DNA constructed thus is complementary to *m*RNA, the former is called *complementary*- or *copy* DNA (cDNA) and the collection, by analogy with the gene bank, *cDNA library* or *cDNA bank*.

The synthesis of DNA using *m*RNA as the *template* is called *reverse transcription*. The enzyme catalyzing this reaction is called *reverse transcriptase*. This enzyme is obtained from the RNA virus called AMV (Avian Mycoblastosis Virus). This enzyme performs reactions similar to that of DNA polymerase and has an absolute requirement for a *primer* with a free 3'-OH. See Fig. 5.2 for the outline of the preparation of cDNA.

After the cDNA has been prepared in large amounts, rest of the protocol is similar to that for constructing gene bank.

5.4.1.3 DNA by chemical / enzymatic synthesis

The method invokes a *mental process* called *reverse translation* which does not occur *in vivo*. Before proceeding, however, the investigator must have a clear objective as to which protein he is attempting to synthesize. Stated differently, the primary structure of the protein to be prepared should be worked out beforehand. The corresponding *codon* in *m*RNA is then found out from the *universal codon*. Finally, artificial synthesis of DNA (complementary to *m*RNA) is carried out. The method is not foolproof, as

the problem due to *degeneracy* in codons cannot be overlooked. As of now, there are instruments available (called oligonucleotide synthesizer or *gene machine*) that can polymerize up to 50 nucleotides in a few days. Since the complete gene is usually several folds longer, several such pieces are later joined in order to construct the complete sequence.

Whatever the source of DNA, a large number of copies of DNA *inserts* is needed. This can be achieved by using *Polymerase Chain Reaction* (PCR).

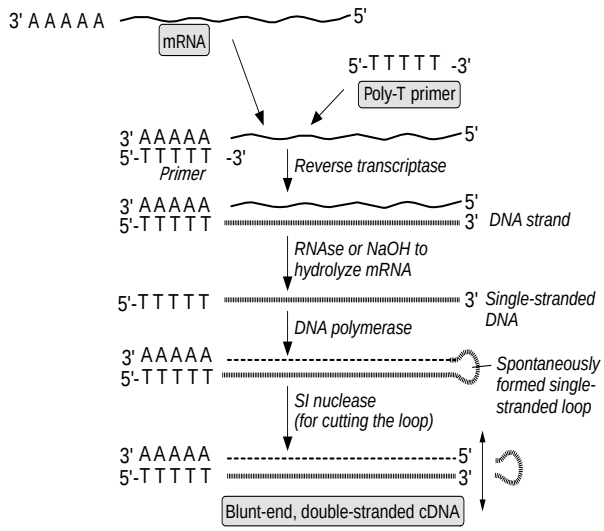


Fig. 5.2 Preparation of cDNA

5.4.2 THE POLYMERASE CHAIN REACTION

The polymerase chain reaction (PCR) was developed by Kary Mullis in 1985. It is an extremely powerful technique for gene amplification. The developer received Nobel Prize in 1993. Fully automated machines are now available that can produce billions of copies within an hour. The device is commonly called *thermocycler* (Fig. 5.3b). Nowadays, several versions of PCR are available, e.g., anchored PCR, inverse PCR, etc., each of which is designed for a specific purpose. The PCR is carried out *in vitro*. The basic requirements for running a PCR are:

- DNA to be amplified
- Two nucleotide *primers* (about 20 nucleotides long). These primers are complementary to the 3' ends of the DNA to be amplified.
- Four nucleotides (deoxy), *viz*., dTTP, dATP, dGTP, and dCTP.
- Heat-stable DNA polymerase

The heat-stable DNA polymerases used in PCR are from *hyperthermophilic archeans*. Some of the important DNA polymerases and their source are given in Table 5.2.

Table 5.2 Some important DNA polymerases and their source

Enzyme	Source organism
Taq	*Thermus aquaticus*
Vent	*Thermococcus litoralis*
Pfu	*Pyrococcus furiosus*
Dynazyme	*Thermus brockianus*

Dynazyme® has been claimed to be the most efficient of the above enzymes. It has a *half-life* of >3 hrs at 96°C. It also has *proofreading* activity, strand-displacement activity, and *deep vent* component. Deep vent component is responsible for separation the G:C-rich regions that tend to form very strong loop within the single strands.

5.4.2.1 The basic procedure of PCR

At the start of the PCR, all the ingredients are added in a reaction mixture. Primers and the deoxynucleoside triphosphates should be in large excess. The step-by-step illustration appears in Fig. 5.3a.

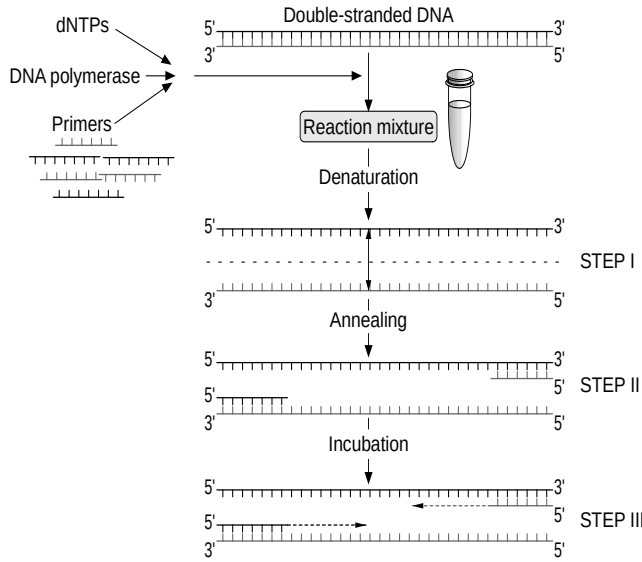


Fig. 5.3a The basic steps of PCR

STEP-I:

The reaction mixture is heated to 96-98ºC to denature DNA. The DNA double helix separates as a result of heating. The step is also called *denaturation* and is analogous to the term *melting* used to indicate DNA unwinding.

STEP-II

The mixture is cooled to 40-60ºC (the step is called *annealing*). Annealing permits the primers to base-pair with complementary 3' ends of each of the separated DNA strands.

STEP-III

Incubation is done at 70-75ºC so that the DNA strands are replicated as usual by the enzyme by elongating the primers

The incubation marks the completion of the cycle. The next cycle starts from step-I where the two DNA duplexes are denatured. The second cycle results in 4 DNA duplexes from two duplexes. Since the enzyme is thermostable it need not be added in each cycle. Thus, at the end of *n*th cycles 2^n copies of DNA duplexes can be expected. In programmed automatic thermocyclers, 20-30 cycles are usually adequate. This produces 10^6-10^9 fold increase in the number of duplexes. The machine takes less than 5 min for a cycle and 1.5-2.5 hrs is all that is needed to go through 20-30 cycles.

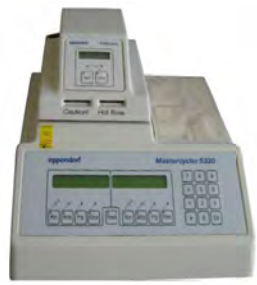


Fig. 5.3b Thermocycler (PCR machine)

In gene cloning, only the desired genes are taken out from a large, complex genome. This removal is achieved with the help of *molecular scissors* called *restriction enzymes*. These enzymes cut the DNA at some specific base sequence termed *recognition site* or *restriction site*. The tailored gene can now be joined to a cloning vector (described next) with the help of an enzyme *DNA ligase*.

5.4.3 JOINING THE DNA INTO CLONING VECTORS

There are several types of restriction enzymes. The microorganisms use these enzymes as natural defense mechanism. They cut the foreign DNA should these enter the *host* cell. Each of these enzymes has specific *base recognition site* where it can cut the DNA. The DNA can be cut in many different ways. Some restriction enzymes cut the DNA from the end. They are commonly called *exonucleases*. Some of them cut the internal section of the DNA and are therefore called *endonucleases*. Further, some cut the DNA straight to produce *blunt* DNA. The most widely used restriction enzymes in genetic engineering are endonucleases that produce a *staggered* cut in the DNA duplex. The cuts are complementary and can again be brought

together through base-pairing. The strands having such ends are *sticky* or *cohesive*. The straight cuts are not cohesive and, by analogy, called *blunt ends* or *flush ends*. Some of the examples of restriction endonucleases appear in Table 5.3.

Table 5.3 Examples of some restriction endonucleases

Enzyme	Source organism	Restriction (cleavage) site and pattern 5' → 3'	
Bam HI	*Bacillus amyliquefaciens H*	cohesive end	-G|G A T C C- -C C T A G|G-
Eco RI	*E. coli RI*	cohesive end	-G|A A T T C- -C T T A A|G-
Hin dIII	*Hemophilus influenza Rd*	cohesive end	-A|A G C T T- -T T C G A|A-
Pst I	*Providencia stuartii 164*	cohesive end	-C T G C A|G- -G|A G C T C-
Sal	*Streptomyces albus G*	cohesive end	-G|T C G A C- -C A G C T|G-
Alu	*Arthrobacter luteus*	blunt end	-A G|C T- -T C|G A-

Note: The staggered cleavage sites have *palindrome* sequence.

5.4.3.1 Linkers

DNA molecules which have staggered cuts can easily join with another complementary base pair but the straight/blunt ends cannot. In such cases linkers may be artificially joined to the blunt ends so that they may become *cohesive*.

Linkers are short, chemically synthesized, self-complementary, double-stranded oligonucleotides which contain within them one or more restriction sites. These linkers can be fused to the blunt-ended DNA, thereby supplying the latter with a site for staggered cut. That is, the ends can now be cut with restriction endonuclease. The joining is catalyzed by DNA ligase. DNA ligase comes from different sources but T_4 ligase is the preferred one. T_4 DNA ligase uses ATP as the cofactor, which serves to adenylate the amino group of a lysine residue in the enzyme. 5' phosphoryl terminus of DNA is also adenylated in the same manner. The DNAs are then joined liberating AMP in the process. See Fig. 5.4.

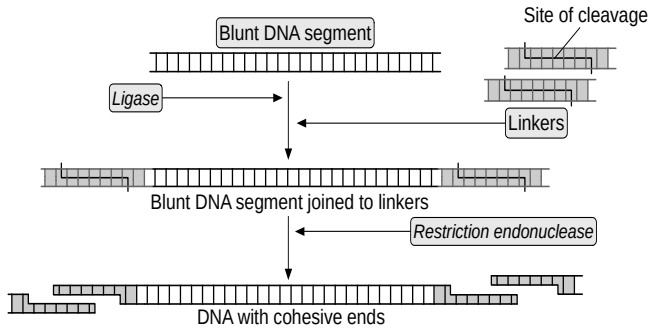


Fig. 5.4 Joining of linkers to the blunt-ended DNA

5.4.3.2 Cloning vectors

A cloning vector is a genetic element into which a foreign gene can be inserted, recombined, and replicated. They are also called *vehicles*. Cloning vectors are generally designed to allow recombination of a foreign DNA at a special site known as *restriction site*. Since the foreign DNA is joined to the self-replicating vector, the replication of the latter automatically ensures the replication of the foreign DNA inserted into it. Cloning vectors also contain *selectable* and *scorable* marker genes and an *attachment site*. In complex vectors like *shuttle vectors* the *ori sites* come from two different organisms. This makes the hybrid (recombinant) gene compatible to both types of organisms. When expression is desired, it also contains *promoters* (for detailed understanding, go through microbial genetics, Chapter 8). The restriction site should not fall within the *ori* site, as this will destroy the replication ability.

Some of the important vectors (all of them genetically engineered) are plasmids (e.g., pBR322, pUC, etc.), λ vectors, cosmids, phagemids, YAC (Yeast Artificial Chromosome), etc. See Fig. 5.5 for the example of plasmid vector. Refer to microbial genetics (page 48) for discussion on plasmids.

Desirable properties of cloning vectors

The fundamental requirements of a cloning vector can be summarized as:

- It should replicate autonomously: must be under *relaxed* control
- It should be easy to isolate and prepare
- It should be easy to deal with in transformation reactions
- It should have suitable markers for easy detection
- In gene transfer, it should integrate either itself or the foreign DNA into chromosome
- It should have as many target sites as possible, with only one site for one enzyme
- The expression vector should have suitable control elements, e.g., promoters, operators, binding sites for ribosomes, etc

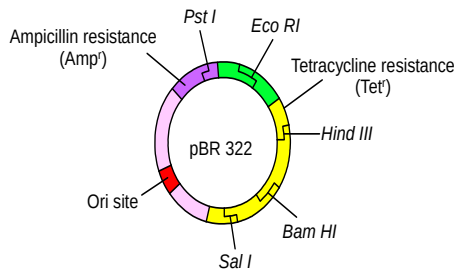


Fig. 5.5 An example of plasmid vector

Charon and λ phages

Wild λ phage has one *cos* site and 5 *Eco* RI sites. A sizeable portion of DNA of this virus is unessential. The phage genome has *att* and *ori* sites. Modified λ phages have been prepared by snipping out unessential regions of DNA. Charon 16 is one such modified vector which has one *Eco* RI site. Since the foreign DNA has to be inserted into *Eco* RI site of the phage vector, the vector can be called an *insertion* vector. Charon 4A is another modified λ phage vector that has 3 *Eco* RI sites. During enzymatic digestion some portion of the vector therefore gets removed for accommodating the foreign DNA. Charon 4A is therefore a *replacement* vector. While including the restriction sites in vectors, some *markers* are used to ascertain whether chimerization has occurred or not. The restriction sites generally fall within such markers. Markers that are responsible for resistance to antibiotics are called *selectable* markers. If the resistance to antibiotics due to the vector gene is lost it can be concluded that chimerization has occurred because the insertion of foreign gene into the resistance gene inactivates the latter. On the other hand, if resistance persists, chimerization has not taken place. In other words, the resistance gene is *still intact* and no insertional inactivation has taken place.

Another type of marker, called *scorable* marker, is also used in cloning vectors. One such marker gene is *lac* Z gene. It is responsible for β-galactosidase activity (see later).

λ phage contains double stranded DNA. The DNA is linear inside the particle but becomes circular as it enters the host. The recircularization is due to the presence of *cohesive* ends (termed *cos* site) of about 12 nucleotides at each end. The *cos* site may also lead to linear linking between the λ DNAs to form *concatemers*. The *cos* sequence is also involved in the packaging of viral DNA into the protein head. The packaging is promoted by viral proteins Nu 1 and A. The phage has the uniqueness of being able to package DNA of sizes between 38-52 kilobase pairs (kbp). Since about 40% of the phage DNA can be snipped out (because it is unessential) an equivalent size (about 25 kbp) of foreign DNA can be inserted or replaced into the phage DNA. Plasmids can incorporate only about 15 kbp, which means λ phages can allow packaging of much bigger sizes of foreign DNA.

Cosmid vectors

These are essentially plasmids that contain a minimum of 250 bp of λ DNA. The vector has an *ori* site, restriction site, *cos* site and selectable markers. Cosmids can take up to 45 kbp long DNA inserts. The packaged cosmids infect the host cells like λ particles, but once inside the host they replicate and propagate like plasmids.

5.4.4 JOINING PASSENGER DNA (FOREIGN DNA)

Usually, the double stranded foreign DNA as well as the chosen vector is cut with the same restriction enzyme. Both should have the restriction site for the enzyme used. Thus, similar cuts are obtained in both of them. The two can therefore form complementary strands for automatic pairing. The sealing is achieved with DNA ligase either from *E. coli* or T_4. T_4 DNA ligase is more active and it uses ATP.

During mixing, a large number of foreign DNA is used to increase the probability of base-pairing to make chimeric DNA. All three types of molecules may react to form any one or more of the following products: *circularized foreign DNA*, *circularized vector*, and *circularized recombinant*. Circularized foreign DNA does not pose problem as it lacks origin of replication. Recircularization of vector is undesirable in that chimerization efficiency is decreased. To prevent recircularization of the vector, its DNA is treated with *alkaline phosphatase*. The enzyme digests 5' phosphoryl group of the vector DNA thereby preventing reclosure. The digestion leaves a *nick* even after reclosure or chimerization. The nick can later be repaired by the host's system.

When foreign DNA with blunt ends is to be used, it is first joined at both the ends with appropriate linkers with the help of T_4 DNA ligase. *Adaptors* can also be used to make chimeric DNAs. Adaptors differ from linkers in that they have restriction sites for two (or more) different enzymes. With the use of two different enzymes, two different sticky ends in the same DNA can be produced. This comes handy when the restriction site of foreign DNA is different from that of the cloning vector. The adaptor serves here as a joining factor. See Fig. 5.5, 5.6 and 5.7.

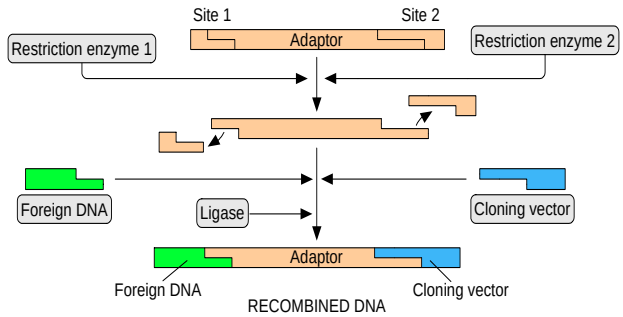


Fig. 5.6 Linking with adaptor molecules

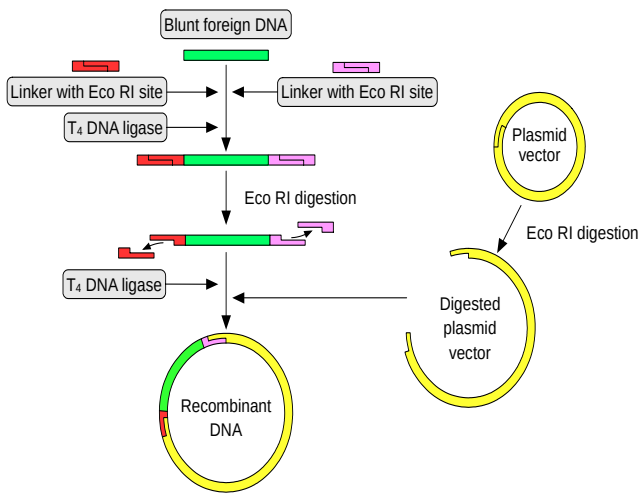


Fig. 5.7 *In vitro* production of recombinant DNA

5.4.5 INTRODUCTION OF RECOMBINANT VECTOR INTO HOST

A good host must have following properties:

- Is easy to transform
- Supports replication of rDNA.
- Lacks restriction enzymes, e.g., strains of *E. coli* K12.
- Is deficient in normal recombination function so that the DNA insert is not altered by natural recombination events.

The most widely used hosts are the special strains of *E. coli* and *Bacillus subtilis*. *Bacillus subtilis* has certain demerits: it cannot store the DNA insert in a stable state for a long time. The organism is nevertheless used as a good host for study related to expression of extracellular cell products such as enzymes.

5.4.5.1 Plasmid introduction

E. coli cells are generally poorly accessible to DNA molecules, but treatment with $CaCl_2$ makes them permeable to DNA. The process involved is poorly understood. Growing *E. coli* cells are suspended in 50 mM $CaCl_2$ at a concentration of 10^8-10^{10} cells/ml. The cells may be incubated for 12-24 hrs for increasing the frequency of transformation. The recombinant vector is then added; efficient transformation requires only a few minutes. The frequency of transformed cells is 10^6-10^7 per μg of plasmid DNA (1 transformation out of 10,000 plasmids). Molecular weight of chimeric DNA in the range 3×10^5 to 8×10^6 can result in successful transformation.

Another method being used is *electroporation* technique. In this technique, electric pulse is used to facilitate entry of foreign DNA into the host cell.

5.4.5.2 Introduction via λ phage vectors, cosmids, etc.

λ phage vectors have *cos* site needed for the packaging of recombinant vector into phage heads. When recombination is carried out *in vitro*, DNA inserts of varying lengths can recombine (see Fig. 5.8).

The phage has the ability to pack a total DNA size of between 38-52kbp only. Depending on the actual size of the λ phage vector, the foreign DNA recombined and packed is of about 25 kbp. Cosmids, being smaller, can recombine and pack up to 45 kbp of foreign DNA. When the foreign DNA is mixed with specially prepared head and tail proteins in cell extracts, packaging of recombinant DNA and assembly of the particle occur under the influence of viral proteins Nu 1 and A.

It must be noted again, only the right size of DNA gets packed. The frequency of recombination is much higher than in $CaCl_2$ method. Special techniques and mutants are required for the preparation of empty heads, tails, and DNA-free terminase. The packed particles are then allowed to infect special strains of *E. coli*. When λ vector is

used, the recombinant DNA integrates to the host DNA. When cosmids are used, they replicate like plasmids.

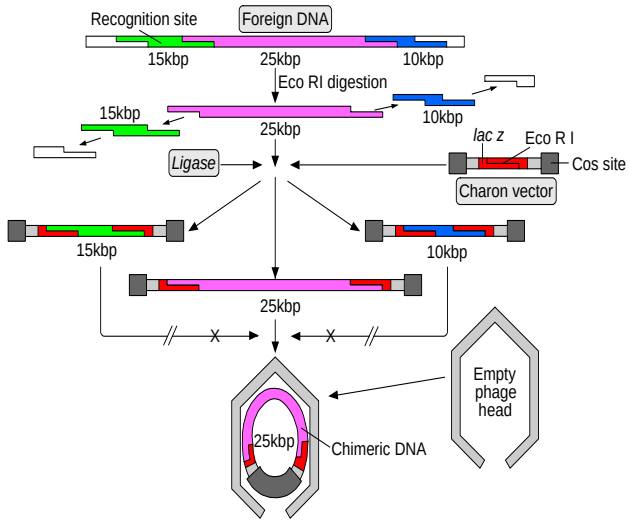


Fig. 5.8 *In vitro* recombination using phage vector

5.4.6 DETECTION OF RECOMBINANT CELLS (CLONES)

Because of markers present in vectors (spanning the restriction sites) and the special host used, identification of the transformants is quite easy. During recombination, two things can happen: (i) *vector will recircularize without taking the foreign DNA*, or (ii) *vector becomes chimeric by taking up the foreign DNA*. During transformation, three things can happen: (i) *the host receives only the vectors*, (ii) *the host receives recombinant vectors*, or (iii) *the host does not receive anything (or only the foreign DNA)*. The sorting out of all these is quite interesting. An example of selection of transformants is provided in the following paragraphs.

5.4.6.1 Examples of recombinant identification

When plasmid is used

- *Vector used*: pBR 322
- *Host used:* selected strain of *E. coli* (sensitive to Ampicillin and Tetracycline)
- *Restriction enzyme used*: *Pst* I.
- *Selectable markers used*: Ampicillin resistance (Amp^r) and Tetracycline resistance (Tet^r)

After reaction, the host cells are grown on a normal medium. The growth is then *replicated* (replica-plated) on a medium amended with ampicillin and tetracycline. The growths on all the plates are compared. The normal plate is called *master plate*. The master plate supports, ideally, the growth of all host cells. The original host cell cannot grow on either of the replica plates because the organism is sensitive to

antibiotics. Following observations (Table 5.4) and conclusions can be drawn after comparing the growth on different plates. See Fig. 5.9 for clarity.

Since the restriction enzyme we have used is *Pst* I, recombination at Tet^r is out of question. Therefore, only 1, 2 and 4 (Table 5.4) are possible.

Table 5.4 Observation and conclusion of test

Observation	*Conclusion*
Growth in $Ampicillin^+$ and $Tetracycline^+$ media	Entry of intact plasmid
Growth in $Tetcycline^+$ media but not in $Ampicillin^+$ media	Recombination in Amp^r gene
Growth $Ampicillin^+$ media but not in $Tetracycline^+$ media	Recombination in Tet^r gene (but not possible with pBR 322 in the above case because we have used *Pst* I as the restriction enzyme)
No growth in both $Ampicillin^+$ and $Tetracycline^+$ media	Host cell did not receive anything or received only foreign DNA

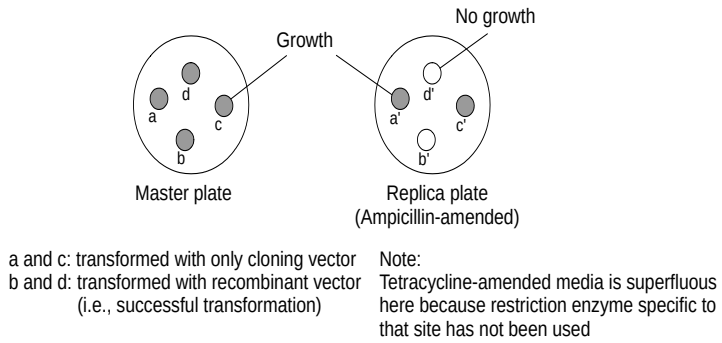


Fig. 5.9 Detection of recombinant cells with the help of markers

When charon-16 is used

Charon-16 has a scorable marker, *lac* Z. Lactase-negative (lac^-) strain of *E. coli* is chosen as the host. The host cells are first spread over a special medium (called *X-gal* medium) to form a *lawn* (rich growth). The medium contains a compound, *5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside*. Over this lawn of *E. coli*, the reconstituted phage particles are spread and the color of lysis (*plaques*) observed. Recombinant *E. coli* cells yield colorless plaques. This is because of the insertional inactivation of *lac Z* gene spanning the restriction site. Cells that have received intact charon vector (i.e., only the phage DNA packed) produce plaques of blue color. This is because the intact *lac Z* has β-galactosidase activity. The organism bearing this gene (passed from phage) can break down *X-gal* (lactose analog) to produce color. Thus, only the colorless transformants are the true transformants.

5.4.7 SELECTION OF SPECIFIC CLONES

Selection of transformants is relatively easy. However, selection of the desired transformants is complex. Their selection is very important because we are interested in only those transformants that have picked up the right gene. There are two basic approaches used for this: (i) *looking for the gene products*, and (ii) *looking for the gene itself.*

5.4.7.1 Looking for the gene product

One basic requirement of this technique is that the host cell itself should not produce the desired gene product. There are several methods for testing this gene product. One common method is to use *antigen-antibody* reaction. The process is also known as *immunological test.* See Fig. 5.10 for the outline of the protocol.

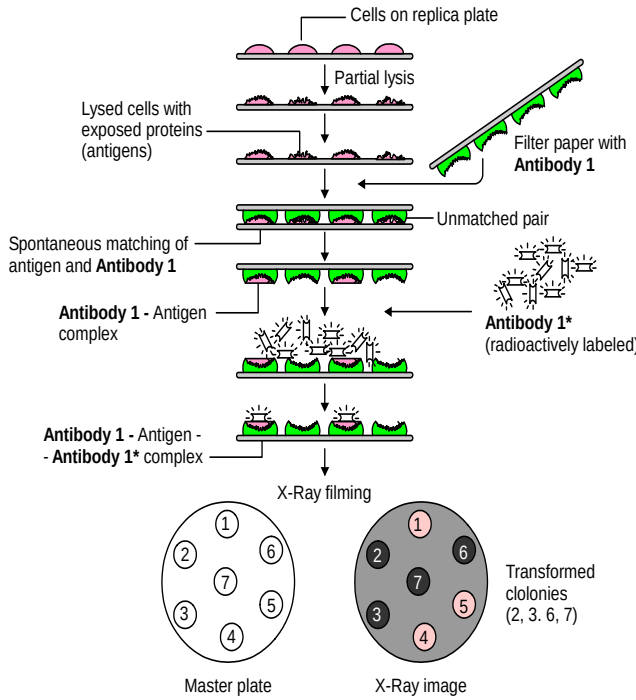


Fig. 5.10 Antigen-antibody reaction for detecting clones

First of all, replica-plates of the colonies are prepared from the master plate. The cells in the replica plates are then lysed by exposure to chloroform or high temperature. Then a cellulose filter (or polyvinyl filter) onto which specific antibody has been fixed is pressed over the lysed cells. If the antibody finds its corresponding antigen (the gene product) in the lysed cells, it will bind to it. The filter is taken out, along with the bound antigen, and incubated with yet another *radioactively labeled* antibody. The gene product (i.e., antigen) gets sandwiched between two antibodies. After reaction, the excess antibody is washed away. The filter is now X-ray imaged to determine the position (and presence) of gene product in the lysed colonies.

Comparison of the image with the master plate will reveal the presence and position of cells (clones) containing the desired gene. The desired colonies can now be picked up from the master plate for subculturing.

5.4.7.2 Searching for the gene itself

This is commonly known as *colony hybridization*. Replica plates are prepared on a *nitrocellulose* filter disc. The disc is placed on a gelled medium (nutrient) and incubated to develop colonies. Cells growing on the disc are nourished through the gelled medium. The disc is then placed on a blotting paper soaked in 0.5 *N* NaOH solution. The alkali diffuses into the nitrocellulose, lyses the cells, and denatures their DNA. Thereafter, the disc is neutralized with *tris* (hydroxymethyl) aminomethane-HCl buffer. This results in binding of the DNA in the same position as the colony. To fix the DNA properly, the disc is baked at 80°C. It is then incubated with a radioactively labeled probe (20-40 nucleotide, labeled with P^{32}). The unbound probes are washed away and the discs are X-ray imaged to compare with the master plate for determining the colony and position of cells containing the desired gene.

5.4.7.3 Hybrid Arrested Translation (HART)

HART is a fusion of two techniques, *viz*., *immunological* and *colony hybridization*. HART can identify a given DNA in a clone even if neither DNA nor RNA probe is available, so long as one knows which protein one is looking for!

In this method, each clone's DNA is extracted and purified and the two strands are separated from one another. Raw RNA is also extracted from the donor cell (the cell from which the foreign DNA was taken). The extracted DNA and RNA are then mixed in a *cell-free protein synthesis system* where everything needed for protein synthesis is present. If the foreign DNA is present in the extract it will hybridize with the complementary RNA extracted from the donor cell. This particular RNA being hybridized will not be free for taking part in protein synthesis. In other words, all the other *m*RNAs in the mixture begin busily directing protein synthesis except for those bound to DNA. So, if the protein one is looking for is expressed in the mixture, the gene one wanted is not present in the clone. To find protein, one can use antigen-antibody reaction as described in immunological method.

CHAPTER 6

PRESERVATION AND MAINTENANCE OF INDUSTRIAL CULTURES

6.1 INTRODUCTION

Microorganisms for the production of industrially important products are useful only if they can be maintained indefinitely in healthy, pure, and genetically stable form.

Industrial culture collection consists of *stock cultures*. A stock culture may be simply defined as a culture which serves as a source of inoculum. Stock cultures are of two types:

1. Working stock culture
2. Primary stock culture

Working stock cultures are maintained in a vigorous and uncontaminated condition and are used frequently. They must be checked frequently for characteristic features and contamination. In contrast, primary stock cultures are kept for long-term storage for later use: they are used to produce new working stock cultures as per need.

6.2 PRIMARY STOCK CULTURE

As this culture is meant for reserve, it is stored in an uncontaminated state for prolonged periods. The cultures are maintained in a state of *low physiological activity* (suspended animation). There are several primary stock culture methods available, ranging from *low risk* to *high risk*. High-risk methods, as evident, use risky albeit simple methods, often requiring frequent subculturing. Should this be the case, selection must be made of the methods that specify a minimum number of transfers, as with each transfer the chances of contamination and mutation increase significantly. Some of the general methods of preservation and maintenance of primary stocks are:

- Periodic transfer
- Oil overlay
- Preservation in soil
- Desiccation
- Lyophilization (freeze-drying)
- Use of low temperatures

6.2.1 PERIODIC TRANSFER

This method is also called *active transfer*, s*ubculturing*, or *serial transfer*. The temperature of storage and the type of medium chosen should support a slow, rather than rapid, growth. This reduces the frequency of transfers. Slants (agar slopes) and broth cultures are the most widely used methods. The culture, after maturation, can be preserved (and maintained) in the refrigerator for a period of 2-6 months. Although simple, these methods are the least desirable. Mutation cannot be prevented and the frequent transfers increase the chances of contamination and genetic changes.

6.2.2 OIL OVERLAY METHOD.

Many bacteria and other organisms can be preserved by this method for 1 month to 2 years. In this method, an agar *slant* growth is overlayed with sterile and inert mineral oil (½" above the tip of the slant surface: see Fig. 6.1) and stored cold. The unique advantage of this method is that some of the growth can be removed to inoculate a fresh medium without contaminating the stock. In other words, the stock can be used for a number of times. This is not possible in *subculture* method (= serial transfer method). Another advantage of this method is the control of water and gas exchanges in the medium. There are some limitations also. For example, this method is not suitable for the preservation of sporulating molds because it sometimes leads to loss of sporulation property (and sometimes, biochemical activity also). The most common mineral oil used is the liquid paraffin. It is sterilized at 180°C in hot air oven for an hour before using for overlay.

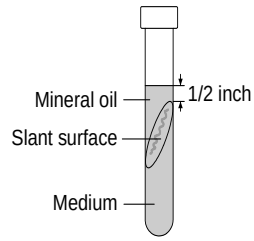


Fig. 6.1 Mineral oil overlay

6.2.3 LYOPHILIZATION

Lyophilization is probably the most satisfactory method for long-term preservation of those microorganisms which can withstand the rigor of the process. In this process, a dense cell suspension (in stationary phase) is placed in small vials (ampules or ampoules), frozen under vacuum (200 µm Hg) at -60 to -70°C, and vials sealed using oxygen-natural gas flame (Fig 6.2). The suspension is prepared in various protective media such as bovine serum, media containing sugars, or skim milk. It is also advantageous to include inositol (5%). The whole is stored under refrigeration in dark. The organism remains viable for as long as 30 years.

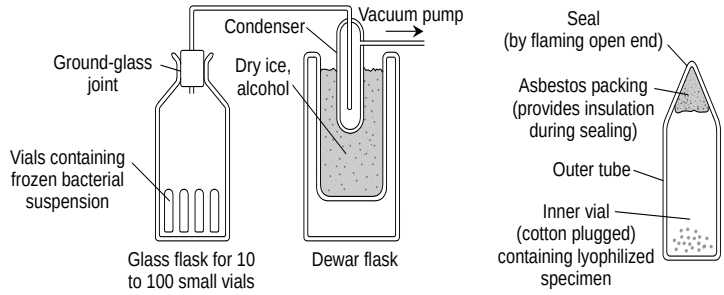


Fig. 6.2 Lyophilization process for the preservation of cultures

The advantages of lyophilization are as follows:

- Requires no subculturing
- Ease of transportation
- Genetic stability
- Low cost
- Less storage space

The only disadvantage of lyophilization is that not all microorganisms can be subjected to this method.

6.2.4 PRESERVATION IN SOIL

Soil can be used either as a carrier or a growth medium. If used as a carrier, abundant spores of bacterial or fungal species must be prepared in advance. The spores are then placed in sterile soil and the resulting preparation dried in air or under vacuum.

For many fungi, maintenance by use of soil is very useful. Moist soil may be inoculated with the fungus and may be allowed to grow until sporulation has completed (see Fig. 6.3). This type of culture may be used both as primary and as working stock culture. Preparation of *murcha* (an amylolytic starter culture indigenous to Nepal) is very much similar to this, differing only in the use of starchy material in place of soil. An example of preservation in soil is given in Fig. 6.3.

6.2.5 USE OF LOW TEMPERATURE

Cells are prepared as a dense suspension in a medium containing a cryoprotectant such glycerol or dimethylsulfoxide. Ampules are prepared and frozen at a controlled rate to -150°C. Initially, the temperature is allowed to go down approximately at the rate of 1°C/min up to -20°C. Thereafter the temperature is brought down as rapidly as is possible. Electrolytes should be kept at as low a concentration as possible. Finally, the ampules are stored in liquid nitrogen refrigerator either by immersing (-196°C) or in a gas phase (-150°C). This method has been successful with many species, including human cells, which cannot be preserved by lyophilization. Most species remain viable for 20-30 years. The method, however, is very expensive. The

expense arises from the amount of nitrogen needed for replenishment. A common laboratory method for preservation by low temperature is given in Fig. 6.4.

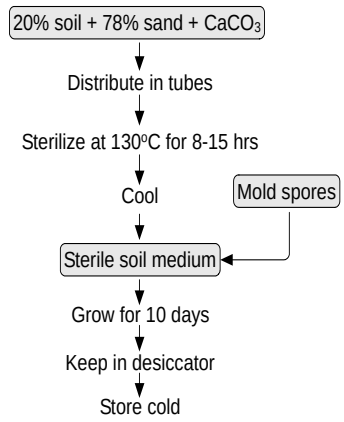


Fig. 6.3 Preservation of mold in soil

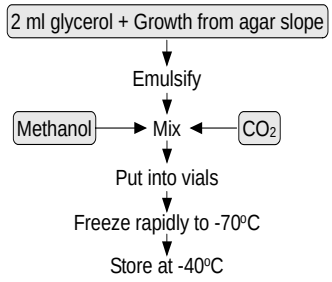


Fig. 6.4 Simple laboratory method for low-temperature preservation

6.2.6 DESICCATION OR DRYING

This method is useful for preserving yeast cells. Cells are kept directly in contact with silica gel desiccant. Silica gel is kept in a screw-capped container (to a depth of about 1 cm, sterilized in hot air oven). The culture is grown to a stationary phase in a suitable medium and then suspended in 5% skim milk medium. The latter is finally transferred to gel without saturating it (the gel). The heat developed should be dissipated quickly. The gel is dried at room temperature for 2 weeks and finally put in an airtight container for storage at 4°C. The culture is stable for several years.

Alternatively, cells may also be preserved by desiccating them on a filter paper kept over silica gel in a desiccator.

6.3 MAINTENANCE OF PURITY OF CULTURE

A variety of chemicals and antibiotics are used for maintaining purity in culture collection. Some common and easily available compounds and their application are given in Table 6.1.

Table 6.1 Some common and easily available compounds and their application

Compound	Concentration (mg/L)	Use
Rose Bengal	35	Inhibits colony growth
Neomycin sulfate	20	Inhibits actinomycetes
Cycloheximide (actidione)	20-200	Inhibits yeasts and some fungi
Penicillin	40	Inhibits bacteria
Griseofulvin	2-20	Inhibits fungi

Invasion of fungal culture by mites is a very common problem. For this, low risk methods such as mineral oil overlay, lyophilization, or liquid nitrogen storage can be used. Rooms for storing cultures as well as those for subculturing should be easily cleanable, have low microbial load, and provided with filtered air. The surfaces and walls should withstand the harshness of cleaning agents. Periodic disinfection should be carried out with UV radiation and other disinfectants such as formaldehyde, ethanol, isopropanol, hypochlorite, etc. In case the contamination is very high, entire room must be fumigated. Fumigation can be achieved by boiling formalin to release formaldehyde. Approximately 0.5 ml formalin should be used for each cubic feet of air space.

6.5 SOME CULTURE COLLECTION CENTERS

- American Type Culture Collection Center (ATCC)
- Commonwealth Mycological Institute (CMI)
- Northern Region Research Laboratory (NRRL)

CHAPTER 7

CONCEPT OF BIOTECHNOLOGY

7.1 INTRODUCTION

The term *biotechnology* came into being in the 1970's when scientists learned to alter precisely the genetic constitution of living organisms by processes without traditional breeding practices. Biotechnology is defined by different persons and organizations in different ways: for example, those of US National Foundation, European Federation of Biotechnology, the IUPAC, etc. This is natural because, by its nature, the area covered under biotechnology is very vast and the techniques used are highly divergent. This has often made a precise definition of the subject rather difficult. Although different definitions of biotechnology differ in their approach, content and emphasis, the two main features common to them all are: (i) *utilization of biological entities (microorganisms, cells of higher organisms – either living or dead), their components or constituents (e.g., enzymes),* in such a way that (ii) *some products or service is generated.* This product or service should, obviously, enhance human welfare. In essence, therefore, biotechnology concerns with the exploitation of biological agent for generating useful products/services. Production technologies pertaining to agriculture, horticulture, and animal husbandry also utilize biological entities to generate useful products. But these activities are not regarded as biotechnology since they are long recognized and established disciplines in their own right. However, the exploitation of animal and plant cells cultured *in vitro* as well as their constituents for the generation of products/services is an integral part of biotechnology.

Although the term biotechnology is of recent origin the discipline itself is very old. Man has long been in association with microorganisms and has gainfully employed them since time immemorial. Historically, biotechnology was an art rather than a science where techniques of manufacture were well worked out and reproducible, as exemplified in by the production of wines, cheeses, beers, etc., but the molecular mechanisms were not understood. For the sake of clarity, the chronology of some important developments in biotechnology is given in Table 7.1

It is ironical that the major advances in biotechnology were the aftermath of post-wars. During the First World War, Germans were forced to develop the technology for glycerol (needed for manufacturing explosives) production when their supply of vegetable oil was disrupted due to the British naval blockade. Similarly, British resorted to acetone-butanol fermentation using *Clostridium acetobutylicum* due to the German interferences with the normal supply of these chemicals. The First World War also left citrus orchards of countries like Italy in ruins; this resulted in a great jump in the prices of citric acid which was extracted from citrus juice. As a result, the technology for citric acid production using *Aspergillus niger* was developed. The production of the antibiotic penicillin by *Penicillium notatum* was discovered in 1930 by Alexander Fleming, but its commercial production began, again, only during

Second World War. But subsequent developments in chemical pharmaceutical production using microorganisms have been very rapid and have covered a very wide range indeed.

Man has continued his quest for improving the natural capabilities of microorganisms, making them capable of novel processes, and discovering microorganisms with new capabilities. This has led to the development of *recombinant DNA technology*, which allows man to modify microorganisms and other organisms to create in them highly valuable, novel and naturally non-existent capabilities.

With the major advances in our understanding of microbiology and biochemistry, genetics, chemistry, chemical- and process-engineering, several molecular innovations have now become possible. Unprecedented changes can now be brought about in living system. Transgenic plants and animals are heralding a new age in agriculture, and gene therapy in humans may eradicate previously incapacitating diseases. In the environment, biotechnology is allowing major improvements in water and land management and also remediating the pollution generated by over-industrialization.

Table 7.1 Chronology of important developments in biotechnology

Activity	Year
Yeasts used to make wine and beer	~ 6000 BC
Yeasts used for making leavened bread	~ 4000 BC
Sewage treatment systems using microbes developed/established	1910 AD
Large-scale microbial production of acetone, butanol and glycerol	1912-14
Large-scale production of penicillin	1944
Mining of uranium with the aid of bacteria	1962
First successful genetic engineering experiments	1973
Marketing of human food of fungal origin (United Kingdom)	1980
The use of monoclonal antibodies for diagnosis approved (USA)	1981
Approval of the use of insulin produced by genetically engineered microbes (USA and UK)	1983
Animal interferon produced from genetically engineered microbes (GEMS) approved for the protection against diseases	1984
Animal experiments by British scientists	

Thus, in its simplest form, biotechnology employs microorganisms to convert simple organic molecules (e.g., sugar) into more useful products (e.g., alcohol) and to produce some unique biochemicals (e.g., antibiotics). On the other extreme of the spectrum are ranged the sophisticated techniques of recombinant DNA technology, hybridoma technology, enzyme technology and enzyme engineering, etc.

7.2 BIOTECHNOLOGY AS AN INTERDISCIPLINARY ACTIVITY

Biotechnology is truly interdisciplinary in nature, exhibiting a bewildering array of sub-disciplines: microbiology, chemistry, biochemistry, genetics, molecular biology, immunology, cell- and tissue culture, physiology, chemical-, biochemical-, and process engineering. A distinction needs to be made between *interdisciplinary* and *multidisciplinary* natures. The term *multidisciplinary* describes a quantitative extension of approaches to problems that commonly occur within a given area. It involves the marshalling of concepts and methodologies from a number of separate disciplines and applying them to a specific problem in another area. In contrast, *interdisciplinary* application occurs when the blending of idea that occurs during multidisciplinary cooperation leads to the crystallization of a new disciplinary area with its own concepts and methodologies. In practice, multidisciplinary enterprises are almost invariably mission-oriented. However, when true interdisciplinary synthesis occurs the new area will open up a novel spectrum of investigations. Fig. 7.1 shows the interdisciplinary nature of biotechnology.

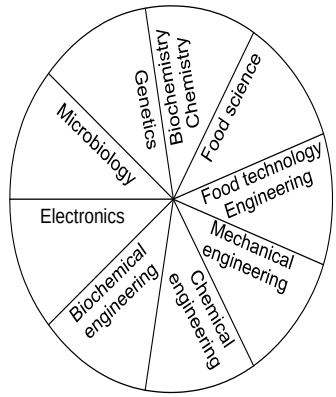


Fig. 7.1 The interdisciplinary nature of biotechnology

7.3 SCOPE AND IMPORTANCE OF BIOTECHNOLOGY

Biotechnology has rapidly emerged as an area of activity having a marked realized as well as potential impact on virtually all domains of human welfare, ranging from food processing, protecting the environment, to human health. As a result, it now plays a very important role in employment, production and productivity, trade, economics and economy. This is clearly reflected in the emergence of numerous biotechnology companies throughout the world, including India, and the movement of noted scientists, including Nobel Laureates, to some of these companies. The total volume of trade in biotechnology products is increasing sharply every year, and it is realistically accepted that by the early 21st century it will be contributing trillions of dollars to the world market. Unfortunately, very few people realize that biotechnology affects over 30% of global economic turnover by way of health care, food and energy, agriculture and forestry and this economic impact will grow as biotechnology provides new ways of influencing raw material processing. Many commentators are confident that the 21st century will be the century of

biotechnology, just as the 20th century was the era of electronics. A summary of the main areas of applications of biotechnology is given in the following paragraphs:

7.3.1 Bioprocess technology

Historically, the most important area of biotechnology, namely brewing, antibiotics, mammalian cell culture, etc.; extensive development in progress with new products envisaged, namely polysaccharides, medically important drugs, solvents, protein-enhanced foods; novel fermenter designs to optimize productivity.

7.3.2 Enzyme technology

Use for the catalysis of extremely specific chemical reactions; immobilization of enzymes; to create specific molecular converters (bioreactors). Products formed include L-amino acids, high fructose syrup, semi-synthetic penicillins, starch and cellulose hydrolysis, etc. Enzyme probes for bioassay.

7.3.3 Waste technology

Long historical importance but more emphasis now being made to couple these processes with the conservation and recycling of resources; foods and fertilizers, biological fuels.

7.3.4 Environmental technology

Great scope exists for the application of biotechnological concepts for solving many environmental problems – pollution control, removing toxic wastes; recovery of metals from mining wastes and low-grade ores.

7.3.5 Renewable resources technology

The use of renewable energy sources, in particular, lignocellulose to generate new sources of chemical raw materials and energy ethanol, methane and hydrogen. Total utilization of plant and animal material.

7.3.6 Plant and animal agriculture

Genetically engineered plants to improve nutrition, disease resistance, keeping quality, improved yields and stress tolerance will become increasingly commercially available. Improved productivity, etc., for animal farming. Improved food quality, flavor, taste and microbial safety.

It is being argued that, although the global production of food may be sufficient to feed the world's population, problems of poverty and distribution of food resources mean that hunger and malnutrition are still endemic in the developing world. In the face of these issues, biotechnology should be used to develop food crops which offer greater nutritional benefits so that the poorest people can obtain adequate nourishment from smaller quantities of food.

7.3.7 Health care

New drugs and better treatment for delivering medicines to diseased parts. Improved disease diagnosis, understanding of human genome.

Biotechnology, thus, has unlimited potential in view of its capability to generate an unlimited range of valuable and useful products/services concerned with virtually every aspect of human existence. For example, in the mid-1991, over 130 biotechnologically derived pharmaceuticals, aimed at everything from *hemophilus* to *AIDS*, from anemia to leukemia, were under regulatory review in the US.

The *new* biotechnology, however, demands very high expertise and skill, and continued heavy funding coupled with dedicated effort. Therefore, highly industrialized countries have a dramatic edge over the less industrialized ones, more so, over the developing countries both with regard to the research and development activities, and the exploitation of market potential.

7.4 POTENTIAL HAZARDS OF BIOTECHNOLOGY

The early studies on gene manipulation provoked wide discussion and concern at the possible risks that could arise with certain types of experiments. Thus, it was believed by some people that the construction of recombinant DNA molecules and their insertion into microorganisms could create novel organisms that might inadvertently be released from the laboratory and become a *biohazard* to human beings or the environment. In contrast, others considered that the newly designed organisms, with their additional genetic material, would not be able to compete with the normal strains present in nature. The present views of gene manipulation are becoming more moderate as the experiments have shown that this work can proceed within a strict safety code. The standards of containment enforced in the early years for recombinant DNA studies were unnecessarily restrictive and there has been a steady relaxation of the regulation governing much of the routine genetic engineering activities. However, for many types of study, particularly with pathogenic organisms, the standards will remain stringent. The physical containment in rDNA studies includes the use of sterile techniques, containment hoods, and specially designed laboratory to prevent vector containing the rDNA form being transferred or *escaping* from the containment to natural ecosystems. Biological containment involves the use of organisms with specially constructed, *weakened* genotypes as vectors in cloning experiments. Ideally, these organisms should be unable to survive under conditions existing in natural ecosystems.

One of the greatest risks of genetic engineering is the possible abuse of the technology for biological warfare. The recent anthrax episode after the 11th December (2001) bombing of the World Trade Center in America is an example of how dangerous genetically engineered microbes (GEMS) could be. Biological weapons are prohibited by International Treaty, but such treaty may of course be disregarded by independent groups (guerilla movements, terrorists, organized crime). The governments that watch over the Biological Weapons Treaty should therefore closely monitor the consequences of genetic engineering. With all these risks in view, National Institutes of Health (NIH) have established guidelines for researches involving rDNA molecules. Under these guidelines the NIHs serve an overseeing

role by sponsoring risk-assessment program, certifying new host systems, serving as information clearing house, and coordinating Federal and local activities.

There is also concern regarding the use of GEMS in vaccines. The poliovirus can be taken as an example. The oral polio vaccine (OPV) consists of weakened form of poliovirus. *Salk* vaccine, an alternative form, consists of killed virus. Because of low cost and ease of administration, OPV is the most widely used polio vaccine. OPV viruses differ from the virulent virus by only a few mutations and the condition in the gut can favor their reversion to pathogenic form. Besides, the OPV can persist for years in people with impaired immune systems, where they mutate faster than in normal populations. HIV-infected people worldwide in particular can serve as a reservoir. Another danger from OPV is the vaccine plant itself. Since vaccines are produced from virulent forms, an escape from the plant can wreak havoc. So factories would have to use high levels of containment, making the vaccine expensive.

There has been growing concern in the domain of transgenic plants also. The main concern relates to the possibility (i) *of transgenic plants becoming persistent weeds*, (ii) *of gene transfers from them to other plants making the latter more persistent and invasive*, (iii) *of their being detrimental to environment*, and (iv) *leading to resistance build-up overtime in pathogens*. The impact on plant ecosystem is of considerable concern. The use of transgenic plant in mass scale not only interferes with the diversity that has its own natural management system but also may adversely affect the environment by bringing about genetic changes in wild types and even wiping out some of the species. The safety of produce is also of considerable debate. Since the assessment of risk is a time-consuming process, it would be nothing if not hazardous to use a *wait-and-watch* approach after introducing the transgenic plants.

As of now, there isn't any perceivable hazard from animal biotechnology. Problems of course exist. The social opinion on transgenic animal research is almost divided in the middle. The main opposition stems from ridiculous assumptions like empathy between humans and animals while at the same time trying to maintain a divide by considering *transgression* for inter-animal transfers of genes.

CHAPTER 8

MICROBIAL GENETICS

8.1 INTRODUCTION

Genetics is the science of inheritance and variability. It attempts to explain the differences and similarities between organisms and the way in which characters are passed from parents to offsprings.

8.2 GENETIC MATERIAL

These are elements responsible for the transfer of genetic traits (characteristics/ properties/information) from parent(s) to the progeny (offspring/daughter or sister cell).

8.2.1 TYPES OF GENETIC MATERIALS

Nucleic acids are the basic units of genetic materials. Deoxyribonucleic acid (DNA, see Fig. 8.1) are the universal units but RNA can also play the same role when RNA is the sole *genome*: this is especially true in the case of RNA viruses such as Tobacco Mosaic Virus (TMV), tumor viruses, etc. RNAs in all other organisms are non-genetic materials (because they are not transferred to the progeny).

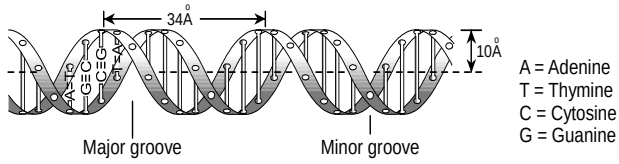


Fig. 8.1 Simplified diagram of normal DNA

The most conclusive proof of DNA as the genetic element came from the elegant experiment performed by Fredrick Griffith in 1928 (transformation phenomenon in pneumococci). In 1953, with Watson and Crick's work, DNA was universally accepted as the genetic element. The evidence that RNA could also serve as genetic material came from A. Gierer and G. Schram's work (1956) with TMV, an RNA-virus.

8.2.1.1 Chromosomes

Chromosome refers to the bulk of DNA present in an organism. Prokaryotic and eukaryotic chromosomes differ from each other by several counts. Prokaryotic chromosomes consist of a single, circular, double-stranded DNA existing in a highly

condensed (folded, negatively super-coiled) form. The size of the chromosome (and also the size of gene or any DNA segment) is usually measured in terms of *base pair* (bp), or *kilobase pair* (kbp) for much larger segments. An idea about the chromosome size can be gathered from examples given in Table 8.1.

8.2.1.2 Mini-chromosomes

These refer to extra-chromosomal genetic elements more widely known as *plasmids* and *episomes*. Episomes are special types of plasmids which can reversibly integrate (fuse) with the main chromosome. Plasmids (and also episomes) are extra-chromosomal self-replicating, double-stranded, closed, circular DNA present in the cytoplasm. The number in a single cell may range from zero to several. A number of host properties are specified by them. A single plasmid may contain 3 to several hundred genes.

Table 8.1 Chromosome sizes of different organisms

Organism	Chromosome size (bp)	No. of genes
Escherichia coli	4.7×10^6	4000
Mycoplasma genitalis	--	470
Human	3×10^9	80,000

The autonomous replication ability of plasmid is due to a unique DNA sequence (gene) from where the replication starts. The site of this origin of replication is denoted by *ori*. Similarly, some plasmids integrate with the main chromosome due to yet another DNA sequence (the site being denoted by *att* for attachment). It is therefore important to note that all plasmids must have an *ori* site. Such and other sites in plasmids are commonly referred to as *modules*. Modules, then, are DNA segments performing a specific function. Each module may contain one or more genes. A simplified diagram of plasmid with modules is shown in Fig. 8.2.

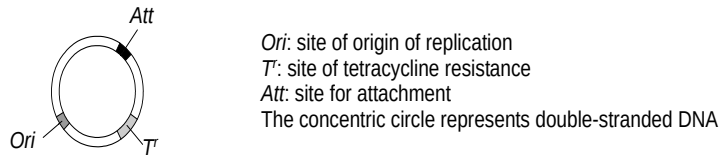


Fig. 8.2 Simplified diagram of plasmid

A number of different types of plasmids have been shown to occur in organisms. Some of the more important ones are:

F-plasmid or F-factor (F for fertility)

This is possessed by microorganisms such as strains of *E. coli*. The module in the plasmid is responsible for sexual reproduction by *conjugation*. The strain having this

plasmid produces a hollow conjugation tube (*pili*) to attach to F-negative strain (the female) and passes varying proportions of the plasmid through this tube into female.

Col-plasmid or Col-factor

This plasmid carries genes responsible for producing *colicin*, which is a bacterial toxin that kills colicin-negative strains. Col-factor also provides immunity to colicin-positive strains against their own colicin.

R-plasmid

This plasmid is responsible for the resistance to antibiotics.

Ti-plasmid

This induces tumor in plants. The plasmid integrates (through wound in plant) stably with the plant DNA. The plasmid codes for enzymes responsible for the synthesis of plant growth hormones. As a result, callus, galls and tumors are formed. *Agrobacterium tumefaciens*, a soil bacterium is responsible for this plasmid.

Ri-plasmid

It is present in *Agrobacterium rhizogenes*, another soil bacterium. The plasmid is responsible for root induction.

Yeast plasmid

This plasmid may be of integrating, episomal, or replicating type. It may be circular or linear. 2 μm-plasmids (pronounced *two micrometer plasmids*) are the most important plasmids present in many strains of *Saccharomyces cerevisiae* and *Saccharomyces uvarum*. These plasmids are covalently closed, and exist in 50-100 copies.

8.2.1.3 Transposable genetic elements

They are also called *jumping genes, cassettes, transposons, mobile genes,* or *insertion sequence.* They are small mobile DNA sequences that move around chromosomes with no regard to homology. The random insertion and ejection of these genes in the host chromosome may lead to mutation. This mutation produces a range of effects, from benign to lethal.

8.2.1.4 Viral DNA

The viral DNA can be single- or double stranded. Further, they can be either linear or circular. Single-stranded DNAs are usually circular. Some examples are given in Table 8.2.

Table 8.2 Some examples of viral DNA

DNA type	Representative virus
Single-stranded, circular	Coliphages M13, fd, Φ X174
Double-stranded	Coliphages T_1 to T_7. λ, μ

8.2.1.5 Mitochondrial DNA

Eukaryotic microorganisms (such as yeasts) have mitochondria. Mitochondria have their own machinery. During cell division mitochondria must also be passed to the progeny, which means that mitochondrial DNA is also passed. This is how the progeny faithfully inherits mitochondrial DNA.

8.2.1.6 DNA in endosymbionts

Certain intracellular parasites such as bacteria and virus maintain symbiotic relationship with host cells. The symbiotic forms are self-reproducing and look like cytoplasmic inclusions. Sometimes, they exhibit an infection-like transmission with a hereditary continuity of their own. Such endosymbionts are usually denoted by the letter σ, κ, μ, etc. A good example of κ particles is the endosymbionts called *Lyticum flagellatum*. The organism consists of a protozoan, *Paramecium tetraurelia* and κ particles. κ particles in turn consist of symbiotic bacterium *Caedobacter taenospiralis* and an infectious virus. The virus controls the toxic viral protein that makes the endosymbiont protozoa a *killer* strain. The protozoan in turn synthesizes folic cid required for the κ particle. Conservation of this killer property from one generation to another implies that DNA in these particles also serves as a genetic element. It must be noted that the killer strain attacks only the sensitive strains.

8.2.1.7. RNA genetic material

*m*RNA, *t*RNA, and *r*RNA are non-genetic RNAs in eukaryotes and prokaryotes. In RNA viruses, however, RNA constitutes their sole genome. They therefore use RNA as the genetic element. Examples of RNA genetic elements are given in Table 8.3.

Table 8.3 Examples of RNA genetic elements

RNA virus	RNA type
Coliphages MS2, F_2, R17, QB	Messenger, single stranded
Raus sarcoma virus	Messenger, single stranded
Polio virus	Messenger, single stranded
Rabies virus	Non-messenger, single stranded

8.3 THE GENETIC CODE

DNA, and in some cases RNA, are the genetic materials. DNA in particular, is the master carrier of hereditary characters. It carries the genetic information in *coded language*. The four common bases, *viz*., Adenine, Guanine, Cytosine, and Thymine can be likened to *alphabet* and the sequential *triplet* arrangement out of these bases can be likened to a *code word*. Assortment of these code words leads to *code language*. Although the code is primarily preserved in DNA it is customarily represented in terms of *m*RNA language. The code words then become *codons*. Combination of these four bases taken three at a time yields 64 theoretical codons. When the code words are translated into decipherable words, they mean amino acids. Out of the 64 codons, however, only 61 codons imply amino acids. They are thus called *sense* codons. The rest 3 are *nonsense* codons, as they do not code for any amino acid. They are also called *stop* codons.

The genetic code is therefore *the system of mRNA sequences that designate particular amino acid during the process of translation*.

The triplet AUG (and sometimes GUG) is called *initiator* codon, as it is the first codon to be encountered during translation (see later). UAA (also termed *ochre*), UAG (*amber*) and UGA (*umber*) are the three *nonsense* codons. The *initiator* and the *nonsense* (or *stop*) codons are collectively called *punctuation* codons.

Codons are grouped into 16 families, each with four codons characterized by its first two bases only. The codon families differ from each other with respect to their third (i.e., 3') base only. There are 8 *unmixed* codon families, *viz*., UC, CU, CC, CG, AC, GU, GC, and GG. See Table 8.4 and 8.5 (the shaded boxes represent unmixed codon family) for detail.

Table 8.4 The unmixed codon family

Codon family	Amino acid specified	Codon family	Amino acid specified
UC	Serine	AC	Threonine
CU	Leucine	GU	Valine
CC	Proline	GC	Alanine
CG	Arginine	GG	Glycine

In the unmixed family (Table 8.5), the third base can be any one of A, U, G, and C. The third base does not alter the specification for amino acid. There are 8 *mixed* codon families, each specifying more than one amino acid, depending on the third base of the codon. In the UU family, for example, UUU specifies for phenylalanine while UUC specifies for leucine.

Table 8.5 The universal codon

1st base (5' base)	2nd base U	2nd base C	2nd base A	2nd base G	3rd base
U	UUU, UUC } Phe	UCU, UCC, UCA, UCG } Ser	UAU, UAC } Tyr	UGU, UGC } Cys	U
	UUA, UUG } Leu		UAA, UAG } Stop	UGA } Stop, UGG } Trp	C, A, G
C	CUU, CUC, CUA, CUG } Leu	CCU, CCC, CCA, CCG } Pro	CAU, CAC } His	CGU, CGC, CGA, CGG } Arg	U, C, A, G
			CAA, CAG } Gly		
A	AUU, AUC } Ile	ACU, ACC, ACA, ACG } Thr	AAU, AAC } Asn	AGU, AGC } Ser	U, C
	AUA, AUG } Met		AAA, AAG } Lys	AGA, AGG } Arg	A, G
G	GUU, GUC, GUA, GUG } Val	GCU, GCC, GCA, GCG } Ala	GAU, GAC } Asp	GGU, GAC, GAA, GAG } Gly	U, C, A, G
			GAA, GAG } Glu		

8.3.1 CRACKING THE GENETIC CODE

In 1961, Nirenberg and Matthaei carried out a decisive experiment to crack the much-hypothesized genetic code. They used artificially synthesized *poly U m*RNA in a cell-free extract. The extract contained the entire requirement for protein synthesis. With poly U *m*RNA, they obtained a polypeptide *polyphenylalanine* as the end product. This showed that the triplet of U codes for phenylalanine. Similar experiments were carried out with poly A, poly C, and poly G to crack the codes for lysine, proline, and glycine respectively. Nirenberg also showed that a codon consisted of base triplet and that the reading of *m*RNA during translation was sequential and non-overlapping. A simple illustration of the principle of his experiment is:

Nirenberg used polyribonucleotides of alternate nucleotide of the sequence CACACACACACACACACACA. Since only two amino acids (histidine and threonine) were obtained in an alternating sequence during translation experiment in cell-free system, it was clear that the triplets CAC and ACA were used. Note above that the combination of triplet can be either ACA or CAC irrespective of where the reading started. If a codon of duplet is used, only one combination (either CA or AC) is possible, which means that the synthesis of two different amino acids would be impossible. A codon of quartet would also have the same result. This study thus showed that a codon consists of a *base triplet* and the reading is *sequential* and *without a break*.

He also used *m*RNA of the sequence AAGAAGAAGAAGAAGAAGAAG. Note that the triplet combination can be either AAG or AGA or GAA. In other words, the *m*RNA consisted of three possible codons. When experimented in a cell-free system a homopolypeptide of lysine was obtained. It was clear that the reading was sequential and without a break. Note again in the above discussion, lysine was coded by AAA. Since lysine was obtained from the triplet AAG as well, it is clear that a single amino acid can be coded by more than one codon.

By 1966, all the 64 codons were cracked. That is, codons for all the 20 amino acids were deciphered. The universal codon, which appears in Table 8.5, is a standard form for most microorganisms for their chromosomal DNA. The codons are slightly different for DNA present in organelles like mitochondria, chloroplast, etc.

8.3.2 CHARACTERISTICS OF GENETIC CODE

There are certain properties of codons that have helped us generalize the genetic code and the codons. Some of these properties (called characteristics hereafter) are:

- *The code is triplet*
- *The codon is non-overlapping:* the same base cannot serve in common for two juxtaposed codons during translation. There are some exceptions though, for example, ØX174 (a virus) codes for more proteins than would be predicted by their nucleotide content, which obviously means overlapping.
- *The codon has polarity:* it is always read in the direction 5'→3'.
- *The genetic code has punctuation codons*
- *The genetic code is universal:* there are exceptions though, for example, UGA refers to *stop* in mammals but *tryptophan* in yeast mitochondria.
- *The code is unambiguous:* generally, the same codon will not code for another amino acid. There is exception here also. For example, GUG codes for methionine as well as valine.
- *The code is degenerate:* there are 61 sense codons whereas only 20 amino acids. It is obvious that the same amino acid is coded by more than one codon (often called *synonyms* or *synonymous codons*). In fact, except methionine and tryptophan all amino acids have synonyms. Such a code is then said to be *degenerate*. Leucine, serine, and arginine have as many as 6 synonyms (see Table 8.5).
- *The code is commaless:* the code is read continuously, without a spacer. In other words, the enzymes translate the code without skipping certain sections of codons.

8.3.3 DEGENERACY AND THE BIOLOGICAL ADVANTAGE

Degeneracy can be of two types, (i) *complete*, and (ii) *partial*. In complete degeneracy, the synonyms are not of the same codon family, e.g., CGG and AGA for arginine. Partial degeneracy occurs in unmixed codon families, e.g., CGG and CGA for arginine.

Degeneracy of genetic code has certain biological advantages. For example, it permits the synthesis by organisms of essentially the same complement enzymes and other proteins varying widely in amino acid composition. Degeneracy also provides the organism with a mechanism for surviving mutagenic effects. For example, transversion of ACC to AGA has no lethal effect on the organism as both the triplets code for the same amino acid, threonine.

8.3.4 WOBBLE HYPOTHESIS

Since there are 61 codons but only 20 amino acids, some amino acids must be coded by more than one codon. Such codons are called *synonyms* and the code is called *degenerate*. Also, since the number of *t*RNA (that works as an adaptor molecule during translation) is more than 20 but less than 61, some amino acids can be carried by more than one *t*RNA. Such *t*RNAs are called *isoacceptors*. The *t*RNA forms a complementary base pairing with codons using its corresponding *anticodon* present in the molecule. However, since the number of *t*RNAs is smaller than that of codons, some of the *t*RNAs must form base-pairing with more than one codon. Such a base pairing leads to *abnormal* bonding.

According to Crick (1966), the ability of the same anticodon of the *t*RNA to read different codons (synonyms) is due to *flexibility* or *wobble* in the pairing among bases of the codon and anticodon. The hypothesis is known as *wobble hypothesis* and the exceptional pairing, *wobble pairing* (Fig. 8.3 and 8.4). Wobble pairing does not follow standard base pairing rule. The hypothesis states that the base at the 5' end of the anticodon is not spatially confined because the other two bases help it to form hydrogen bond with any of several bases located at the 3' end of the codon. However, in most cases, it is essential that the first two bases of the codon should form standard base pair. This state is called *two out of three* condition and is particularly true of unmixed codon families (Table 8.5).

Table 8.6 Allowed base-pairing combination according to wobble hypothesis

5' base of the anticodon	3' base of the codon	
C	G	
A	U	
U	A	G
G	C	U
I		U, C, A
	Normal	Abnormal

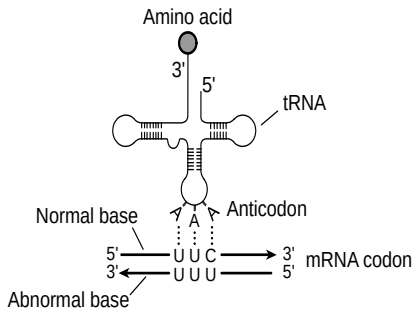


Fig. 8.3 Normal and wobble pairing

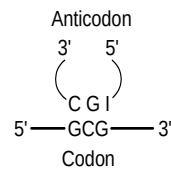


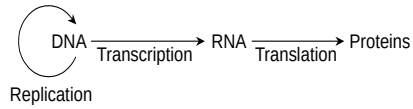
Fig. 8.4: Wobble pairing

The first two bases are of capital importance in the base pairing. They imply factor of genetic stability because a *point mutation* at the third position of the base will have

no influence in its coding ability. Nevertheless, the codon has altered, and this particular mutation is called *silent mutation*. However, the wobble hypothesis fails to explain degeneracy of genetic code in some cases. For example, it cannot explain some of the observed pairing such as in Fig. 8.8.

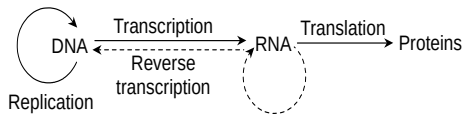
8.4 CENTRAL DOGMA AND TEMINISM

Forwarded by Crick (1958), the *central dogma* of protein synthesis states that genetic information flows from nucleic acids to proteins and not vice-versa. The first step of the central dogma is known as *transcription*. In this step, the message flows from DNA to *m*RNA and does not involve a change of code since DNA and RNA are complementary. The second step involves a change of code from nucleotide sequence to decipherable amino acid sequence. This step is called *translation*. The scheme can be illustrated as:



8.5 TEMINISM (CENTRAL DOGMA REVERSE)

In 1970, Temin and Baltimore discovered an enzyme that could use *m*RNA as the template for the synthesis of DNA. The enzyme is now called *RNA-dependent DNA polymerase* or *reverse transcriptase*. He showed that RNA virus could replicate RNA with the enzyme RNA-dependent-RNA polymerase. This finding showed that the information does not necessarily flow DNA → RNA in one direction but can also be reversed. The improved version of central dogma can therefore be represented as:



8.6 TRANSCRIPTION

8.6.1. THE RNA

The RNAs are formed using DNA as the template, by the normal polymerization mechanism. The complementary RNA produced initially is called a *primary* transcript. There are 4 broad classes of RNAs, *viz*., (i) *transfer RNA* (*t*RNA), (ii) *messenger RNA* (*m*RNA), (iii) *ribosomal RNA* (*r*RNA), and (iv) *heteronuclear* RNA. The last one is found in eukaryotes only.

Transfer RNA

This is also called *soluble* RNA and accounts for 15% of total RNAs in a cell. The *t*RNA molecules serve a number of functions, the most important of which is to act as a specific carrier of *activated* amino acids (see later). Thus, there are at least 20

*t*RNAs in every cell to account for the 20 amino acids. The number of *t*RNAs is variable between 20-64 (both not inclusive), depending on the organism.

All the *t*RNAs have a common design and consist of 3 folds giving each a *cloverleaf* structure (Fig. 8.5). The base sequence at the 3' end of all *t*RNAs is cytosine-cytosine-adenine. During translation, amino acids bind to their terminal adenosine via 3' OH group of its ribose. Each *t*RNA also has a recognition site on which the anticodon is present. The anticodon forms hydrogen bonding with respective codon on the *m*RNA. In general, *t*RNAs contain 70-90 nucleotides only. They may sometimes contain rare bases such as *inosine* and methylated bases. All *t*RNAs have secondary structures which are responsible for the characteristic function they carry out.

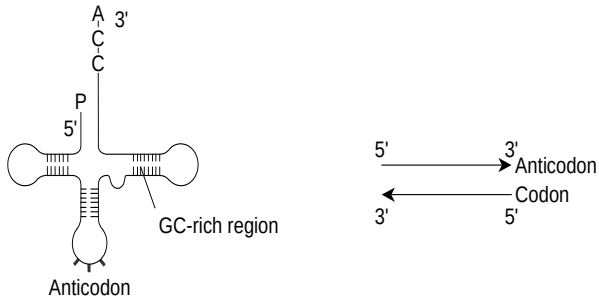


Fig. 8.5 Schematic diagram of *t*RNA

Messenger RNA

It is also called template RNA. Messenger RNA is the most heterogeneous of all the RNA types with respect to size and stability. It accounts for 5% of total RNA in a cell. It is synthesized on the surface of DNA template. Thus, it has base sequence complementary to DNA and carries genetic information or *message* (hence its name) for the assembly of amino acids. *m*RNAs are very unstable in bacterial systems: they have a half-life of about 2 min. Mammalian *m*RNAs have half-lives ranging from few hours to one day. The short half-life of prokaryotic *m*RNA is related to the need for these organisms to adapt to quickly changing surrounding environment.

Ribosomal RNA

It is the most stable form of RNA and is found in association with ribosome. It is also the most abundant and accounts for 80% of total cellular RNA. *r*RNA represents about 40-60% of ribosome by weight. It combines with some 55 different proteins to form complex structures called *ribosome* whose molecular weight is about 3 million dalton. Ribosomes are sites of protein synthesis. Unlike *m*RNA, *r*RNA is not an informational element (does not carry genetic information).

The ribosomes, as also the *r*RNAs, are classified according to their sedimentation coefficient expressed in Svedberg unit, S. Svedberg unit (also called *sedimentation coefficient*) is defined as the sedimentation velocity per unit field of centrifugal force. It

has a unit of cm/sec given by: $S = v/r\omega^2$ where S is the Svedberg unit, v is the sedimentation velocity (i.e., rate of migration, cm/sec) of the solute particles in the direction of centrifugal force, r is the distance in cm of the solution from the rotational center, and ω is the angular velocity of rotation. A sedimentation coefficient of 1×10^{-13} cm/sec is expressed as one Svedberg unit.

The characteristics of ribosomes vary somewhat in different organisms. The ribosomes from animals and eukaryotes consist of 40*S* and 60*S* subunits, which together form an intact 80*S* particle. Bacterial ribosomes consist of 30*S* and 50*S* subunits that can be dissociated into ribosomal and *r*RNA particles. Combination of 30*S* and 50*S* particles gives an intact 70*S* particle (*not the arithmetic sum*). See Fig. 8.6(a) and 8.6(b) for the summary. It has been shown that prokaryotes have 21 small proteins and 33 large proteins.

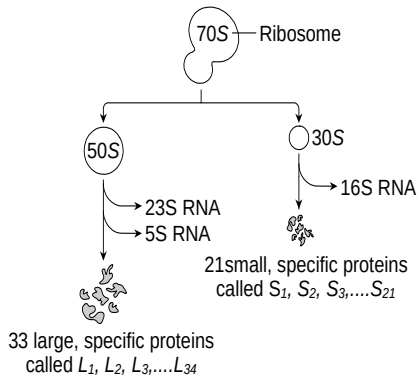


Fig. 8.6(a) The make up of bacterial ribosome

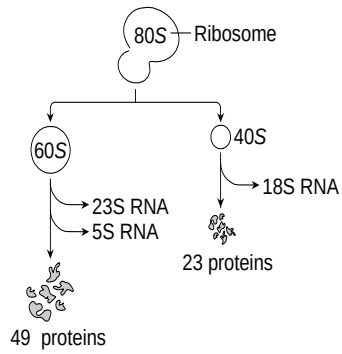


Fig. 8.6(b) The make up of mammalian ribosomes

8.6.2 MECHANISM OF TRANSCRIPTION

The synthesis of RNA using DNA as the template is called transcription. Here, the coded message preserved in the DNA is transcribed into *m*RNA language. The transcription process is similar to replication of DNA in following aspects:

- Transcription as well as replication has *polarity*. The polymerization takes place in the 5'→3' direction
- Replication as well as transcription cannot occur without a template DNA
- The basic event in replication as well as transcription is the polymerization of respective nucleotides

There are fundamental differences, though. The differences mentioned in the following paragraph must be carefully borne in mind by the students. See Fig. 8.7 for an idea about the basic mechanism of polymerization of RNA during transcription.

- Replication cannot start without the presence of a *primer* but transcription does not require a primer
- Replication is a *symmetrical* process. Both the strands are duplicated simultaneously. In transcription, the process is almost always *asymmetrical*. Only one of the strands of the double stranded DNA is used as the template. The other strand is primarily for stabilization. The strand that is involved in coding is called *sense strand*. It is used for transcribing different species of RNAs. The corresponding regions of the complementary DNA are non-coding regions or *antisense* as they do not serve as template for any RNA transcription. But other regions of the second strand can function as the coding or sense strand for other *t*RNA species. Such transcription of two different sets of RNA species on the templates of two different DNA strands of a DNA duplex is called asymmetric transcription.
- The enzymes used in replication and transcription are quite different. Prokaryotic replication uses DNA polymerases (*Pol I*, *Pol II*, *Pol III*, etc.) but the transcription requires a *single* RNA polymerase
- Replication has high fidelity because of the presence of *proofreading* enzyme (*Pol I)*. Transcription has no mechanism for proofreading. Proofreading mechanism in replication is related to genetic stability, which is essential for the survival of the organism. On the other hand, proofreading in transcription need not be of high fidelity. The errors that occur in transcription are not inherited by the offsprings because RNA is not a genetic material

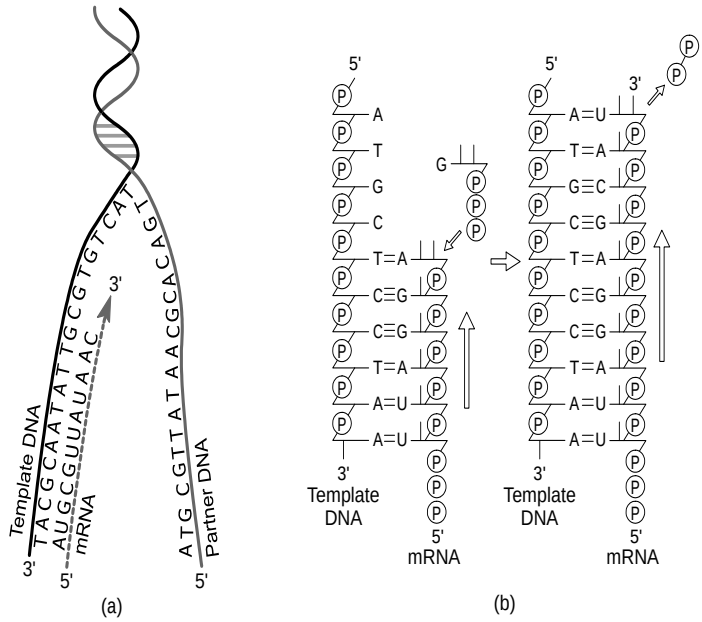


Fig. 8.7 Polymerization of *m*RNA

8.6.3 BASIC STRUCTURAL FEATURES OF PRIMARY RNA TRANSCRIPT

The following are true of any primary RNA transcript in prokaryotes:

- Presence of pppA or pppG at the 5' end
- Presence of 5' UTR (*untranslated region,* also called *5′ leader region*)
- Presence of 3' UTR (also called *3′ leader region*)
- *Polycistronic* nature of *m*RNA
- Presence of *spacers* in the transcript

Prokaryotic *m*RNAs are *polycistronic*. That is, they carry the information for the production of multiple polypeptides. Each region of *m*RNA responsible for the coding of polypeptide is called *cistron*. In polycistronic *m*RNA, the cistrons are separated by non-coding sequences called *spacers*. See Fig. 8.8 for the schematic representation of prokaryotic primary RNA transcript and Fig. 8.9 for the eukaryotic primary RNA transcript.

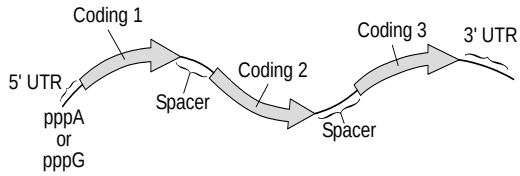


Fig. 8.8 Prokaryotic primary RNA transcript

The primary RNA transcript of eukaryotes is different from that of prokaryotes. The main features in eukaryotic primary RNA transcript are: (i) Presence of *cap* at the 5' UTR (ii) *Monocistronic* nature, and (iii) Poly A tail.

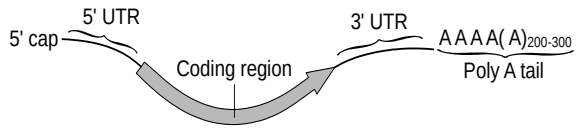


Fig. 8.9 Eukaryotic primary RNA transcript

The 5' ends of eukaryotic *m*RNAs have a 7-methyl guanylate attached by 5' to 5' triphosphate linkage. This structure is called *cap*. The functions of cap are (i) to make the translation efficient, and (ii) protect the RNA from exonucleases. See Fig. 8.10 for the structure of cap.

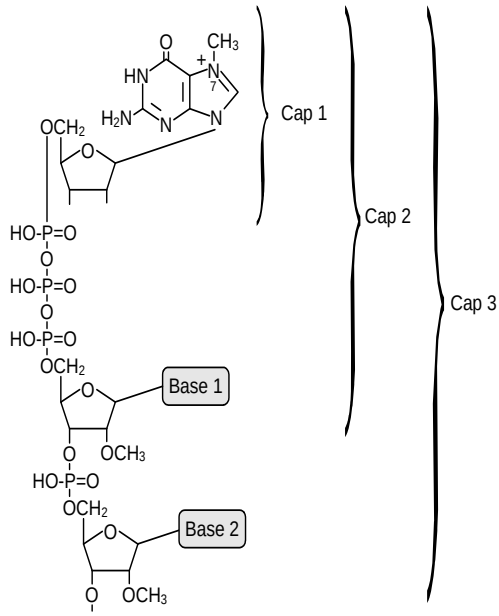


Fig. 8.10 The cap of the eukaryotic *m*RNA

8.6.4 PROKARYOTIC RIBOSOMAL RNA

There are three kinds of prokaryotic *r*RNA, *viz*,, (i) 23*S r*RNA (2904 nucleotides) that is a component of 50*S* ribosome, (ii) 16*S r*RNA (1541 nucleotides) that is a component of 30*S* ribosome, and (iii) 5*S r*RNA (120 nucleotides) that is a component of 50*S* ribosome.

8.6.5 ORIGIN AND PROCESSING OF *r*RNA TRANSCRIPT

Prokaryotic *r*RNAs arise from the processing of a large, 30*S* precursor *r*RNA. Seven genes produce *r*RNA. Each gene produces 30*S* precursor *r*RNA that is processed to discrete, functional *r*RNA. All seven genes contain the sequences that become 23*S*, 16*S*, and 5*S r*RNA. Within the transcribed portion of these genes are some of the *t*RNA genes. Different *r*RNA genes contain different *t*RNA genes. Upon formation of the 30*S* precursor, the non-functional spacer sequences are removed by a series of specific *endonucleolytic* cleavages by the enzyme *ribonuclease P* and *ribonuclease III*. After removal from the spacer sequences, some of the bases in the final *r*RNA undergo *base modification*: they are methylated. This modification is needed for the *r*RNAs to be fully functional.

Many *t*RNAs are formed from the processing of precursor *r*RNA. Those that are not formed in this manner arise from large precursor transcripts. The *t*RNA genes are clustered, and each of the transcripts that form functional *t*RNAs is removed by the enzymes *ribonuclease P* and *ribonuclease D*. All *t*RNAs have CCA at their 3' end. Some *t*RNA transcripts do not contain CCA. This sequence is added to those lacking by the enzyme *tRNA nucleotidyl transferase*. Many of the bases of *t*RNA are modified. These modifications consist of various methylations and other more extensive modifications of some of the bases. The modifications are needed for the *t*RNAs to adopt their unique, functional conformations. See Fig. 8.11 for the mechanism of *t*RNA and *r*RNA formation.

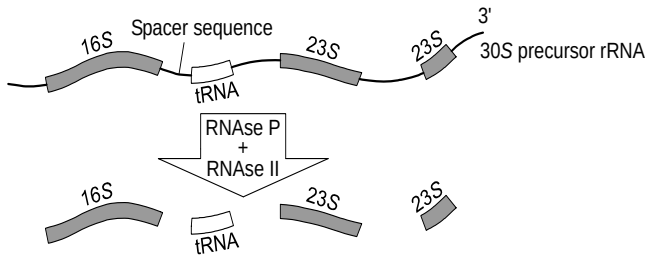


Fig. 8.11 The processing of prokaryotic RNA from the primary transcript

8.6.6 THE TRANSCRIPTION PROCESS IN PROKARYOTES

The process of RNA synthesis directed by DNA template is termed transcription. It occurs in 3 steps, *viz*., (i) *Initiation*, (ii) *Elongation*, and (iii) *Termination*.

8.6.6.1 Initiation

This step implies the recognition of a region of the helicoidal double-stranded DNA molecule (the region is called *promoter*), the binding of RNA polymerase (holoenzyme) to the DNA, localized separation of the two strands producing a short single-stranded region (about 17 base pairs at a time), one of which will serve as a template for the pairing of ribonucleotides, and the selection of the first ribonucleotide of the RNA chain.

Unlike DNA polymerases, this enzyme does not have a proofreading activity. Bacteria have a single polymerase (called *DNA-dependent RNA polymerase* or simply, *RNA polymerase*) responsible for the synthesis of all cellular RNAs (*t*RNA, *r*RNA, *m*RNA). It contains six subunits, *viz.*, α, α, β, β', ω, and σ. The complete enzyme (*holoenzyme*) can be represented by $\alpha_2\beta\beta'\omega\sigma$. The σ factor has a loose attachment. The *core* enzyme therefore consists of only $\alpha_2\beta\beta'\omega$.

There are several different σ factors in *E. coli*, each being specific for binding to the promoter site at 3' end of a specific coding part of the template DNA. The core enzyme is primarily responsible for elongation. Fig. 8.12 is an oversimplified illustration of the core and holoenzyme.

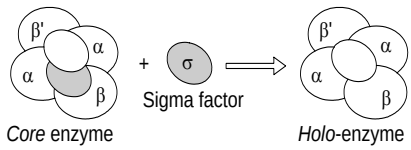


Fig. 8.12 Model of prokaryotic RNA polymerase

Unlike replication, transcriptional initiation does not require a primer. Promoter sequences are responsible for directing RNA polymerase to initiate transcription at a particular point of the template DNA. In bacteria, a promoter consists of a sequence of about 40 bp on the *upstream* site of the transcription initiation site. There are two common patterns (*consensus*) consisting of 6 bp each, called (respectively) *Sequence -10* (or *Pribnow-* or *TATA box*) and *Sequence -35*. There are about 200 promoters in *E. coli*, the model organism. See Fig. 8.13 for the schematic representation of a typical prokaryotic promoter sequence.

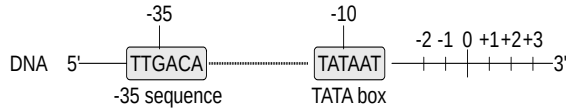


Fig. 8.13 Promoter sequence in prokaryotic DNA

By convention, the starting point of the transcription is numbered +1. The bases situated *downstream* (right hand side from the starting point) are numbered +2, +3, +4, etc. The sequence (*consensus*) shown above is not the actual sequence of the sense strand. They are from the *partner* DNA and are therefore *representative of RNA transcript*.

The core enzyme has the ability to bind to any section of the DNA. It is the σ factor that causes the holoenzyme to bind preferentially and more tightly to the promoter sequence. The σ factor therefore initiates transcription by enabling the holoenzyme to recognize the proper binding site. The binding of σ (as a part of the holoenzyme) to the promoter site facilitates partial opening or *melting* of DNA double helix. The

part that melts is generally rich in A:T bases so that the opening is easier compared to the G:C counterpart. The binding of holoenzyme immediately opens the double-stranded DNA over an 11 base-pair region immediately downstream the initiation site. This gives rise to a transcription bubble (Fig. 8.14) around the initiation site and provides the *holopolymerase* an easy access to the nucleotides in the unwound stretch of the DNA sense strand.

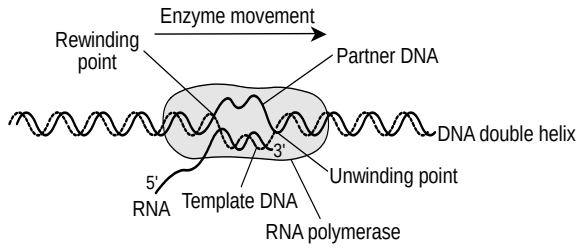


Fig. 8.14 The initiation of transcription

After the transcription initiation site has been exposed by the formation of the transcription bubble, an appropriate ribonucleoside 5' triphosphate from the surrounding medium gets its base hydrogen-bonded to the corresponding base of the coding strand of the template DNA. The first nucleotide is usually pppA, or sometimes, pppG, *but always a purine nucleotide*. Thereafter the next complementary ribonucleoside triphosphate comes for the polymerization. The β and γ phosphates are eliminated from the second ribonucleotide in the process. During the polymerization, α_2 binds to the promoter, β to the ribonucleotide, and β' to the template DNA.

If *rifampicin* is administered to the prokaryotes, the compound binds to β subunit and inhibits initiation. Once initiated, elongation (polymerization) cannot be inhibited.

8.6.6.2 Elongation

During or immediately following the formation of the first phosphodiester bond, the polymerase releases its σ factor and changes into core enzyme. Because of the lowered affinity of the core enzyme to the promoter (because σ factor has been released) the former is now no longer restricted. It is free to move along the coding strands towards 5' direction.

The released σ factor may join another core polymerase to form a holopolymerase for fresh chain initiation. As the polymerase is translocated progressively along the coding strand in the 3'→5' direction, the transcription bubble also moves in the same direction while RNA elongation proceeds in 5'→3' direction. The holoenzyme covers about 60 bp, and the bubble comprises about 17 bp. The hybrid DNA-RNA covers about 12 bp. The passage of the enzyme is followed by the displacement of the preformed RNA chain from the hybrid state and the previously-open DNA helix is reconstituted.

In *E. coli*, at 37°C, the *m*RNA is synthesized at a rate of 40-50 nucleotides/sec. That of T_3 and T_7 is 200/sec at 37°C.

As each RNA polymerase molecule moves downside along the gene to transcribe a progressively elongating RNA transcript, other RNA polymerase molecules follow it in close succession in binding to the promoter, in moving downstream along the same gene and transcribing successive RNA transcripts. The onward translocation of successive polymerase molecules lends an arrowhead appearance to the electron micrograph of gene undergoing transcription (Fig. 8.15).

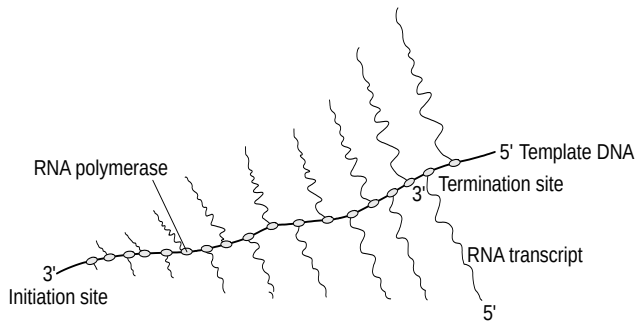


Fig. 8.15 Transcription in prokaryotes

8.6.6.3 Termination

In prokaryotes, e.g., *E. coli*, there are two basic methods of termination, *viz*., (i) *factor-independent termination*, and (ii) *factor-dependent termination*.

Factor-independent termination

A number of *E. coli* genes carry a transcription termination signal in the form of specific consensus base-pair sequences in the DNA duplex. The signal consists of a *palindromic* sequence rich in G:C followed by a region rich in A:T. The RNA transcript of this signal region consequently has a *poly U* 3' tail preceded by a *hairpin loop*. The loop causes the RNA polymerase to slow down. The RNA-DNA hybrid beyond the loop is unstable because of weak A:U bonds and consequently the RNA falls off the template. The DNA reconstitutes and the enzyme is liberated. See Fig. 8.16 for the diagrammatic representation.

Factor-dependent termination

Particular sequences act as the termination sequence in the presence of a factor called *rho* (ρ), a hexameric protein with an *ATPase* activity. A short hairpin loop is formed in ρ-dependent termination also. The RNA polymerase pauses on the DNA on reaching its termination site, which is not well defined yet for such genes. The ρ protein meanwhile binds to a recognition sequence on the RNA transcript in 5'→3' direction. On reaching the RNA polymerase halted at the termination site, the ρ factor unwinds the RNA-DNA hybrid double helix with the energy of the hydrolysis of ATP. This helps release the polymerase from the DNA.

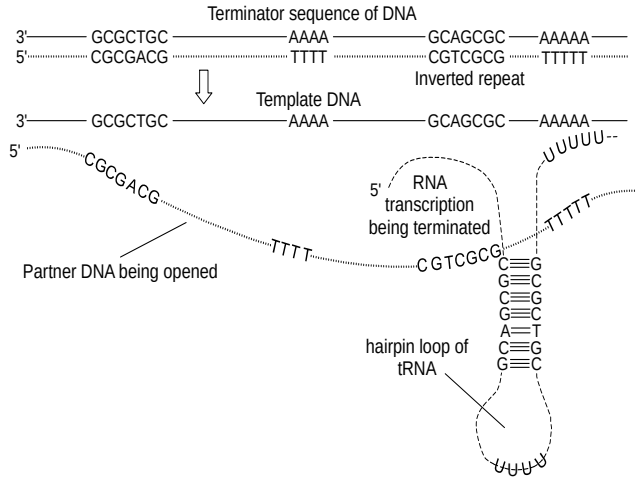


Fig. 8.16 Factor-independent termination of transcription

8.7 TRANSLATION

Translation is the synthesis of proteins under the direction of specific genetic codes (represented as codons in *m*RNA). Ribosomes and *t*RNA participate in translating the message of the genetic code into amino acid sequence of the nascent peptide. Amino acids are polymerized sequentially into a peptide, starting from its N-terminal end following the 5'→3' sequence of codons in the *m*RNA.

Prokaryotes translate the cytoplasmic proteins in the cytosol and the exportable- and membrane proteins on the cytoplasmic surface of the plasma membrane.

Protein synthesis consists of 3 distinct steps, *viz*., (i) *Initiation*, (ii) *Elongation*, and (iii) *Termination*. But before these steps are followed sequentially, the amino acids have to be *activated*: in other words, the amino acids do not spontaneously link together. The activation involves formation of *aminoacyl tRNA*, a "charged" amino acid.

8.7.1 FORMATION OF AMINOACYL *t*RNA (CHARGED *t*RNA)

*t*RNA serves as an adaptor molecule by bringing the specific amino acid coded by the *m*RNA. The joining of the *t*RNA with the specified amino acid is illustrated in Fig. 8.17.

The respective *synthetases* ensure that only the correct amino acids are bound to the corresponding *t*RNA. This specificity apart, the enzymes also have proofreading activity. Most synthetases have a *synthetic* site that does not accept amino acids larger than the specified size and a *hydrolytic* site for degrading amino acids smaller than the specified size.

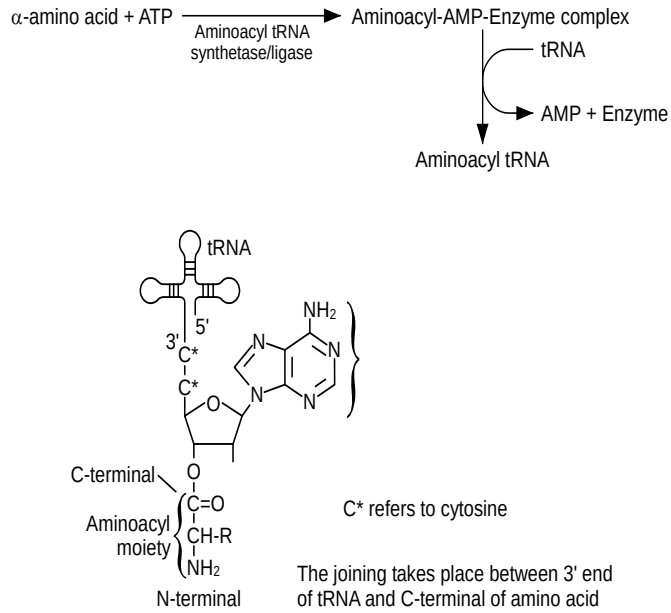


Fig. 8.17 Activation of amino acid before actual translation

In translation, the first codon to be read is AUG, which is for methionine. The methionine brought in the *first step* of the translation (as against *within* the peptide chain) is of a different type, *viz*., *N-formyl methionine*. The specific initiating *t*RNA used here is called *N-formyl methionine tRNA* or *t*RNA$^{f^{met}}$, which is formed according to Fig. 8.18.

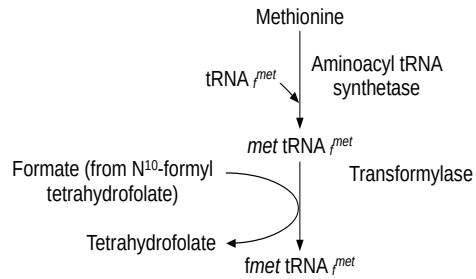


Fig. 8.18 Formation of formylated methionine *t*RNA

8.7.2 INITIATION

The main features of the initiation step are: (i) *binding of mRNA to ribosomes*, (ii) *selection of initiation site*, (iii) *formation of charged tRNA*, (iv) *binding of the tRNA* (to *m*RNA) *bearing the first amino acid*.

The first event is the formation of a *30S initiation complex*. Since prokaryotic ribosome exists as 70*S* unit, it must first be dissociated into 30*S* and 50*S* subunits. This

dissociation requires the action of *initiation factors* IF-1 and IF-3. See Fig. 8.19 for illustrated representation.

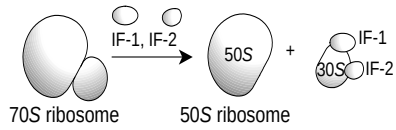


Fig. 8.19 Formation of 30*S* initiation complex

The initiation factor remains bound to 30*S*. IF-3 now helps 30*S* complex to bind to the initiation site in *m*RNA. A purine-rich sequence called *Shine-Dalgarno sequence* (also referred to as *SD sequence, leader sequence, etc.*) within the *m*RNA (within 10 nucleotides upstream the initiation codon AUG) base-pairs with 16*S r*RNA. The *m*RNA base sequence is AGGAGGU or similar to it. The binding of 30*S* also needs S_1, S_{18}, and S_{21}. See Fig. 8.20 for the illustration of mechanism of binding.

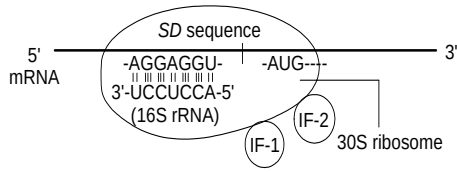


Fig. 8.20 Transient base-pairing between *m*RNA and *r*RNA

IF-2 (another initiation factor) in combination with GTP, brings the f*met*tRNA$_f^{met}$ to the initiator codon. IF-3 dissociates upon binding of f*met*tRNA$_f^{met}$. See details in Fig. 8.21.

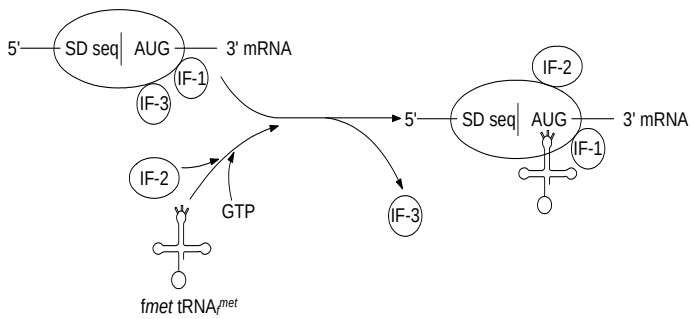


Fig. 8.21 The binding of first amino acid

The release of IF-3 allows 50*S* to combine with 30*S* to form 70*S* initiation complex. This new formation causes release of IF-1 and IF-2. GTP is removed by hydrolysis into GDP + P_i. See Fig. 8.22 for detailed illustration.

The ribosome covers a region of 35-40 nucleotides. This can be shown by a method called *foot-printing*, which is analogous to DNA *finger-printing*. The region covered by the ribosome is protected against enzymatic degradation.

The 70*S* complex is now thought to consist of 2 sites, *viz*., P *site* and A *site*. The P *site* refers to *peptidyl site*, where peptide chain grows, and A *site* refers to *aminoacyl site*, where the new incoming *charged* amino acid is accepted. The first activated *t*RNA, however, is bound to the P site rather than the A site as just described. See Fig. 8.23 for detail.

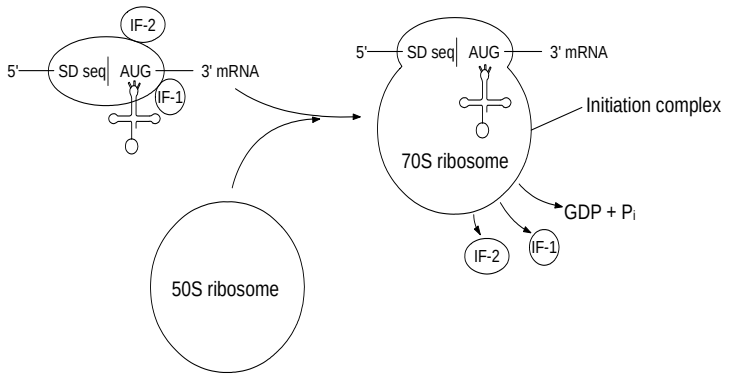


Fig. 8.22 Formation of 70*S* initiation complex

8.7.3 ELONGATION

Elongation entails: (i) *attachment of a new Aminoacyl tRNA to site A*, (ii) *joining together of two amino acids* (polymerization) *by peptide bond formation* – the process called *transpeptidation*, and (iii) *moving of one codon towards 3' end of mRNA* – the process called *translocation*.

Three *elongation proteins* are required for the process, *viz*., *EF-Tu*, *EF-Ts*, and *EF-G*, where *EF* refers to elongation factor, *Ts* to *thermostable* nature of the factor, and *Tu* to the *thermounstable* nature of the factor. *EF-G* is a factor responsible for catalyzing translocation. Elongation factors account for 5-10% of all proteins. The involvement of the elongation factors is quite interesting. The delivery of the new charged *t*RNA to the site A is affected by *EF-Tu*-GTP complex. After being bound, *EF-Tu*-GTP breaks into *EF-Tu*-GDP + P*i*. Another factor, *EF-Ts* now replaces GDP form *EF-Tu*-GDP to form a new complex *EF-Tu-EF-Ts*. Intervention by GTP again dissociates the new complex to *EF-Tu*-GTP and *EF-Ts*. *EF-Tu*-GTP is free to perform another cycle again. See Fig. 8.23 for detail.

The activated amino acid attached to the *t*RNA in the P site, initially f*met*tRNA^{met}, is transferred to the amino group of the Aminoacyl *t*RNA in the site A. This is catalyzed by *peptidyl transferase*, which is an integral part of 50*S* subunit (i.e., L_2, and L_{16}). The P site becomes empty of amino acid. The reaction is also called *transpeptidation*. See Fig. 8.24 for detail.

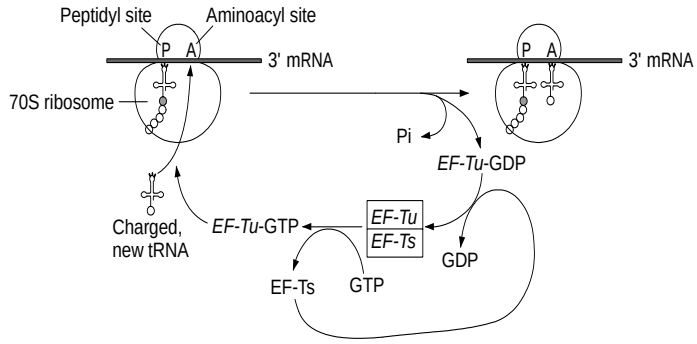


Fig. 8.23 Involvement of elongation factors in translation

After transpeptidation follows *translocation*. The ribosome moves one codon in the 5'→3' direction along the *m*RNA. This movement releases the uncharged *t*RNA from ribosome. Very soon, this *t*RNA falls off the *m*RNA and enters the *t*RNA pool. The relative position of the site A is translocated to that of site P, leaving again a new site for the Aminoacyl *t*RNA. The translocation is catalyzed by *EF-G*.

Another ribosome assembly may occur immediately the previous assembly moves some 80 nucleotides ahead. Beads of such ribosomes seen attached to an *m*RNA are called *polyribosome* or *polysome*. The rate of translation in *E. coli* is about 15 amino acids per sec.

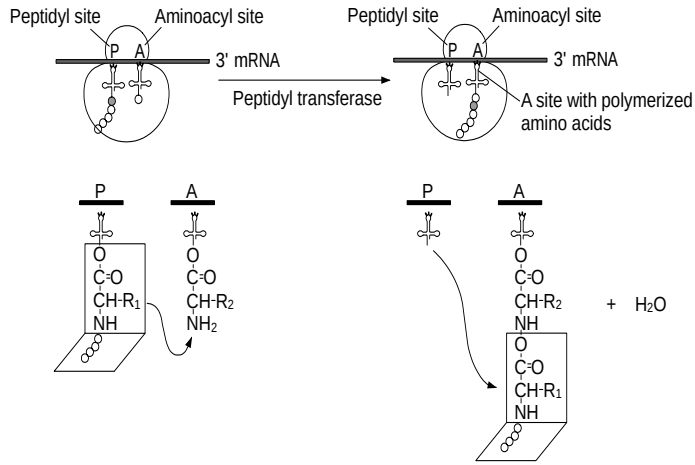


Fig. 8.24 Mechanism of elongation of the peptide chain

8.7.4 TERMINATION

No *t*RNA pairs with *stop codons*. Instead, the stop codons are recognized by release factors *RF-1* and *RF-2*. The former recognizes UAA and UAG, the latter, UAA and UGA. A third factor in association with GTP promotes termination. The binding of release factors induces peptidyl transferase to release the polypeptide in the *t*RNA in

the P site by hydrolysis. The ribosomal unit then separates in a GTP-dependent manner. The 30*S* ribosome may move along the *m*RNA until another Shine-Dalgarno sequence is encountered and the translation resumes, or it may completely dissociate from the *m*RNA. The dissociation of 70*S* unit needs *IF-3*. The natural termination of protein synthesis uses *nonsense* codons but it appears that the signal is not limited to a single nonsense triplet: the serious disadvantage in dependence on a single nonsense codon is that it can at any time mutate to sense codon thereby upsetting the process.

During termination, the release factors bind to site A. The same peptidyl transferase now serves as the hydrolase. With the usual mechanism, the empty *t*RNA comes out of the assembly and eventually falls off the *m*RNA. See Fig. 8.25 for an idea about movement of ribosome and termination of protein synthesis. See Fig. 8.26 for the recapitulative diagram of transcription and translation.

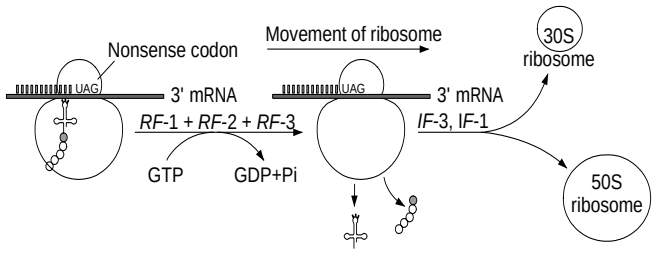


Fig. 8.25 Termination of translation

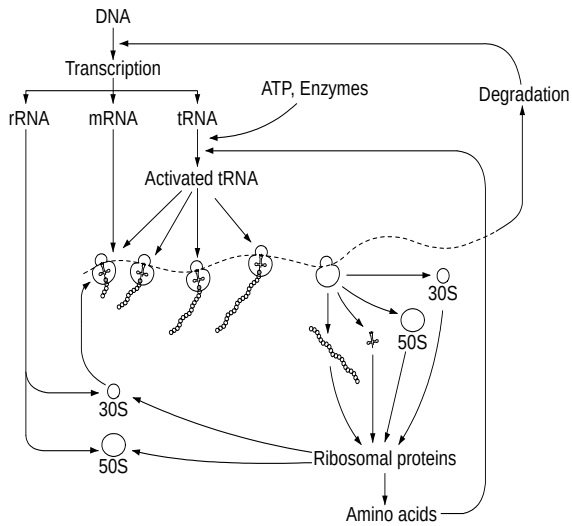


Fig. 8.26 Recapitulative diagram of transcription and translation

Note:

- Streptomycin affects S_{12} and causes misreading of *m*RNA
- Tetracycline inhibits binding of charged *t*RNA to the codon by binding itself to 30*S* ribosome
- Chloramphenicol binds to 50*S* and blocks transpeptidation
- Erythromycin inhibits translocation
- The above drugs affect only prokaryotes
- The fidelity in *E. coli* is 1 error in 2000

8.8 METABOLIC REGULATION

A living cell is in a *dynamic* state. For proper growth and maintenance, the cell requires a highly integrated coordination between *anabolic* and *catabolic* processes. Since the genomic DNA is the key to every expression in the cell, a highly efficient mechanism must be available to achieve the delicate balance of many processes. The mechanism is collectively called *gene regulation*. Although cells contain the genetic capacity to synthesize a large number of proteins, not all of these are present (or synthesized) at any given time. Many of these are synthesized only as and when required. The regulation may range from a modest repair of DNA to complex reactions. Besides, gene regulation is also responsible for orchestration and maintaining functional difference that exist in cells during development.

The control of metabolism in a cell depends on: (i) *Enzyme concentration*, (ii) *Substrate (co-substrate) concentration*, (iii) *Action of activators and inhibitors*, and (iv) *Modification of enzymes*.

The concentration of enzyme is controlled at two levels, *viz*., *transcription* and *translation*. The transcription is operated through protein factors which interact with particular nucleotide sequence (generally located upstream of genes) and the RNA polymerase itself. Modulation of transcription of a gene is performed by the joint action of RNA polymerase and a series of regulatory proteins (*activators, repressors, terminators, anti-terminators*), which in the presence of co-factors, interact with the DNA (or *m*RNA) at the level of particular nucleotide sequences (targets).

In the following paragraphs, regulation of protein synthesis (also enzyme synthesis) will be discussed. There are 6 points at which the amount of protein can be regulated, *viz*., (i) *synthesis of primary transcript*, (ii) *post transcriptional processing*, (iii) *mRNA degradation*, (iv) *translation*, (v) *post-translational modification of proteins*, and (vi) *protein degradation*. See Fig. 8.27 for the summary.

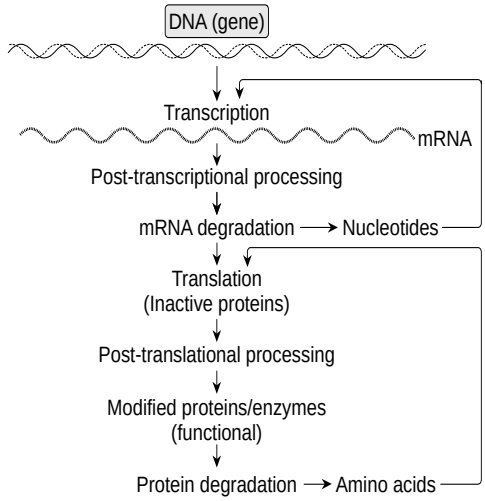


Fig. 8.27 Summary of regulation of protein synthesis

8.8.1 REGULATION OF GENE EXPRESSION

The synthesis of particular gene product is controlled by mechanisms collectively called *gene regulation*. Evidently, although cells have an enormous genetic capacity not all the proteins are synthesized all the time. Many of them are produced only on special occasions and in response to some environmental stimuli. For example, antibodies are produced in response to *antigens*. Such controlled and regulated synthesis of gene products leads not only to *cellular economy* but also to *homeostasis*. It is obvious that genes have myriad activities. Consequently, genes have been variously named:

- *Structural genes*: they code for enzymes, *t*RNA, *r*RNA, *m*RNA, proteins, indeed anything that has a structure
- *Regulatory genes*: they code for products that regulate expression by structural genes. They may or may not be located near the structural gene. Regulatory genes are usually not considered as part of *operon*
- *Architectural genes*: they are responsible for integration of proteins into the structure of the cell
- *Temporal genes*: they control the time and place of action of other genes and largely control the differentiation of the cells and tissues of the body

The regulation of metabolic process can be possible by regulation of enzyme concentration, coenzyme concentration, concentration of activator and inhibitor, and by varying the specific molecular activity of the enzymes by covalent modification of the proteins.

Exhaustive investigations have established that regulation of gene expression in all life forms may occur at three levels: (i) *transcription*, (ii) *translation*, and (iii) *post-translation*.

8.8.1.1 Regulation at the level of transcription

In bacteria, there occur several mechanisms of gene regulation at the level of transcription. A notable method depends on whether the enzyme being regulated act in *catabolic* or *anabolic* pathway. For example, in a multi-step catabolic system the availability of the molecule to be degraded commonly determines whether the enzymes of the pathway will be synthesized or not. In contrast, in biosynthetic pathway, the final product is often the regulatory molecule.

The molecular mechanisms for each of the regulatory patterns differ greatly and are of one of the two types: (i) *negative regulation*, or (ii) *positive regulation*.

In negative regulation, transcription is inhibited through the binding of an inhibitor molecule to the DNA. To remove the effect of this inhibitor molecule, an *antagonistic* molecule (called *inducer*) is required. Thus, the resumption of transcription requires neutralization of the effect of inhibitor.

In positive regulation, an affector molecule activates a *promoter* by binding to the DNA. See Fig. 8.28 for the illustrated explanation of positive- and negative regulations.

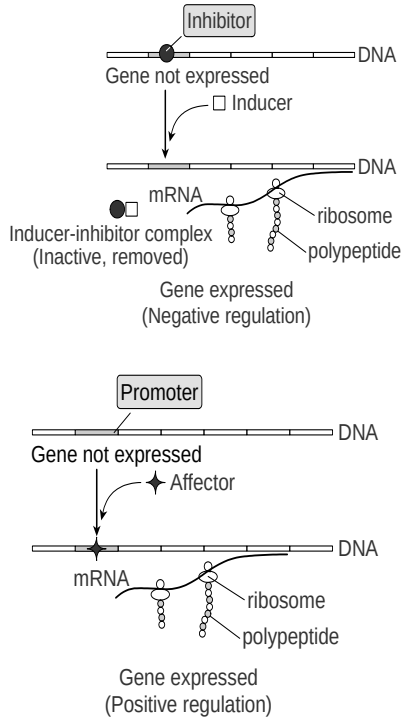


Fig. 8.28 Summary of Negative-and Positive regulation

The lac operon

lac operon is an example of negative regulation. Before we proceed, let us become familiar with some terminologies of operon.

- *Operon*: an operon is a group of coordinately regulated genes (i.e., polycistronic), the products of which typically catalyze a multienzymatic pathway and its controlling elements. An operon is operated through a common promoter.
- *Inducible genes*: they are genes used for the production of inducible enzymes when a specific inducer is present. For example, production of the enzyme *β-galactosidase* is induced by the presence of its substrate, lactose, in the medium. Ordinarily, when lactose is not supplied, the organism does not synthesize the enzyme: it would be a waste!
- *Constitutive genes*: these refer to prokaryotic genes whose expression is not regulated. The products of the genes are produced at a constant, often low, rate.

The purpose of lac operon

The purpose of *lac* operon in *E. coli* is to make enzymes needed to metabolize lactose. In *E. coli*, *lac* operon consists of two classes of genes, *viz*., *structural*- and *regulatory* genes. The schematic representation of *lac* operon in *E. coli* is given in Fig. 8.29.

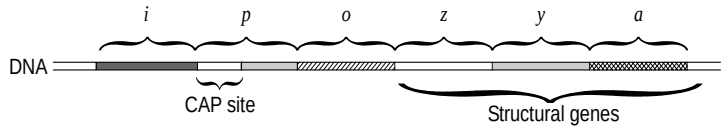


Fig. 8.29 Schematic representation of *lac* operon genes in *E. coli*

Notations:

i = *lac* repressor (regulator), which has its own promoter in DNA and SD sequence in *m*RNA
p = promoter
CAP site = site within the promoter needed for binding affector molecule for positive regulation (promotes transcription; see catabolite repression, page 76)
o = operator, the site where the repressor due to *i* gene binds for inhibiting transcription
z = structural gene that codes for β-galactosidase
y = structural gene that codes for galactoside permease
a = structural gene that codes for thiogalactoside transacetylase (uncertain function)
z, y, and *a* all have separate SD sequences with stop codons in between.

In the absence of lactose in the medium, *lac* operon does not function. This is because *lac* repressor synthesized by gene *i* tightly binds to the operator thereby blocking the transcription. *Lac* repressor is a constitutive molecule that is continuously expressed in low amounts. It is a diffusible *tetrameric* protein that diffuses along the DNA to reach the operator. It has one binding site per unit. The site is specific for inducer.

The presence of inducers such as *allolactose* (true inducer: see Fig. 8.31) or *isopropyl thiogalactoside* (*IPTG*, also referred to as *gratuitous inducer*: see Fig. 8.31) the negative regulation by *lac* repressor is relieved. Upon binding of the repressor and inducer to form *inducer-repressor* complex, the structure not only prevents the binding of other repressors to *o* site but also causes the ones already bound to fall off. The RNA polymerase is now free to move ahead for transcription. Lactose serves as an inducer indirectly, in the form of allolactose. A small amount of lactose is converted to allolactose form. With the depletion of lactose, allolactose will also deplete. The repressor again binds to the operator thereby quickly stopping the transcription. The unstable *m*RNA is now rapidly degraded. See Fig. 8.30 for detailed illustration.

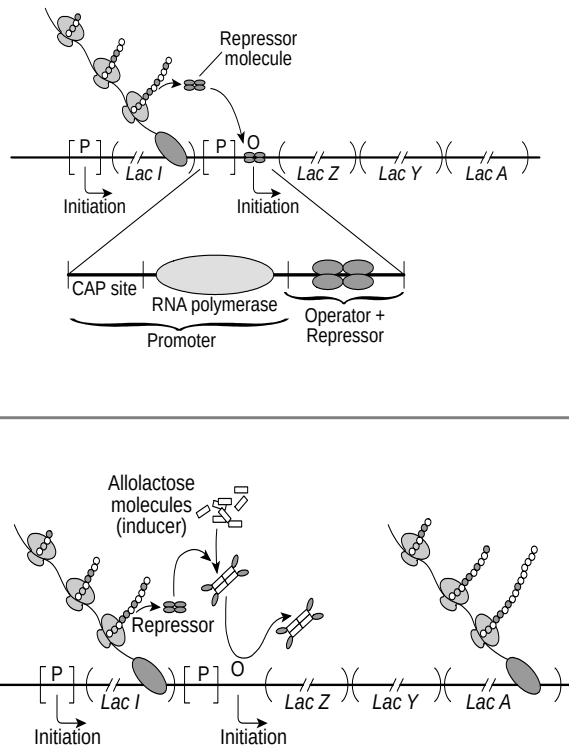


Fig. 8.30 Schematic representation of *lac* operon in *E. coli*

Catabolite repression (positive regulation)

When lactose is supplied to *E. coli*, lactose-degrading enzymes are rapidly synthesized (5000 per cell in several minutes). However, if glucose is supplied, the preference switches back to glucose. This switching to the preferred fuel is known as *catabolite repression*, as it is due to the catabolite glucose that repression has occurred. It is important to note, however, *the presence of glucose does not so much repress the expression of the lac operon as does the absence of glucose enhance the expression.*

Functioning of *lac* operon not only requires an inducer but also a *positive regulatory system*. The system consists of cAMP (cyclic AMP), a site within the promoter called CAP site, a CAP (*Catabolite Activator Protein: dimeric in nature*) factor, and CAP gene *remote* to the operon. CAP is a regulatory factor synthesized separately. cAMP is inversely related to glucose concentration. Depletion of glucose increases the level of cAMP, which then complexes with the constitutively produced regulator CAP to form cAMP-CAP complex. Upon binding of this complex to the CAP site, it stimulates β-galactosidase production by 50 fold.

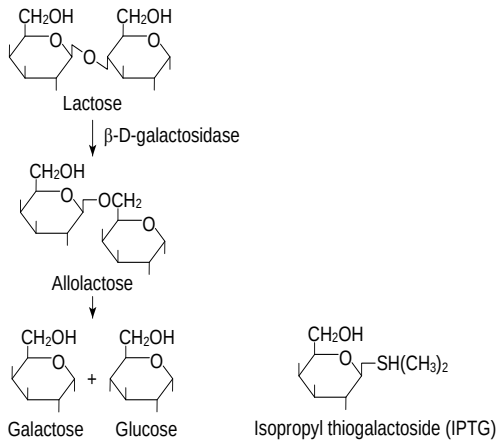


Fig. 8.31 Substrates of β-galactosidase

Tryptophan operon

The tryptophan operon in *E. coli* is responsible for the synthesis of the amino acid tryptophan. Regulation of this operon occurs in such a way that when tryptophan is present in the growth medium, *trp* operon is not active. That is, when adequate tryptophan is present, transcription of the operon is inhibited; however, when its supply is insufficient, transcription occurs. The *trp* operon is quite different from *lac* operon in that tryptophan acts directly in the repressor system rather than as an inducer.

Tryptophan synthesis is controlled by two mechanisms, *viz*., (i) *Negative repression by corepression*, and (ii) *Control by attenuation*. Brief descriptions of both the control mechanisms are given in the paragraphs to follow.

Negative regulation by corepression

The regulatory protein of the repressor system of the *trp* operon is the *trp* R gene product. This protein is called *trp aporepressor* and does not bind to the operator unless tryptophan is present. The *trp* R gene has its own promoter and is remote from the operon. When tryptophan concentration is high the same tryptophan functions as corepressor: it binds with the aporepressor (i.e., *trp* R gene product) to form an active *trp* repressor. The latter binds to the operator of the *trp* operon and blocks transcription by blocking the movement of RNA polymerase. This type of regulation by the cell is used for rough control of tryptophan synthesis. Fine control is possible with yet another mechanism called *attenuation.*

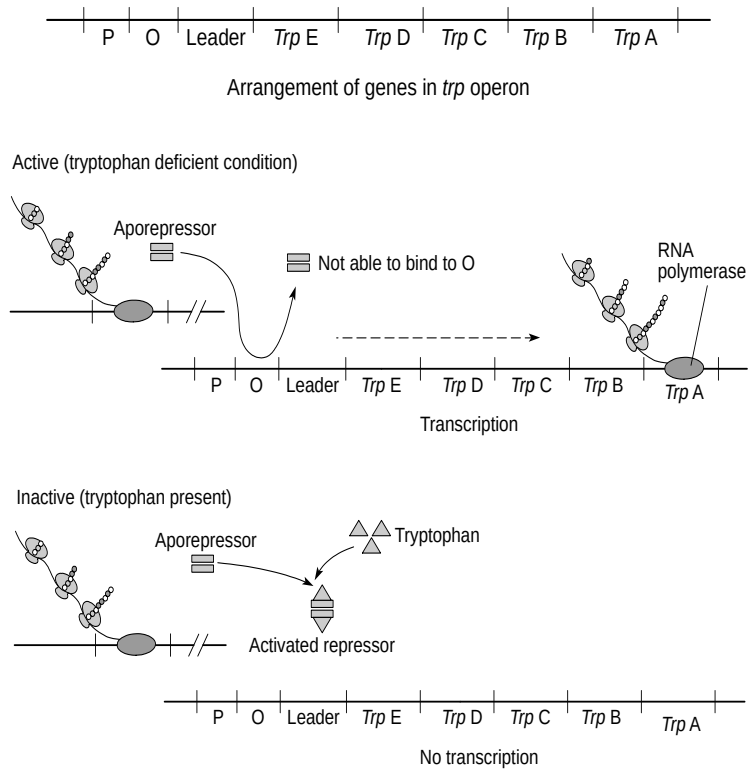


Fig. 8.32 Negative regulation by corepression

The structure of trp operon

Tryptophan is synthesized in five steps from *chorismic* acid, each requiring a particular enzyme. In *E. coli* chromosome the genes encoding these enzymes are located adjacent to one another in the same order as they are in the biosynthetic pathway; they are translated from a single polycistronic *m*RNA. The genes are called *trp* E, *trp* D, *trp* C, *trp* B, and *trp* A. They are structural genes. Just upstream the first structural gene (i.e., *trp* E), are attenuator (*trp* a), leader (*trp* L), operator (O), and promoter (P) genes. See Fig. 8.32 for detail.

This method of regulation can occur only in prokaryotes, because of the *coupling* of transcription and translation. The fundamental feature of attenuation is that, at low tryptophan concentrations, the operon makes full-length *m*RNA; but at high tryptophan concentrations, transcription is prematurely terminated.

The transcript of *trp* L has four complementary segments (1, 2, 3, and 4). Segment 2 is complementary both to 1 and 3 (see Fig. 8.33). Segment 3 is complementary to both 2 and 4. On this transcript, 3 types of hairpin loops (1:2, 2:3, 3:4) are thus possible. In *vivo*, however, one of the last two (2:3 or 3:4) occurs. This is because the ribosomes closely follow the RNA polymerase and segment 1 is not free from hairpin loop. The loop 3:4 acts as the transcription terminator because RNA polymerase cannot cross this loop. The transcription prematurely terminated in this way is called attenuation. Such a transcript has (in *trp* attenuation) an incomplete 140-nucleotide RNA transcript of L gene, which is eventually released.

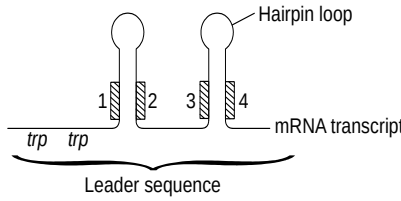


Fig. 8.33(a) Transcript of *trp* L

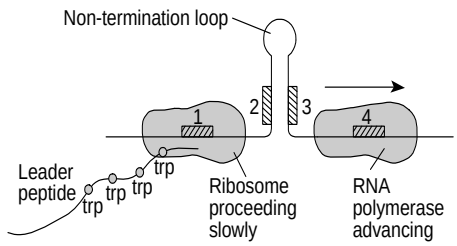


Fig. 8.33(b) Attenuation regulation at low tryptophan concentration

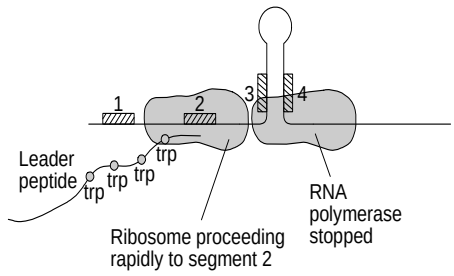


Fig. 8.33(c) Attenuation regulation at high tryptophan concentration

8.8.1.2 Translational control

In prokaryotic gene regulation at the translational level, the lifetime of *m*RNA molecule may be genetically determined. Enzymatic degradation of *m*RNA is from the 5'→3'. The average lifetime of many *m*RNAs of *E. coli* is only two minutes at 37°C. The specific nucleotide sequence at the 5' end may influence its susceptibility to enzymatic digestion. Further, catabolic enzymes are denied access to *m*RNA when the ribosome coats them. Hence, the lifetime of *m*RNAs may also be correlated with the number of free ribosomes available at any given moment to translate *m*RNA molecules. Bacteria vary their rates of protein synthesis by varying their ribosomal content rather than by varying the translational rate.

CHAPTER 9

CONCEPT OF FERMENTATION TECHNOLOGY

9.1 INTRODUCTION

The term *fermentation* came from the Latin verb 'fervere', which means *to boil*. It is clear now that the boiling impression was due to carbon dioxide bubbles quickly moving up to the surface of the liquid medium.

Fermentation has many definitions. In a biochemical sense, fermentation is an anaerobic process in which substrate is utilized for energy production without the involvement of molecular oxygen. In industrial microbiology, fermentation is simply a large-scale microbial process. This implies that the process can be both aerobic and anaerobic. Indeed, almost all of the industrial fermentations are highly aerobic processes Thus, production of beer, sake, *jand*, wine, penicillin, etc., all are examples of fermentation.

Fermentation process requires the presence of both the substrate and the conversion agent (microorganism and/or enzymes). For large scale production, the process also requires a large vessel for accommodating the organism and the substrate. For improved yield, a carefully controlled environmental- and cultural condition is required. Since the yield and productivity is a function of microbial property, selection and improvement of microbial strains has become a norm in industrial fermentations. The overall fermentation event can be presented as:

$$\text{Substrate} \xrightarrow{\text{Microorganisms or Enzymes}} \text{Products or Services}$$

9.2 FERMENTER AND FERMENTATION PROCESS

The tank or vessel in which industrial fermentation is carried out is called *fermenter* (*fermentor*) or *bioreactor*. It is a vessel/system which provides the organism with favorable place for multiplication and product formation. There are several types of fermenters, some of which you will come across in the paragraphs to follow. The spellings *fermenter* and *fermentor* are interchangeably used, although the term *fermenter* refers to (lexically) the causative agent of fermentation, viz., the microorganism. In industrial microbiology, many terms have contextual implications. For instance, when talking about microbial production of alcoholic beverages, the term *alcohol fermentation* does not mean the microbial conversion of alcohol into other products. Rather, it refers to microbial conversion of substrates into alcohol.

The terms fermenter and bioreactor are often used interchangeably. In a very strict sense, all fermenters are bioreactors but not all bioreactors are fermenters. Although biochemical conversion occurs in both of them, the term fermenter has been conventionally associated with product generation for human, animal, or plant use.

Bioreactor, on the other hand encompasses all the biochemical conversion processes, including those that generate service, e.g., waste treatment.

9.2.1 THE COMPONENT PARTS OF A FERMENTATION PROCESS

Regardless of the type of fermentation (with possible exception of some transformation processes) an established process may be divided into six basic component parts (see Fig. 9.4 also), namely:

1. Formulation of the medium for culture propagation (inoculum build up) and the main fermentation
2. Sterilization of the medium, fermenter, and ancillary equipment
3. Production of active pure culture in sufficient quantity to inoculate the production vessel
4. Growth of the microorganism in the production fermenter under optimum condition for the product formation
5. Extraction of product and its purification
6. Disposal of effluents produced by the process

9.2.1.1 Medium formulation

Formulation of medium is a very important part of the fermentation process. The composition of media for pre-fermentation is quite different from that for main fermentation. The pre-fermentation stage implies propagation of the pure culture from the stock culture (which may be in slants, vials, flasks, plates, etc.). This stage is also known as inoculum build-up (see later). The inoculum build-up phase is concerned with rapid propagation of the cells and so the medium is generally richer in nutrient level.

The main fermentation is concerned with accumulation of the desired metabolite/product. In most cases, the organism does not accumulate the metabolite in very rich growth medium. Sometimes they have to be starved and sometimes some key compounds must be added or removed from the medium to force the organisms to synthesize the metabolite. This is where the importance of medium formulation comes in. For this reason, industries spend a good amount of resources on medium formulation. The findings from the researches have been very useful for fermentation industries. Several excellent examples of medium formulation (e.g., for glutamic acid, citric acid, tetracycline, etc.) have been given elsewhere in this book (pages 328, 319, and 289).

9.2.1.2 Sterilization and asepsis

Microbiological processes cannot run successfully if contaminations occur. Contaminants may come from various sources and due to various reasons. Therefore all the potential sources of contamination must be sterilized prior to fermentation.

Sterilization refers to freedom from all life forms. This can be achieved by physical means (heat, radiation, filtration, etc.) or with chemical agents (e.g., sodium

hypochlorite). In almost all cases, the medium used for the fermentation should be either pasteurized or sterilized. Very often, the fermenter itself and the ancillary parts must be sterilized. The air that is to be supplied during the fermentation should also be sterile. Some "protective" fermentations (e.g., alcohol and wine production) may not have stringent requirements for the main fermentation but there are many fermentations which are very sensitive to contamination.

Medium is usually sterilized in batch or continuous process. The continuous process normally utilizes plate-heat exchangers or flash sterilizers, using hot water or steam as the heating agent. Air sterilization can be done by passing through filter beds of glass wool, fiber, etc., of 5-15 μm pore size. The packing material gets contaminated in due course and this is rendered sterile by injecting steam (Fig. 9.1).

Asepsis refers to measures adopted to create a germ-free condition. In microbiology, this relates to prevention of contamination of the sterilized material. In fermentation industries, aseptic techniques are used to avoid contamination during subculturing, inoculum build up, fermentation, and sampling. A few examples of aseptic techniques are described in the following paragraphs.

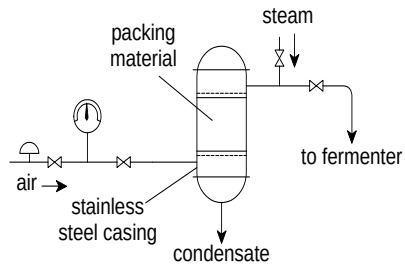


Fig. 9.1 Sterilization of air

Aseptic sampling

During fermentation, samples are regularly taken out of the fermenter to monitor the progress of the process. This interruption can result in contamination of the fermentation medium if aseptic techniques are not used. The pipelines and valves for handling sample should be rendered sterile both before and after sampling. Fig. 9.2 gives an example of the arrangement for aseptic sampling.

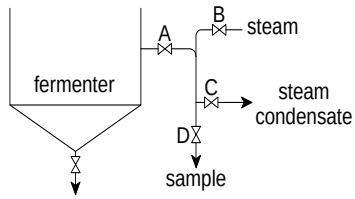


Fig. 9.2 Protocol for aseptic sampling

During the normal fermentation process, valves A, B, C, and D remain closed. When sampling is to be done, valve B is opened to let in steam through valves C and D. After passing steam for a specified period, the valves are again closed. Valve A is now opened and the sample allowed to fill up the space between the valves A, B, C and D. Valve A is now closed and then valve D opened to receive the sample. After receiving the sample, the pipeline is sterilized once again as described before.

Aseptic inoculation

Inoculum for the final fermentation is prepared separately and aseptically. Once prepared, it is transferred to the main fermenter aseptically so that the final fermentation remains as contamination-free as possible.

Usually, the final inoculum tank is directly linked to the main fermenter and the transfer takes place under pressure created by air supplied to the inoculum tank. There are provisions also for steam-sterilizing the pipelines. See Fig. 9.3 for an idea.

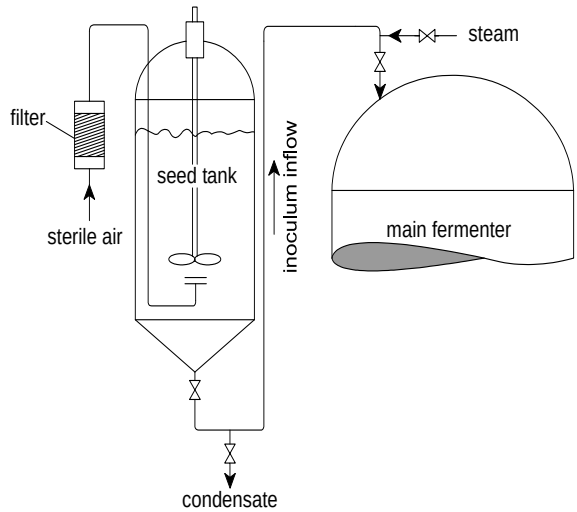


Fig. 9.3 Protocol for aseptic inoculation

9.2.1.3 Inoculum build-up

The preparation of a population of microorganisms from a dormant stock culture to an active state of growth that is suitable in the final production stage is called *inoculum development* or *inoculum build up*. Inoculum build up generally starts in flask cultures (called *shaker flask* or *shake flask*, Fig. 9.4).

The culture is grown in a rich medium for 2 to several days under aerobic condition. Thereafter the culture is transferred aseptically to a bigger vessel for propagation. Once again growth takes place for 2 to several days. The transfers are continued for a few more times until the desired amount of inoculum is produced. At each step, the volume is increased by 20-200 times. The culture should be in log phase. Everything must be carried out aseptically. As mentioned earlier, the medium for

inoculum build up is quite different from the one used for the final fermentation. You will find several examples of inoculum build up and medium formulation in the later chapters (pages 151, 217, 279, etc.)

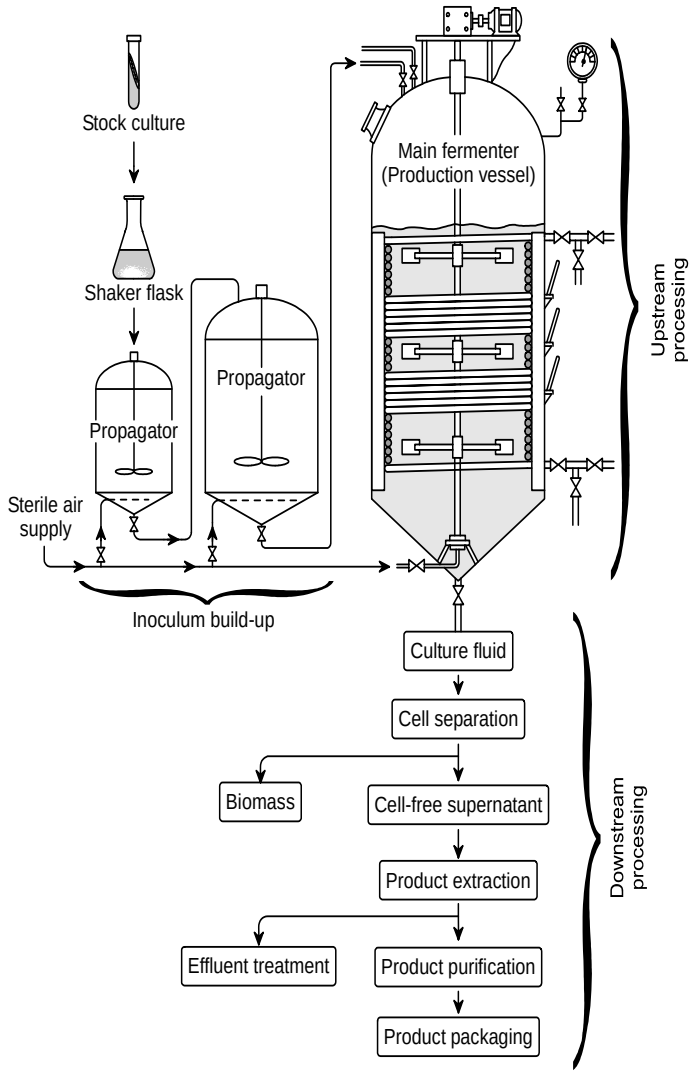


Fig. 9.4 The component parts of a fermentation process

9.2.2 TYPES OF FERMENTERS / FERMENTATION PROCESSES

Various schemes have been used for classifying fermentation process. Some of the commonly used schemes and the classifications are:

- Aerobic and Anaerobic
- Batch, Continuous, and Fed-batch
- Solid state culture (= Surface culture) and Submerged culture

You will see considerable overlapping in the above schemes. For example, solid state (also called *solid substrate*) and surface culture are batch processes while submerged fermentation can be either batch or continuous. It must also be noted that there are several variations in each category. For example, a continuous fermenter can be of tower type, cascade type, air-lift type, etc. A brief description of basic types of fermentation processes is given in the following paragraphs.

9.2.2.1 Solid-state fermentation

It is a fermentation in which the microbial growth occurs on moist, non-soluble substrate in the absence or near-absence of free-flowing water. The solid substrate acts as a source of carbon, nitrogen, minerals and a growth surface which absorbs water necessary for microbial growth. In addition, the solid substrate provides anchor points for the growth and propagation of microorganisms. Solid state fermentation (also called *koji* process, see later) is a highly aerobic process. The microorganisms get their nutrients by hydrolyzing (breaking down) the substrates. In other words the microorganisms are good producers of extracellular (i.e., produced and sent out of cell) hydrolytic enzymes. Most indigenous fermented foods and a few enzymes are produced by this method. Some of the important examples of solid-state fermentation are mushroom cultivation, *jand, kinema, natto, sauerkraut, tempeh, sake,* and *miso* production. All solid-state fermentations are batch processes. The process cannot handle more than about 1000 kg per batch. This is because the medium is not liquid and the control of temperature, pH, nutrient distribution, and aeration is very not easy.

The solid substrate chosen are usually wheat, maize, soybean, rice, and wheat bran. The substrate is initially rendered sterile either by autoclaving or cooking and cooled before inoculating with the organism. The inoculum is prepared aseptically separately. When the inoculum is a mold, it is called *koji*. The substrate, which may or may not need to be supplemented, serves as a rich source of nutrients. Such substrates support the growth of mycelial organisms which can grow at high nutrient concentrations and produce a variety of extracellular enzymes.

The fermentation may take place in trays (rotary or stationary), compartments, or even in a room. When large-scale production is used, temperature and humidity is controlled by passing conditioned air. Some examples of solid-state fermentation are given in later sections.

The main advantages of solid substrate fermentation are:

1. Low cost (due to simple device and less manpower)
2. Readily carried out on home scale
3. Reduction of the fermentation- and liquid effluent volumes
4. Reduced risk of bacterial contamination because of low moisture level

The limitations of solid substrate fermentation are:

1. Difficult to scale up

2. Difficult to control and monitor different factors, e.g., pH, temperature, nutrient distribution, etc.
3. Gas exchange is difficult (O_2 supply and CO_2 removal, that is)
4. Problem of heat exchange

More recently, solid substrate fermentations have been used to produce extracellular enzymes, fungal toxins, and fungal spores.

9.2.2.2 Submerged fermentation

It is a process in which organisms are forced to grow in a submerged state (that is, state in which cells are dispersed in liquid medium). Submersion is carried out using suitable mixing device or technique. The oxygen required by the microorganism is supplied by passing forced air through the medium. Thus, although the microorganisms are aerobic, they do not experience lack of oxygen. The advantages of submerged fermentation are:

1. Can be operated in large volumes and even in a continuous mode
2. Temperature, pH, oxygen concentration, etc., can be closely controlled

Some of the examples of submerged fermentation are: production of beer, vinegar, ethanol, baker's yeast, etc. For beer and ethanol, only the initial phase is truly submerged. During the latter part, fermentative metabolism is maintained simply by not supplying oxygen. The organism quickly consumes the available oxygen and then shifts the metabolism toward ethanol production. The intimate mixing is possible even without an agitator. The CO_2 evolved during alcohol generation rises to the surface and sets the whole broth in continuous motion.

9.2.2.3 Batch process

This is the simplest type of culture in which microorganisms grow in a vessel with limited amounts of nutrients under optimum environmental condition. Unlike continuous culture, the microorganisms pass through all stages of microbial growth cycle, *viz.*, lag phase, log phase (exponential phase), stationary phase, and decline phase, exhibiting a sigmoid growth curve (Fig. 9.5). Completion of each cycle constitutes a batch. The downstream processing (Fig. 9.4) is done at the end of each cycle. The vessel is then prepared for receiving the next batch. This preparation requires certain time period, called *down time*, which is equivalent to unproductive period. This is what makes batch process less efficient than the continuous process.

9.2.2.4 Continuous fermentation

In this, the microorganisms are maintained in exponential phase (log phase) so that the growth remains constant throughout the fermentation period (which may even run for years). This is possible by continuous feeding (of fresh medium) and withdrawal (of finished product). Both these activities need very delicate balance. You will find several examples of continuous fermentation (See microbial production of ethanol, page 219; and semisynthetic penicillins, page 133).

Continuous process is far more efficient and economical than any other processes because:

1. There is no down time as in batch process
2. Since the culture is in log phase there are no lag-, stationary-, or decline phases
3. The process can be automated, which means less manpower is needed

There are certain limitations, however. For example, since the process runs for very long periods of time contamination can be a problem. Besides, the source of raw material must not dry up.

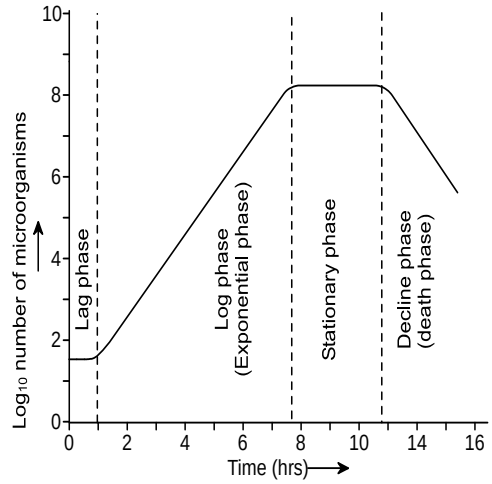


Fig. 9.5 The bacterial growth curve

9.2.2.5 Fed-batch fermentation

Basically, it is a batch culture that is fed continuously with fresh medium without simultaneous removal of the original culture medium from the fermenter. This results in continuous increase in volume of the medium in the fermenter. The feeding and growth rate is adjusted such that the fermentation is complete by the time volume reaches the predetermined level. This method is used when high concentrations of nutrients turn out to be counter-productive due to shift in the pattern of metabolism, catabolite repression, etc. Bakers yeast is produced by this method (see page 153).

9.3 FERMENTER DESIGN

The basic function of a fermenter is to provide a controlled environment for the growth of a microorganism or a defined mixture of organisms to obtain the desired product. This is where development and design of fermenter comes in. Let us now look at some important aspects fermenter design.

The problem

1. Selection of the best type of reactor for the particular reaction
2. Determination of the best operating conditions

The objective of design

The objective of design is to be able to describe the effects of operating conditions on the performance of a bioreactor and to compare alternative designs with economic criteria. For this, an in-depth knowledge of (and experience in) microbiology, biochemistry, thermodynamics, microbial and biochemical kinetics, fluid mechanics, mass and heat transfer, and economics is essential. Even with such knowledge and experience an ideal fermenter can rarely be made. Fermenter designing, ultimately, is a matter of compromise.

The designer's job

The designer's primary job is to construct, at the lowest possible cost, a fermenter, and design in features such that control will be possible over reasonable ranges of the important process variables (e.g., oxygen concentration, temperature, pH, etc.) and that the operation will be reliable and contamination-free. To achieve these ends, he must, at the minimum provide for:

1. Adequate heat and oxygen transfer
2. Aseptic and sterilization procedures
3. Reliable foam control
4. Good spatial definition of environmental conditions
5. Simple, rapid, and thorough cleaning systems
6. Responsive, reliable, and appropriate monitoring and control systems
7. Appropriate materials for construction and reliable fabrication methods
8. Possibility that the fermenter will be used for more than one product (flexibility)

9.3.1 FERMENTER CONFIGURATIONS

There are different types of fermenters. The structure and design of fermenter is called 'fermenter configuration'. Fermenters vary both with respect to configuration and capacity. In the former case, the primary variations are in geometric ratios and the types and number of impellers used. There are many fermenters which cannot be covered by general rule (e.g., tower fermenter, air-lift fermenter, tubular fermenter, etc.). Generally, the design decisions are made on the basis of conventional 'wisdom', professional advice from equipment manufacturers, personal experience, and hearsay. Stated differently, it is very difficult to design a fermenter. Its designing needs the involvement of microbiologists, engineers, and technologists. A very common fermenter type is described next.

9.3.1.1 The stirred tank fermenter

The stirred tank fermenter (STF) is used almost universally in fermentation industries. The capacity of the fermenter ranges from 100,000-500,000 gallons (1 US gallon = 3.79 liters, 1 imperial gallon = 4.55 liters). A typical stirred tank fermenter (batch) is shown in Fig. 9.6.

Description of parts

Sight glass: This is used to view what is inside the fermenter. A powerful lamp placed on the opposite side of the fermenter provides light for viewing.

Agitator/impeller: This is a motor-driven mixing device that agitates the liquid medium. Agitation fulfills several functions. It helps breakdown the air bubbles, distributes the nutrients uniformly, and helps remove the heat developed during fermentation.

Cooling/heating coil: This is used for removing or adding heat to the fermenter. To remove heat, cold water is passed from the lower end. To add heat, steam is passed from the upper end.

Baffle: This is placed against the wall of the fermenter. During agitation, the liquid tends to form *vortex* if there is no baffle. Baffle works by foiling formation of vortex. It also helps in better mixing of liquid. Usually, a small gap is left between the wall and the baffle so that the liquid can pass through it. This gap helps in automatic cleaning of the wall of the fermenter by an action referred to as "scouring". A fermenter has at least four baffles.

Steady bearing: This is used to hold the shaft that carries the impeller.

Air duct/pipe: This is used for supplying air in the medium.

Inoculum port: This is used for the introduction of culture

Feed port: This is used for introducing the fermentation medium (= feed).

Pressure gauge: This is use for measuring the air pressure inside the fermenter.

Temperature probe: This is used for continuous measurement and monitoring of temperature of the medium.

pH probe: This is used for the continuous measurement and monitoring of pH of the medium

Dissolved oxygen probe: This is used for the continuous measurement and monitoring of dissolved oxygen concentration of the medium

9.3.1.2 Fabrication of stirred tanks fermenters

The material for construction should withstand sterilization. Mild steel fermenter is often used. For 300,000-400,000 liter capacity, 7 mm plates may be used for the sides of the vessel and 10 mm plate for the top and the bottom. The top (and sometimes bottom) of the vessel should be hemispherical to withstand internal pressure. The shape of the vessel is almost always cylindrical. Quite often, the bottom section is made conical to facilitate product removal. In certain fermentations, wooden, concrete or plastic fermenters are also used.

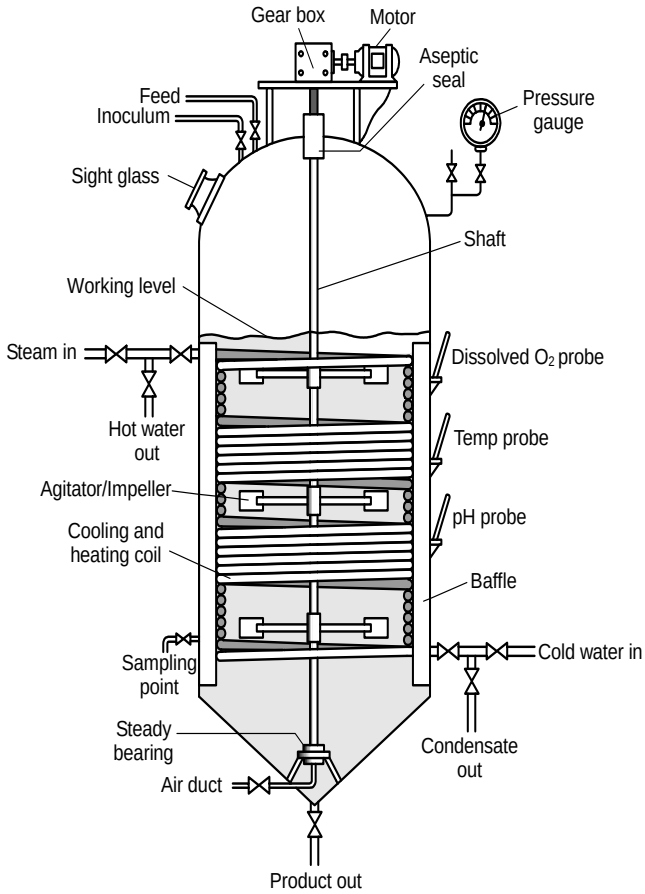


Fig. 9.6 A typical batch, stirred tank fermenter

9.3.1.3 Geometric ratios of stirred tanks fermenters

Geometric ratios are very important in the construction of a fermenter. For a stirred tank fermenter, the commonly used ratios are Liquid height/Tank diameter, Impeller diameter/Tank diameter, Baffle width/Tank diameter, and Impeller height/Tank diameter, etc. Typical data of the ratios and other related details of a stirred tank reactor are given below. See Fig. 9.7 for the notations used in the data.

Operating volume = 170 dm^3	P/V = 0.74
Liquid height, (L) = 150 cm	P/W = 0.77
Liquid height/Tank diameter, (L/D) = 1.7	P/Y = 0.77
Impeller diameter/Tank diameter, (P/D) = 0.33	P/Z = 0.91
Baffle width/Tank diameter = 0.0.098	H/D = 2.95
Impeller height/Tank diameter = 0.37	

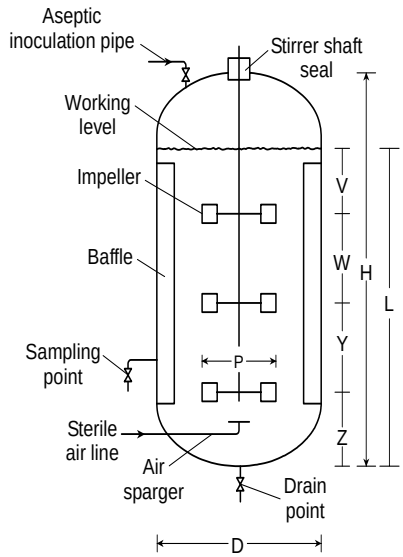


Fig. 9.7 Typical dimension for a stirred tank fermenter

9.4 AERATION AND AGITATION

The purpose of aeration in a submerged fermentation is to supply adequate oxygen to the microorganisms. Oxygen is supplied to the submerged fermentation in the form of sterile air. The aeration rate is in the range 0.25-2.0 vol/vol/min.

Agitation serves a double purpose, namely:

1. Diminishes the size of air bubbles to give a bigger interfacial area for oxygen transfer and to decrease the diffusion path
2. Maintains a uniform environment throughout the vessel content

Based on the method of aeration and agitation, fermenters can be classified as (i) mechanically agitated, and (ii) non-mechanically agitated.

Baker's yeast production is a very good example of non-mechanically agitated fermentation (see page 154). In it, the air passing into the fermenter itself agitates the medium. In such fermenters, the ratio of height to diameter is usually not greater than 5:1. The type of aeration-agitation system used in a particular fermentation depends on the characteristics of fermentation process under consideration. The mechanically agitated aeration system is usually required in fungal and actinomycetous fermentations. Such fermentations develop very complex rheology as the fermentation progresses (due to microbial growth) and may require specially designed impellers.

9.4.1 COMPONENTS OF MECHANICALLY AGITATED SYSTEMS

9.4.1.1 The impeller

There are four basic designs of impellers, *viz.*, (i) Disc turbine, (ii) Marine propeller, (iii) Open turbine, and (iv) Vaned disc (see Fig. 9.8). The most widely used impeller is the open-turbine (= flat-bladed turbine). It can break up a fast air stream without itself becoming flooded with air bubbles. Ideally, the impeller should be 1/3 to 1/2 D_t (tank diameter) above the base.

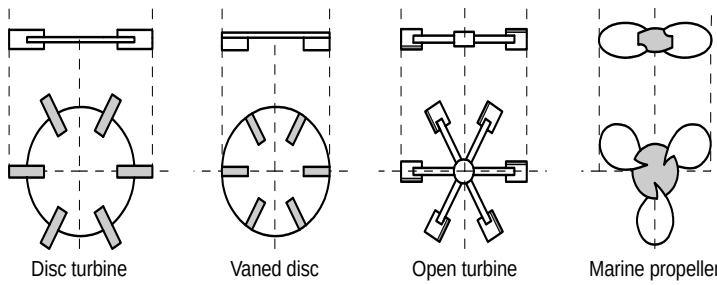


Fig. 9.8 Different types of impellers

9.4.1.2 Stirrer glands and bearings

These are required for the satisfactory sealing of stirrer shaft assembly. Most industrial fermenters have mechanical seal (see Fig. 9.9 for an over-simplified schematic). This seal is composed of two parts, one part is stationary in the bearing house and the other one rotates along with the shaft. The two components are pressed together with expanded bellows. The meeting surface should be precision-machined. Steam condensate is used for the lubrication of mechanical seal.

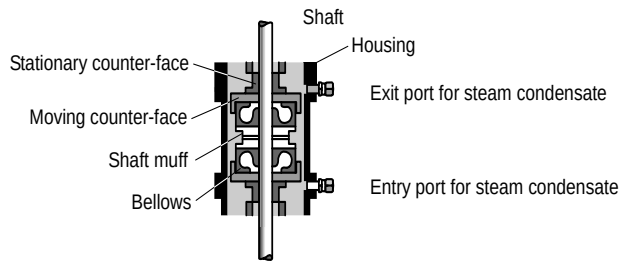


Fig. 9.9 Schematic drawing of mechanical seal

9.4.1.3 Baffles

Baffles improve aeration and prevent vortex formation. Four baffles are normally needed. A small gap is needed between the baffle and the vessel wall for scouring action (which enables self-cleaning of the walls of the vessel). See Fig. 9.5 and 9.7 for an idea about baffles.

9.4.1.4 The air sparger

It is a device for introducing air into the medium in the fermenter. The two most widely used types of spargers are: (i) Nozzle sparger and (ii) Orifice sparger.

Nozzle sparger consists of a single open or partially closed pipe for providing stream of air bubbles. Ideally, the pipe should be positioned centrally below the impeller and as far away as possible to ensure that the impeller is not flooded with air bubbles. See Fig. 9.10 for the schematic drawing.

Orifice sparger system is widely used in yeast production where mechanical agitation is not done. The air sparged in the medium does the mixing. For example, baker's yeast production in a 200 m^3 vessel uses orifice assembly with 24 side tubes containing in all 30000 holes of 1.5 mm diameter each. See Bakers yeast production for the schematic drawing (page 154).

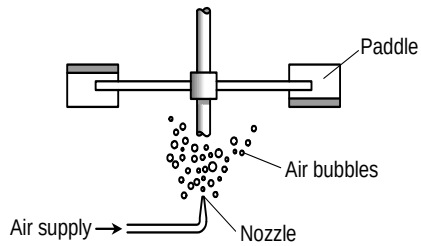


Fig. 9.10 Schematic drawing of nozzle

9.4.1.5 Cooling

Since fermentation is a metabolic process, large amount of heat is generated. This is undesirable for obvious reasons. Internal cooling coil is most satisfactory for faster cooling. Agitator has an important role here too. When cooling coils are used, 50-70 m^2 may be taken as an average contact area for a 55000 dm^3 fermenter.

9.4.1.6 Foam control

Foam is an undesirable aspect. Foams are generated primarily due to denatured microbial- and other proteins. Foaming reduces the vessel capacity and thus the productivity. Foams may also enter the various entry ports of the fermenter if left uncontrolled. This entry in due course leads to contamination. Besides, the product also oxidizes due to increased exposure to air. Foam can be controlled by two main methods, namely, (i) mechanical defoaming, and (ii) chemical defoaming.

Mechanical defoamers rely on discs, propellers, brushes, etc., for defoaming (see vinegar production, page 264 also). In the chemical defoaming method, antifoams such as alcohols, fatty acids, esters, silicones, sulfonates, etc., are added in controlled amounts. They all work by reducing the surface tension and thus suppressing the foam. In automatic control systems, sensing probes are inserted at a suitable distance

above the working level. As soon as the foam touches this probe the electrical circuit closes and this activates systems for the introduction of chemical antifoams.

9.5 BASIC VARIABLES FOR MONITORING FERMENTATION

The most important variables that need control during fermentation are:

1. Temperature
2. Agitation rate
3. Aeration rate
4. Dissolved O_2 activity
5. pH
6. O_2 and CO_2 partial pressure in the exhaust gas

9.5.1 MEASURING / SENSING DEVICES

Temperature

Thermocouples are the cheapest but do not have resolution. Resistance Temperature Detector (RTD, made of platinum) is probably the best for critical applications but it is very expensive. The principle involved in the latter instrument is that electrical resistance changes with change in temperature. The accuracy is ± 0.25%.

Aeration

Rotameters and thermal mass flow meters are used for monitoring the supply of air. Rotameter is a very simple air flow measuring device. It consists of a vertically mounted glass tube with an increasing bore and enclosing a free-moving float (Fig. 9.11 gives an idea about the principle on which the rotameter works). The position of the float in the graduated glass tube is indicative of the flow rate.

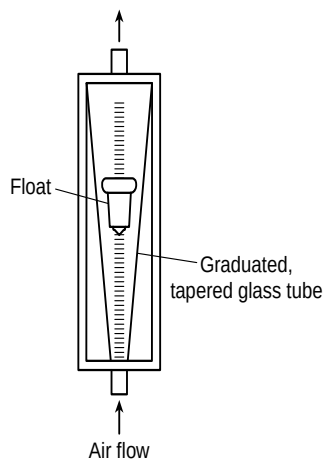


Fig. 9.11 Working principle of rotameter

Agitation rate

The rate of rotation is sensed by tachometers

Dissolved O_2

Electrodes are used for the continuous measure of dissolved O_2. Oxygen selectively diffuses through the membrane and produces signal. The current is directly proportional to the activity of O_2 in the broth but not to the concentration.

Pressure

Normally, pressure is measured by Bourdon tube gauges, which in turn are of different makes.

pH

Electrodes of various types can be used for the continuous measurement of pH.

9.6 FERMENTER SCALE UP

Determination of optimum condition for fermentation, e.g., medium requirements, aeration, agitation, temperature, pH, duration, etc., using a microbe producing a metabolite is called process development. Consideration of cost, labor, time, and space make it mandatory to carry out the process in 3 distinct stages: (i) laboratory process using flasks, (ii) scaling up using small to medium fermenters and, finally, (iii) production scale fermentation experiments.

The information gained from small fermenter is used to predict/determine the proper fermentation conditions for the large fermenters; this is called scale-up of the process. The use of small fermenters saves cost (media, etc.), labor, time, etc., allows replicated studies and keeps the production fermenters free for operations. However, the information generated from small fermenters is not entirely applicable to production fermenters. As a result, some production-scale experiments are always required at the time of process development and later during production. In such cases, a pilot fermenter may be included.

Scale up is extremely important yet one of the most complicated aspects of industrial microbiology. An understanding of the problems of scale up is extremely important because rarely does a microbial process behave the same way in large-scale fermentation as in small scale laboratory equipment. In industrial fermentation, mixing and aeration becomes complex. As the size of the fermenter is increased, the surface-to-volume ratio also changes. Scale up of an industrial process is, therefore, the task of the biochemical engineer, who is familiar with gas transfer, fluid dynamics, mixing, and thermodynamics.

CHAPTER 10

BASICS OF ENZYME TECHNOLOGY

10.1 INTRODUCTION

Enzymes are biocatalysts that catalyze (govern, initiate and control) biochemical reactions with specificity and at a rate compatible with cellular reactions. They are produced by living cells but can act independently of cell if appropriate environmental conditions are created. Almost all enzymes are proteins. As of now, however, partly due to the discoveries of enzyme-like compounds that defy the conventional concept of enzymes in properties and makeup, other newer terms have evolved. To this end, several non-protein molecules can also carry out reactions similar to enzyme-catalyzed reactions. Some of these molecules are *ribozymes*, *synzymes*, etc. There are also quite a few non-traditional enzymes, e.g., *extremozymes*, *abzymes*, etc. A brief treatment of the abovementioned *new* enzymes will shortly follow but it must be noted here that some enzymes are active only when *coenzymes* (cofactors) are present. Such *incomplete* enzymes are called *apoenzymes*. The fully functional form (after combining with coenzyme) is then termed *holoenzyme*. Almost all vitamins of the B group function as coenzymes. The illustration of coenzyme-requiring enzyme is given in Fig. 10.1.

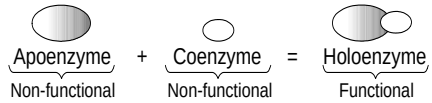


Fig. 10.1 Combination of apoenzyme and coenzyme to make holoenzyme

It must be clear from the very beginning that an enzyme *cannot initiate* a reaction that cannot occur spontaneously. It also does not alter the *overall free energy change* of the reaction.

10.1.1 ABZYMES

Abzymes are antibodies that function as enzymes. They are also called *catmab* (referring to catalytic monoclonal antibodies). Antibodies, by definition, have evolved to recognize and bind to the ground states of the molecules they are specific to. In contrast, enzymes have binding sites that preferentially bind to the transition state of their substrate molecules. A catalytic antibody is produced in response to molecules that have a structure similar to the proposed/expected transition state of the reaction to catalyze which the antibody is sought.

Abzymes are usually artificial constructs but naturally occurring abzymes have also been observed in normal individuals (e.g., anti-vasoactive intestinal autoantibodies) and individuals with autoimmune problems.

10.1.2 RIBOZYMES

These are RNA molecules that have catalytic power. Ribozymes are so far known to catalyze only two reactions: (i) *cleavage of RNA*, and (ii) *cleavage of DNA*. The catalytic power of ribozymes is due to their 3-D structures, which are able to generate in them substrate-specific binding sites.

10.1.3 EXTREMOZYMES

These are enzymes that function optimally only under extreme conditions of temperature, pH, etc., e.g., DNA polymerase from *Pyrococcus furiosus* that has half-life of 20 hrs at 95°C and functions optimally at 90°C.

10.1.3 SYNZYMES

These are generally synthetic polymers, sometimes proteins, which have enzymatic activities. Synzymes must possess two functional sites: one for substrate binding and the other for catalysis. Cyclodextrin is a non-protein molecule in which 6, 7, 8, 9, or 10 α-1,4-linked D-glucose residues are joined head to tail in a ring (called α-, β-, γ-, δ-, and ε-cyclodextrins respectively). When pyridoxal coenzyme is attached to C_6 hydroxyl group of β-cyclodextrin, it acts as a natural transaminase.

10.2 CLASSIFICATION AND NOMENCLATURE OF ENZYMES

Enzymes can be classified according to various schemes, such as:

1. Substrate acted upon

Table 10.1 Examples of enzymes whose names are derived from the substrate acted on

Substrate	Enzyme
Protein	Protease (proteinase)
Carbohydrate	Carbohydrase
Lipid	Lipase
Penicillin	Penicillinase
Sucrose	Sucrase
Polyphenol	Polyphenolase

2. Type of reaction catalyzed

Table 10.2 Examples of enzymes whose names are derived from the reaction they catalyze

Reaction type	Enzyme
Isomerization	Isomerase
Oxidation	Oxidase
Dehydrogenation	Dehydrogenase
Hydrolysis	Hydrolase
Aldolization	Aldolase
Transamination	Transaminase

3. The IUB system

This system of classification takes into account the overall chemical reaction. Although complicated, the IUB (International Union of Biochemistry, 1961) system is precise, descriptive, and informative. Enzymes are classified and named by the Commission on Biochemical Nomenclature. All the enzymes are classified into 6 groups; subclasses also occur. Each enzyme is given a code number, the interpretation of which gives many details regarding the enzyme. An enzyme may be denoted in one of the following three accepted ways: (i) a *four-number* code following the letters EC (for *enzyme commission*), e.g., *EC 3.2.1.26* (this is for *invertase*: the first number refers to enzyme class, the second to subclass, the third to sub-subclass, and the fourth to serial number of the enzyme within a subclass), (ii) *its systematic name* based on the above classification, e.g., *β-D-fructofuranoside fructohydrolase* for what is commonly called *invertase* or even *sucrase*, or (iii) *its recommended name*, e.g., *β-D-fructosidase* (for invertase, that is). The classes and their examples appear in Table 10.3.

Table 10.3 Classes and examples of enzymes (IUB system)

S.N.	Class	Common example
1	Oxidoreductase	Dehydrogenase, oxidase, oxygenase
2	Transferase	Transaminase, kinase
3	Hydrolase	Lipase, peptidase, phosphatase
4	Lyase	Decarboxylase, hydratase
5	Isomerase	Phosphohexoisomerase, mutase
6	Synthetase/Ligase	Acetyl-ScoA synthetse, glutamine synthetase

Hydrolases are the most commonly used enzymes, accounting for nearly 80% of all commercially produced enzymes. A major share of it constitutes proteases (used in detergent, dairy, meat and leather industry) followed by carbohydrases (28%), and

lipases (5%). The usage of enzymes in various industries is as follows: detergents (34%), dairy-related uses (14%), starch processing (12%), textile applications (11%), beverages and brewing (7%), animal feed (7%), bakery (5%), and others (9%).

10.3 CATALYTIC POWER OF AN ENZYME

The *catalytic power* of an enzyme is measured in terms of activity. Some of the terms used for this are *turnover number*, *enzyme unit*, and *specific activity*.

1. The turnover number is defined as the number of substrate molecules converted into product per unit time when the enzyme is fully saturated with substrate. The values of turnover vary widely with different enzymes and depend on conditions in which the reaction is taking place. However, for most enzymes, the turnover numbers fall between 1-10^4/s. The turnover number of 6×10^5 per sec for carbonic anhydrase is one of the largest known.
2. The *enzyme unit* is the amount of enzyme which will catalyze the transformation of one mole of substrate per minute under defined conditions.
3. *Specific activity* is expressed as units of enzyme per milligram of protein.

10.4 FACTORS AFFECTING ENZYME ACTIVITY

The main factors that affect enzyme-catalyzed reaction are (i) *temperature*, (ii) *pH*, (iii) *enzyme concentration*, (iv) *substrate concentration*, (v) *presence of inhibitors*, (vi) *presence of allosteric affectors*, (vii) *covalent modification*, (viii) *metal activators*, and (ix) *redox potential.* Although a full discussion on the above factors is beyond the scope of this book, a brief treatment is given in the following paragraphs.

10.4.1 EFFECT OF TEMPERATURE

Enzymes are sensitive to temperature changes. They have their own optimum temperature at which the catalytic activity is maximum. The optimum temperature for most enzymes, with certain exceptions, is around 40°C. At low temperatures the reaction is slow; and at higher temperatures, the enzymes, being protein, get denatured and cease to function. The effect of temperature (hypothetical) is shown in Fig. 10.2.

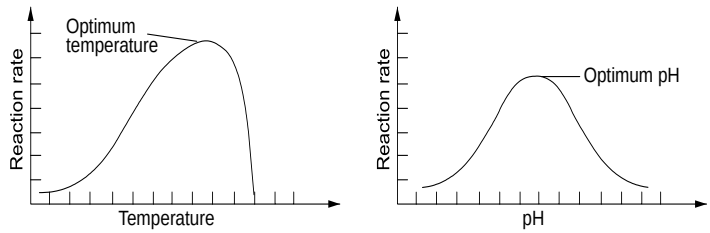


Fig. 10.2 The effects of temperature and pH on enzyme activity

10.4.2 EFFECT OF pH

The pH changes profoundly affect the ionic character of the amino- and carboxylic-groups on the enzyme molecule and therefore markedly affect the catalytic site and conformation of an enzyme. Without an appropriate conformation, an enzyme cannot function as desired (see later). Besides, low or high pH values can cause considerable denaturation and hence inactivation of the enzyme. Like temperature, the pH should also be in optimum range for maximum activity (Fig. 10.2).

10.4.3 EFFECT OF ENZYME CONCENTRATION

As is true of any catalyst, the rate of an enzyme-catalyzed reaction depends directly on the concentration of the enzyme. Provided that there is sufficient amount of substrate, increase in concentration of the enzyme will yield a first order kinetics (Fig. 10.3)

10.4.4 SUBSTRATE CONCENTRATION

With a fixed enzyme concentration, an increase in substrate concentration will at first result in a very rapid rise in the velocity of reaction rate. As the substrate concentration continues to increase, however, the increase in the rate of reaction begins to slow down until, with a large substrate concentration, no further change in velocity is observed. This is because at high substrate concentrations the active sites of the enzymes (see later) are completely filled (saturated) and can catalyze no more reaction than their full capacity.

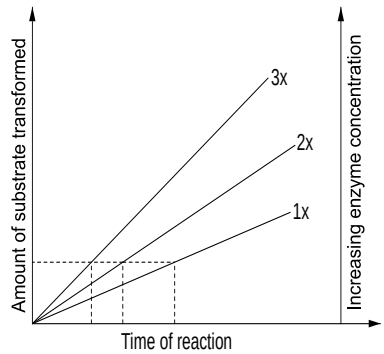


Fig. 10.3 Effect of enzyme concentration on reaction rate

The mathematical equation that defines the quantitative relationship between the rate of enzyme-catalyzed reaction and the substrate concentration for simple system and thus fulfils the requirement of a hyperbolic curve is the *Michaelis-Menten* equation:

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

Where, v = observed velocity (reaction rate) at a given substrate concentration, [S] = substrate concentration at any given instant (expressed in moles/L), K_m = Michaelis-Menten constant (expressed in moles/L), and V_{max} = maximum velocity of reaction (at saturating concentration of the substrate). The hyperbolic curve due to Michaelis-Menten equation appears in Fig. 10.4.

10.4.5 EFFECT OF INHIBITORS

A number of compounds have the ability to combine with certain enzymes, but do not serve as substrates. These compounds therefore block catalysis by the enzyme. Such compounds are called *inhibitors*. Inhibition in enzyme-catalyzed reactions can be grouped into three broad types: (i) *competitive inhibition*, (ii) *non-competitive inhibition*, and (iii) *uncompetitive inhibition*, a brief treatment of which shortly follows.

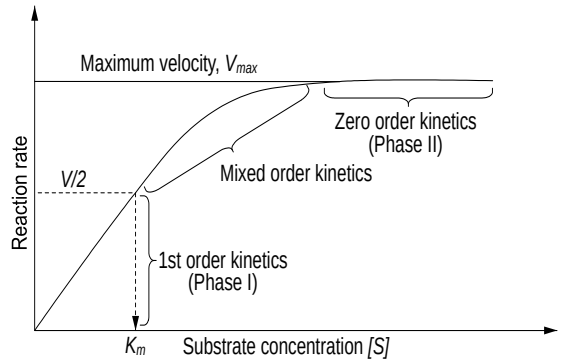
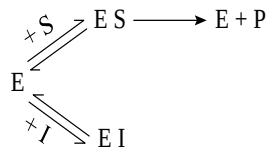


Fig. 10.4 Effect of substrate concentration on reaction rate

10.4.5.1 Competitive inhibition

In this category, the inhibitor has structural analogy with substrate of the enzyme and thus competes with it in order to bind to the *active site* (see later) of the enzyme. This inhibition is also called *substrate analog* inhibition. The enzyme can therefore combine with either the substrate or with the inhibitor and following equilibrium may exist:



Where, I = inhibitor, and S = substrate. The enzyme involved in the formation of EI complex cannot function as a catalyst; only ES (enzyme-substrate complex) will allow the formation of the reaction product.

The phenomenon can also be related to the Michaelis-Menten constant, K_m. Consider a typical enzyme-catalyzed reaction:

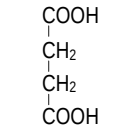
$$E + S \underset{K_2}{\overset{K_1}{\rightleftharpoons}} ES \xrightarrow{K_3} E + P$$

Where P = product, and other notations carry usual meanings. The ratio of dissociation of ES to its formation is given by:

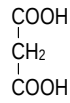
$$K_m = \frac{\text{Dissociation of ES}}{\text{Formation of ES}} = \frac{K_2 + K_3}{K_1}$$

We can thus see from the initial equilibrium that K_m value increases in the presence of inhibitor as some of the enzymes are being simultaneously utilized for the formation of EI complex. This EI reversibly breaks down to E and I producing an effect equivalent to the dissociation of ES.

The maximum velocity, on the other hand, will remain the same. Thus, succinic acid, which is readily oxidized to fumaric acid by succinic dehydrogenase, is competitively inhibited by malonic acid because the structure of malonic acid closely resembles that of succinic acid. See below for the structural analogy of succinic and malonic acid.



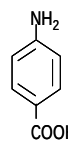
Succinic acid



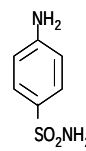
Malonic acid

The inhibition, however, can be reversed by increasing the concentration of the substrate, succinic acid. The proportion of enzyme molecules combining with the inhibitor (and therefore competitive inhibition) depends on: (i) *substrate concentration*, (ii) *inhibitor concentration*, and (iii) *affinity of the enzyme for the substrate and the inhibitor*. Competitive inhibition exists for all enzymes. A non-metabolic structural analog is generally a competitive inhibitor.

Numerous practical applications are based on competitive inhibition, particularly in chemotherapeutics: fight against bacteria, control of weeds, parasites, etc. The basic idea is to inhibit an enzymatic reaction which is of capital importance in microorganisms being fought. A classic example is that of *sulfamides*, analogs of *para*-aminobenzoic acid (a compound indispensable to many bacteria - but not to man - for the synthesis of folic acid).



p-amino benzoic acid



Sulfanilamide

The sulfamides compete with *para*-aminobenzoic acid for the active site of the bacterial enzyme catalyzing the transformation of this derivative into folic acid. This explains the bacteriostatic effect of sulfamides.

Another beautiful example of competitive inhibition is that of methanol on alcohol dehydrogenase. Oxidation of methanol produces formaldehyde, which is toxic to all biological tissues. This accounts for the toxic effect of methanol. As an antidote, ethanol is administered to the methanol-poisoned patient. Ethanol competes with methanol for the active site of alcohol dehydrogenase. Methanol will be slowly excreted away.

10.4.5.2 Non-competitive inhibition

In this, the inhibitor compounds bind either to the enzyme (but to the site other than the active site), or to the ES complex (to form ESI complex), or to both. But the inhibitors are not displaced by increasing the substrate concentration; the inhibition is irreversible. Evidently, enzyme from the inhibitor-enzyme complex cannot be available any more so as to form the final product. This leads to decrease in V_{max} and can even become zero on total inhibition. The K_m value, however, is unaltered.

A good example is the reaction of *iodoacetamide* on triose phosphate dehydrogenase, a sulfhydryl enzyme.

$$\underset{\text{(Iodoacetamide)}}{\text{Enzyme ~ SH} + ICH_2CONH_2} \longrightarrow \underset{\text{(Enzyme-acetamide complex)}}{\text{Enzyme ~ SH} + CH_2CONH_2} + HI$$

Many drugs are based on this principle. Thus, penicillin blocks the cell wall synthesis of bacteria by preventing bridge formation between N-acetylmuramic acids.

A highly dangerous nerve poison *diisopropyl fluorophosphates* works by inhibition of *acetyl cholinesterase*, the enzyme immediately associated with nerve function.

One potential use of non-competitive feature of inhibitors in enzymatic reactions is that they can be added to a reaction mixture to rapidly reduce or arrest the reaction when it has proceeded to the desired stage.

10.4.5.3 Uncompetitive inhibition

Some inhibitors can combine reversibly with ES complex only. They are therefore called *uncompetitive* inhibitors. These inhibitors have no affinity for substrate alone. They also bear no resemblance to substrate and therefore yield no products(s). The reaction can be shown by:

$$E + S \rightleftharpoons ES \xrightarrow{I} ESI$$

Where, the notations carry usual meanings.

This type of inhibition is found in *multisubstrate* reactions. Both K_m and V_{max} are altered but the slope is the same as that of an enzymatic reaction free of inhibitors. Experimentally, the value of K_m is generally obtained from a graph known as *Lineweaver-Burk* plot. It is a reciprocal plot and uses rearranged form of Michaelis-Menten equation given below:

$$\frac{1}{v} = \left(\frac{K_m}{V_{max}}\right)\frac{1}{[S]} + \frac{1}{V_{max}}$$

The nature of graphs for various inhibitions can be shown as in Fig. 10.5.

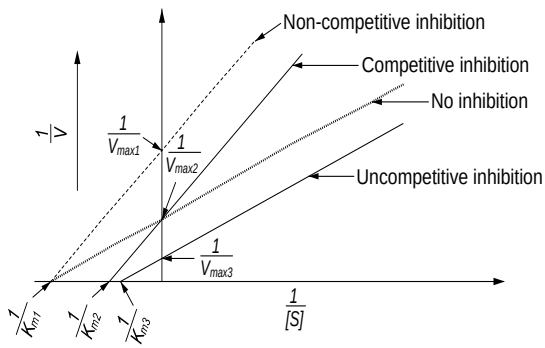


Fig. 10.5 Reciprocal plots of v and [S] in the presence of different types of inhibitors

10.4.6 EFFECT OF ALLOSTERIC AFFECTORS

All *allosteric* enzymes have *quaternary* structures. In addition to catalytic sites where the substrates bind, these enzymes have one or several allosteric sites, which can be located on a different polypeptide chain. The allosteric affectors (*activators* or *inhibitors*) need not have structural analogy with the substrate. A distinct feature of this enzyme is that it does not exactly exhibit Michaelis-Menten kinetics. This is principally because the enzyme is not a simple one that is subject to inhibitions as described before. The kinetics of allosteric enzymes in the presence of allosteric affectors is shown in Fig. 10.6.

Allosteric activator or inhibitor does not change the V_{max}. Only the K_m values are different. When an allosteric activator binds to the allosteric site, there results a slight modification of the conformation of the enzyme – called *allosteric transition* (reversible) – which changes the conformation of the catalytic site. The site, in general, acquires a conformation more favorable to the binding of substrate; the affinity of the affected enzyme for the substrate increases (K_m" < K_m). Even the shape of the curve can change from sigmoid (a characteristic of allosteric enzyme) to the hyperbolic form. The binding of allosteric affectors that activate or increase substrate-binding capacity of enzymes is called *cooperative binding*. If the activator is the substrate itself, for example, as in the case of hemoglobin-oxygen reaction, it is called a *positive homotropic response*.

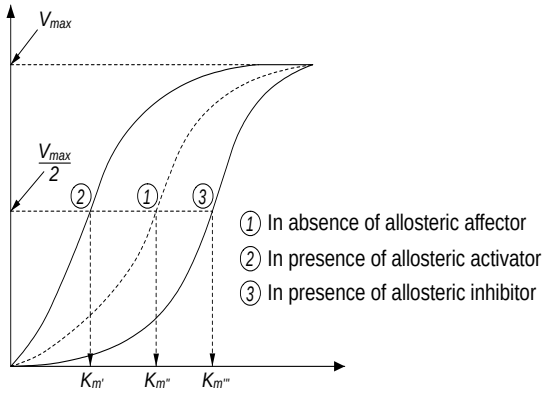


Fig. 10.6 Kinetics of reaction catalyzed by an allosteric enzyme

When the activator is a compound other than the substrate itself, the response is called *heterotropic* (positive) *response*. Hemoglobin is therefore a homotropic enzyme. Phosphofructokinase, which uses AMP as an activator, is a heterotropic enzyme. See Fig. 10.7 for a simplified explanation of allosteric transition caused by allosteric activator.

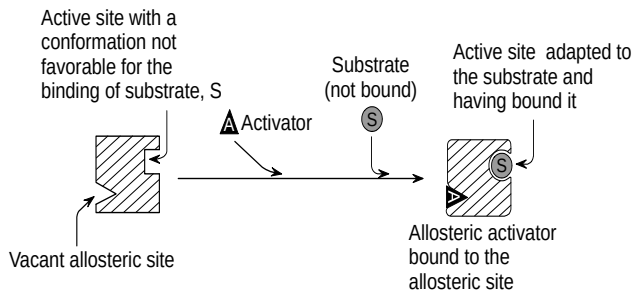


Fig. 10.7 Allosteric transition caused by allosteric activator

When an allosteric inhibitor binds to allosteric site, an allosteric transition (also reversible) takes place causing a change in the active site, which takes a conformation less favorable to the binding of substrate. The affinity of the enzyme for the substrate decreases ($K_{m'''} > K_{m'}$, Fig. 10.7). The curve in this case is a true sigmoid. The V_{max}, however, remains unchanged. Although most allosteric inhibitors are competitive by nature (K_m changed, V_{max} unchanged, and reversible), non-competitive inhibitions also occur. In this case, two or more affectors may compete for the same allosteric site(s). See Fig. 10.8.

Allosteric enzymes play a very important role in metabolic regulation, which occurs through allosteric controls. The sigmoidal curve denotes response which acts, in a sense, as an *off-on* switch and so provides a much more sensitive control than the hyperbolic response.

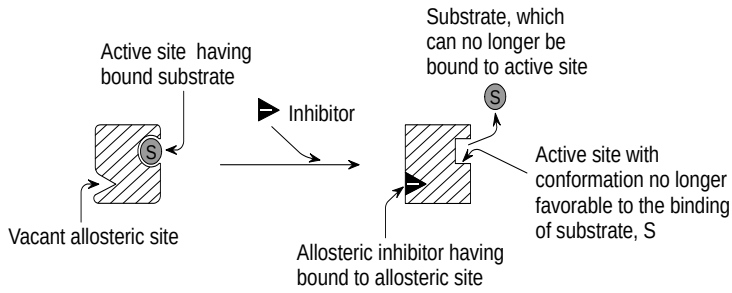


Fig. 10.8 Allosteric transition caused by allosteric inhibitor

10.4.7 EFFECT OF COVALENT MODIFICATION

The activity of numerous enzymes is controlled, not by formation of complexes between enzymes and regulatory molecules, but by covalent modification of the enzyme.

Some *monomeric* enzymes, particularly those responsible for protein digestion (pepsin, trypsin, chymotrypsin, etc.), are synthesized in catalytically inactive forms (called *proenzymes* or *zymogens*). It would be extremely damaging to cells if they were synthesized in active form. Only after they reach the intestine that they are converted into active forms. This conversion consists of cleavage of one or several peptide bonds. The cleavage initiates a reorganization of the spatial structure of the enzyme and the specific hydrolytic reaction occurs.

10.4.8 EFFECT OF METAL ACTIVATORS

The rates of enzyme-catalyzed reactions are altered by certain ions which behave as activators. A large number of enzymes requiring nucleoside di- and triphosphates invariably need divalent metal ions like Mg^{+2} or Mn^{+2}, which are occasionally replaceable. Many enzymes require monovalentpage cations, usually Na^{+}, K^{+}, or NH_4^{+} for maximum catalytic activity. Amylases require Cl^{-} ions for activity while carbonic anhydrase, chymotrypsin and alkaline phosphatase require Zn^{+2}. Usually, these ions interact with the substrate so that the latter's binding with the active site will be most favored. See Fig. 10.9 for example of involvement of metal ions. If metals form only loose and easily dissociable complexes and can easily release the metal without denaturation, the enzymes are called *metal-activated* enzymes. Those enzymes that do not release metals easily are called *metalloenzymes*. Some of the important and familiar enzymes that need metals for catalytic action are *enolase*, *carboxypeptidase*, *hexokinase*, *glutamine synthase*, etc.

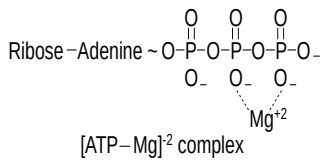


Fig. 10.9 The involvement of Mg^{+2} in favoring substrate binding

10.4.9 EFFECT OF REDOX POTENTIAL

Many enzymes are sensitive to oxidizing or reducing agents. The oxidizing or reducing ability of an enzyme is measured in terms of *redox potential*. The redox potential is the electromotive force, measurable in millivolts developed by the solution when in physical contact with the platinum electrode as compared to normal hydrogen electrode at zero potential. The redox potential of an enzyme is either negative or positive owing to its relative reducing or oxidizing ability in comparison to hydrogen.

10.5 MECHANISM OF CATALYSIS

The mechanism of enzyme-catalyzed reaction is a composite description of all the events that take place at a molecular and atomic level, from the initial binding of the substrate to the release of the product.

The essential steps involved in an enzyme-catalyzed reaction are: (i) *binding of substrate to the enzyme*, (ii) *conformational changes of enzyme or substrate or both as a result of binding*, (iii) *changes in chemical bonding by way of transition states and intermediates*, (iv) *further conformational changes on formation of products*, and (v) *release of product to the solvent*.

Enzymes reduce the overall activation energy, denoted by ΔG. Even the modest reduction in the value of ΔG leads to a very large increase in the reaction rates. For example, ΔG of an uncatalyzed breakdown of H_2O_2 into water and oxygen is 76 kJ/mole. The enzyme *catalase* requires only 30 kJ/mol for the same reaction. This lowering of energy is enough to yield 9×10^8-fold increase in the reaction rate, enough to reduce the time from years to seconds.

An oversimplified summary of the mechanism of an enzyme-catalyzed reaction is given in the following paragraphs:

Binding of substrate is probably the most important step in enzyme catalysis. An enzyme is a unique polypeptide which folds in the solution in a defined 3-D structure. Usually the polar amino acids orient outwards and come in contact with the aqueous medium. Non-polar amino acids are found in the interior side. All enzymes have unique 3-D site(s) for binding substrate(s) in specific orientation. The site is called *active site* (*catalytic site*). To this end, some enzymes have been categorized as *serine* enzymes, *lysine* enzymes, *sulfhydryl* enzymes, according to the essentiality of specific amino acid residues like serine, lysine, or cysteine-SH, respectively, at their active sites.

Substrate molecules are comparatively much smaller than the enzyme molecule. For example, consider invertase of molecular weight 127,000 against its substrate sucrose of molecular weight mere 342!

The side chain groups of the amino acids, e.g., $-NH_2$, -COOH, $-CH_2OH$, etc., serve as the catalytic group. Not all the amino acids involved function alike. One category constitutes the specificity site and enables recognition of the substrate to enable reaction. The second category of amino acids participates in the chemical transformation of the substrate. These two categories form the catalytic part of the

active center. Other participating amino acids are necessary for maintaining the adequate conformation of the allosteric sites (which control functioning of active site) and adequate positioning of the enzyme within the cells (for example, association of the enzyme with another enzyme to form a multienzyme complex).

The configuration of the active center, according to modern hypotheses and evidences, is not rigid. It has great flexibility. The association of the substrate with the active center induces very regularly a change in conformation which results in the *induced-fit* or *induced-adjustment* of the structure of the active center. The side chains, which participate in the functioning of catalytic part of active center, change conformation during attachment of the substrate to form the familiar *enzyme-substrate* complex, but also very often during catalytic transformation of the complex to form reaction products. It is quite obvious that any event leading to the change in natural conformation of the active center will lead to inactivation of the enzyme. The enzyme-substrate complex is formed mainly by non-covalent bonds, such as *hydrogen bond*, *electrostatic bond*, *Van der Waal force*, and *hydrophobic interaction*. Enzymes such as transaldolase use covalent bondings also.

Chemically, the enzymatic catalysis involves interactions between functional groups of substrate and enzyme molecules. This is in fact the central stage of reaction. It is difficult to generalize the events but in essence, amino acid side chains may act as acids or bases and thereby catalyze reactions. Electrons may be transferred during the course of reaction. Covalent and other bonds are formed for the reaction to occur but are later broken to form reaction products. Once the product is formed, the enzyme again changes its conformation to allow release of the product. Although the description is rather mechanical and straightforward, the chemical explanation, again, is quite complicated. Refer to allosteric regulation of enzymes (Fig. 10.7 and 10.8) also.

10.6 ENZYME KINETICS

Enzyme kinetics is the study of reaction rates of enzymes. It is an indispensable part of *enzymology*. Enzyme kinetics has dual purpose, *viz.*, *to understand the normal and abonormal metabolism of organism as a whole*, and *to elucidate the nature of enzyme process itself*. Because of the complexity of the nature, property and function of enzymes, however, no single kinetics can characterize all enzymes. In the following paragraphs is described a classical treatment of enzyme kinetics (for steady state) due to Michaelis and Menten (and therefore called *Michaelis-Menten* equation). The equation is derived employing certain assumptions and using Briggs and Haldane derivation for steady-state kinetics. The equation yields a *saturation hyperbolic curve* typical of the simplest, single-substrate-single displacement enzyme reaction.

The basic assumptions used in the derivation are: (i) *only a single substrate and a single product are involved*, (ii) *the process proceeds essentially to completion*, (iii) *the concentration of the substrate is much greater than that of the enzyme in the system*, (iv) *an intermediate enzyme-substrate complex is formed*, and (v) *the rate of decomposition of substrate is proportional to the concentration of the enzyme-substrate complex*.

The steps for the derivation are as follows:

A typical enzyme-catalyzed reaction involves the reversible formation of an enzyme-substrate complex, ES, which eventually breaks down to form enzyme, E, again and the product, P.

$$E + S \underset{K_2}{\overset{K_1}{\rightleftharpoons}} ES \underset{K_4}{\overset{K_3}{\rightleftharpoons}} E + P$$

Where, S is the substrate, K_1, K_2, K_3, and K_4 are the respective reaction rate constants.

A few milliseconds after the enzyme and the substrate have been mixed, a concentration of ES builds up and does not change as long as S is in large excess and $K_1 >> K_3$. This condition is called the *steady state* of reaction, since the rate of decomposition just balances the rate of its formation. We can therefore safely write, for steady state:

Rate of formation of ES = Rate of decomposition of ES or,

$$K_1[E][S] + K_4[E][P] = K_2[ES] + K_3[ES]$$

$$\Rightarrow \frac{[ES]}{[E]} = \frac{K_1[S] + K_4[P]}{K_2 + K_3} \text{-------(i)}$$

However, at an early stage of reaction, the rate of formation of the product, P is very small; the rate of formation of ES from E + P is even smaller. This would enable us to safely rewrite the initial equation:

$$E + S \underset{K_2}{\overset{K_1}{\rightleftharpoons}} ES \xrightarrow{K_3} E + P$$

Therefore, using K_4 [P] ~ 0 in equation (i), we have,

$$\frac{[ES]}{[E]} = \frac{K_1[S]}{K_2 + K_3}$$

Using $K_m = (K_2 + K_3)/ K_1$, and rearranging,

$$\frac{[E]}{[ES]} = \frac{K_m}{[S]} \text{-------(ii)}$$

But [E] and [ES] are not measurable values. We can resolve equation (ii) if we consider that the total enzyme concentration $[E]_t$ in the reaction consists of free

enzyme [E], and the enzyme-substrate complex, [ES]. The free enzyme concentration is, therefore,

$$[E] = [E]_t - [ES] \text{-------(iii)}$$

Using (iii) in equation (ii),

$$\frac{[E]_t - [ES]}{[ES]} = \frac{K_m}{[S]}$$

$$\frac{[E]_t}{[ES]} = \frac{K_m}{[S]} + 1 \text{-------(iv)}$$

Since the terms cannot still be readily measured, we can take the help of following relation: the maximum velocity, V_{max}, is attained when the total enzyme, $[E]_t$, is completely complexed with saturating amounts of S, or

$$V_{max} \propto [E]_t$$

Moreover, the initial velocity, v, is proportional to the enzyme present as ES complex at a given concentration of S, or,

$$v \propto [ES]$$

$$\Rightarrow \frac{V_{max}}{v} = \frac{[E]_t}{[ES]} \text{-------(v)}$$

Using relation (v) in equation (iv),

$$\Rightarrow \frac{V_{max}}{v} = \frac{K_m}{[S]} + 1$$

$$\Rightarrow v = \frac{V_{max}[S]}{K_m + [S]} \text{-------(vi)}$$

Equation (vi) is the required Michaelis-Menten equation. The graphical representation is given in Fig. 10.4.

10.6.1 UNIT AND SIGNIFICANCE OF K_m

If we choose v = half of V_{max},

$$\frac{V_{max}}{2} = \frac{V_{max}[S]}{K_m + [S]}$$
$$K_m = S$$

This shows that the unit of K_m is the same as that of [S], i.e., moles/L. It can also be seen that K_m represents substrate concentration, [S], at v = ½ V_{max}

K_m can be defined in many ways. It can be considered as substrate concentration when the reaction rate is half of the maximum rate. That is, K_m = [S], when v = ½ V_{max}. Also, since $K_m = (K_2 + K_3)/K_1$, it is a ratio of complex dissociation to complex formation rate. Evidently, at K_m values greater than unity the dissociation of ES complex dominates.

K_m signifies many important meanings, some of which are:

1. If K_m is known, the fraction of sites filled, *f*ES, at any substrate concentration can be calculated from the following equation:

$$f\text{ES} = \frac{v}{V_{max}} = \frac{[S]}{K_m + [S]}$$

2. High K_m values indicate weak binding between E and S to form ES. Low K_m values indicate strong binding of E and S. This is particularly true if $K_2 >> K_3$, which means that the product formation is negligible. Equilibrium exists between E+S and ES and K_m will equal dissociation constant.
3. K_m values can be used to identify whether a given enzymatic reaction is free from inhibitions, as also the type of inhibition.
4. K_m values can be used to predict V_{max} of a reaction at a given substrate concentration. Under practical condition, the observed velocity, v, becomes V_{max} at [S] ≥ $100K_m$. The reaction is then independent of [S] and exhibits *zero order* reaction. The basic assumption is that at [S] >>K_m:

$$v = \frac{V_{max}[S]}{K_m + [S]} \text{ becomes}$$

$$v = \frac{V_{max}[S]}{[S]}$$

$$\Rightarrow v = V_{max}$$

5. K_m value can be used to evaluate dependence of v on [S]. Under practical conditions, this relation can be obtained at [S] $\leq 0.01K_m$. The basic assumption is that at $K_m >>$ [S],

$$v = \frac{V_{max}[S]}{K_m + [S]} \text{ becomes}$$

$$v = \frac{V_{max}[S]}{K_m}$$

$$\Rightarrow v \propto [S]$$

The reaction is then of 1st order.

The K_m values of enzymes differ greatly. However, for most enzymes, the general range is between 10^{-1} and 10^{-6} mole/L. K_m values are not absolute constants; they depend on the source of enzyme, environmental conditions such as temperature, ionic concentration, and particular substrate.

10.6.2 DETERMINATION OF K_m Value

K_m of an enzyme-catalyzed reaction is determined usually by graphical methods such as *Woolftees plot*, *Lineweaver-Burk plot*, etc. For reasons of simplicity and sufficient accuracy, the Lineweaver-Burk plot is more extensively used. In this method, only a small number of experimental points are required. Furthermore, V_{max} can be readily evaluated by extrapolation. The plot is also called *Double reciprocal plot*. The plot is actually a rearranged form of Michaelis-Menten equation:

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

$$\Rightarrow \frac{1}{v} = \frac{K_m + [S]}{V_{max}[S]}$$

$$\Rightarrow \frac{1}{v} = \left(\frac{K_m}{V_{max}}\right)\frac{1}{[S]} + \frac{1}{V_{max}}$$

The last equation is in the form of $y = mx + c$; $1/v$ and $1/[S]$ represent variables y and x respectively; K_m/V_{max} and $1/V_{max}$ represent constants m and c respectively (see Fig. 10.10).

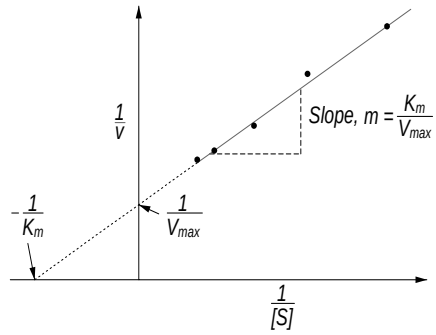


Fig. 10.10 A typical Lineweaver-Burk plot

The experimental results are plotted as follows: a double plot of $1/v$ values on the ordinate and 1/[S] values on the abscissa is made. A straight line is obtained from which the value of K_m is calculated. While plotting, to obtain greater accuracy, the line is extended to a point where $1/v = 0$. It may be noted, mathematically $1/v = 0$ is impossible. The line is further extended to a point where $1/[S] = 0$. These manipulations are required because, under practical condition, the value of 1/[S] never touches the ordinate. For, this would mean that $1/[S] = 0$, which is mathematically impossible.

Note that at $1/v = 0$, $1/[S] = - 1/K_m$, from which the value of K_m can be calculated. Similarly, for V_{max}, we can use $1/[S] = 0$ from the graph, in which case $V_{max} = v$. This is equivalent to saying that at infinitely high substrate concentration, $v = V_{max}$, which has already been stated at the very outset.

10.7 GENERAL PROPERTIES OF PROTEIN ENZYMES

In brief, to suit the context, properties of protein enzymes can be stated as follows:

- They are proteins
- They exhibit specificity in reaction
- They are biocatalysts
- Their activities can be controlled

The very proof for their being protein is the presence of amino acid residues. With some exceptions, all protein enzymes are globular in structure. Like proteins, they all have *primary*-, *secondary*-, *tertiary*-, and sometimes, *quaternary* structures. They get denatured by agents that denature proteins. A very remarkable feature of enzyme makeup is the diversity they exhibit with respect to organization/arrangement. Thus, based on the degree of association and function, protein enzymes are grouped into (i) *monomeric enzymes*, (ii) *oligomeric enzymes*, and (iii) *multienzyme complex*.

Monomeric enzymes are the simplest in makeup. They consist of a single polypeptide. Some of the familiar examples of monomeric enzymes are *pepsin*, *trypsin*, etc.

Oligomeric enzymes contain at least two and as many as 60 or more polypeptide subunits. Oligomeric enzymes have a wide range and diversity of functions. Some of the important categories of oligomeric enzymes are: *isozymes* (e.g., lactate dehydrogenase), *allosteric enzymes* (e.g., aspartate transcarbamylase), *bifunctional enzymes* (e.g., tryptophan synthetase in *E. coli*), etc.

A distinction needs to be made between oligomeric enzymes and multienzyme complex. Multienzyme complexes are actually aggregates of a number of enzymes. They are all engaged in a sequential series of reactions in the transformation of substrates into products(s). The enzymes are tightly associated and all attempts to dissociate them lead to complete inactivation of the enzyme. In essence, multienzyme complex consists of an organized *mosaic* of enzymes in which each of the components is so located as to allow effective coupling of the individual reaction catalyzed by these enzymes. An excellent example of multienzyme complex is the one involved in oxidation of pyruvic acid to acetyl-SCoA and CO_2. The enzyme has a molecular weight of about 4 million and consists of three separate catalytic activities, *viz*., that of *pyruvic dehydrogenase*, *dihydrolipoyl transacetylase*, and a *dihydrolipoyl dehydrogenase*. An illustration appears in Fig. 10.11.

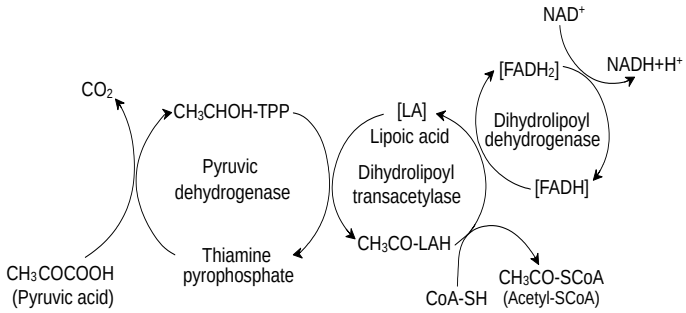


Fig. 10.11 Oxidation of pyruvate to acetyl-SCoA

One of the most important distinctions between a chemical catalyst and the enzyme is the specificity. Unlike chemical catalysts, enzymes are very specific in catalyzing reactions. They can unequivocally select substrates or reactions, which is not possible in chemical catalysis. Specificity may be observed on the one hand in the type of reaction catalyzed and on the other hand in the substrate for reaction. Enzymes also have the ability to distinguish between isomers.

10.8 MECHANISM OF ENZYME BIOSYNTHESIS

Metabolism is principally regulated by a change in the rate of enzyme reaction, which in turn is controlled at the levels of *transcription*, *translation*, and *post-translation*. Enzyme concentration is varied by two mechanisms, *viz*., *controlled synthesis*, and *controlled degradation*.

Enzyme synthesis is controlled by *induction* or *repression*. Many of the enzymes produced/used commercially are of *inducible* type. The biosynthesis of inducible enzymes is triggered only when the corresponding substrate (the inducer) is

presented to the organism. Often, inducers are analogs or derivatives of substrates, e.g., IPTG (isopropyl thiogalactoside) for β-galactosidase. Induction occurs at the level of transcription. The gene is rapidly transcribed and the resulting *m*RNA translates for enzyme production.

Repression can be of two types, *viz*., feedback repression and catabolite repression. Feedback repression results from the accumulation of end products in concentration more than needed by the cell. This is called allosteric regulation. In this, the end product or an intermediate binds to the allosteric site of the enzyme resulting in a conformational change in the enzyme. This is unfavorable for the enzymatic reaction. When the end product diminishes, the system works again (the binding is relieved).

Catabolite repression occurs when an organism is grown in a readily metabolizable carbon source, glucose as against lactose for *E. coli*, for example. The repression is at the level of transcription but the mechanism does not represent an *off-on* system. It is meant only for *fine-tuning* of control. Feedback control, on the other hand is used for rapid control.

10.9 THE KINETICS OF ENZYME BIOSYNTHESIS

Biochemical processes are extremely complex and sensitive to a number of factors and rendering their mathematic modeling is most difficult. This is why commercial fermentation processes have not been significantly optimized in an engineering sense.

Excellent contributions to the development of kinetic models of enzyme formation stem from Terui and associates (1967). The represented models refer to hydrolases produced commercially. They are based on the assumptions that the rate limiting ability of enzyme forming system corresponds to *m*RNA and that the specific rate of enzyme production is proportional to the quantity per cell of *m*RNA. Hence, for growth-associated enzyme production the following hypothetical relation was proposed:

$$\frac{dE}{dt} = a\mu = b\frac{d\mu}{dt} - k$$

Where, μ = specific growth rate (h^{-1}), k = monomolecular decay rate constant of the specific *m*RNA (h^{-1}), a and b = system constants, with b representing rate of growth-associated repression exerted at the level of transcription, E = growth-associated enzyme production.

The equation becomes very complex when the enzyme production occurs at stationary phase.

$$\underbrace{E = E_m e^{-k(t-t_m)}}_{\text{E due to the mRNA carried over from the growth phase}} + \underbrace{K_1\left(e^{\lambda(t-t_m)} - e^{-k(t-t_m)}\right)}_{\text{E due to the turnover synthesis and degradation of mRNA}}$$

10.9.1 MANIPULATION OF BIOSYNTHESIS

For an industrial production of microbial enzymes, of first and foremost importance is the selection of suitable strains. They are then progressively improved in laboratory. A number of methods are available for the improvement, most of then aimed at overcoming the inherent control mechanisms of the microorganisms over enzyme synthesis. The techniques are of two main categories: (i) manipulation of genetic function, and (ii) manipulation of cultural- and process conditions.

10.9.1.1 Manipulation of genetic function

This entails mutation programs for (a) desensitizing controlling enzymes, (b) producing auxotrophic mutants, (c) enzyme engineering, (d) producing leaky cells, (e) inserting multiple genes, and (f) mutation to constitutivity.

10.9.1.2 Manipulation of cultural- and process conditions

This entails following approaches: (a) introduction of inducer, (b) mixed culture to aid removal of end products, (c) use of slowly metabolizable substrates, (d) control on feed rate, and (e) selective removal of corepressor.

10.10 ENZYMES IN VARIOUS INDUSTRIES

Enzymes are obtained from animal tissues, plants, bacteria and fungi (including yeast). The bulk of enzymes, both in terms of quantity and variety, are derived from microorganisms, higher plants being the distant second and animals being the least important. The only animal enzyme to be produced in quantities greater than 2MT/year is *rennin* or *chymosin* obtained from calf stomach. See Table 10.4 for production data as of 1996.

Table 10.4 World production of enzymes

Enzyme	Source	Annual production (MT)
Protease	*Bacillus (*bacteria*)*	550
α-amylase	*Bacillus* (bacteria)	350
Amylase (α and β)	Barley malt	> 200
α-amylase	*Aspergillus* (mold)	20
Glucoamylase	*Aspergillus* (mold)	> 100
Rennet	*Mucor miehei* (mold)	25
Pectinase	*Aspergillus* (mold)	20
Papain	Papaya latex	> 10

10.11 PRODUCTION OF MICROBIAL ENZYMES

At present, over 2,000 enzymes have been isolated and characterized, about 1000 of them are recommended for various applications, and about 50 microbial enzymes

have industrial applications. Based on the area of use, microbial enzymes can be classified as (i) *fine chemicals*, and (ii) *industrial enzymes*. Fine chemicals are confined to laboratory use for research purposes and hence require a very high degree of purification. This is also true if the enzymes are to be used in diagnostic kits, e.g., in biosensors. Industrial enzymes, on the other hand do not warrant such high degree of purification, particularly if the enzymes are to be used in an industrial scale.

10.12 PRODUCTION ASPECTS

Microbial enzymes are relatively high-value, low-volume products. In most cases the fermentation process is largely *secondary* to downstream processing. This is particularly true of intracellular enzymes. If a ready source of enzyme is available, e.g., *Aspergillus niger* mycelia after gluconic acid fermentation (the mycelia are a rich source of glucose oxidase), the fermentation process can be totally circumvented. However, in the case of extracellular enzymes fermentation is obligatory. It must be emphasized here that adequate attention must be given to the design of the fermentor. In general, it is advisable to use small, batch-type, stirred-tank, aerated fermenters designed to allow considerable flexibility in use.

A large-scale enzyme production entails, in the first stage, choice of strains that produce large amounts of the concerned enzyme. It is also important to note here that extracellular enzymes are preferred. This is basically because the downstream processing of intracellular enzymes is very costly. The cells have to be ruptured to release the enzymes, which again is not easy. The enzymes need extensive purification because they come contaminated with, among other things, nucleic acids. Added to these aspects, the enzymes cannot be produced in large amounts under cellular environment: this is because of the repression and feedback inhibition encountered by the synthesizer cell. Thus, for the overproduction of enzymes, some mechanisms must be available for counteracting these inherent feedback inhibition and repression systems.

The criteria for the choice of strains are:

- Extracellular enzymes are preferred
- High yield of enzyme
- Genetic stability
- Ability to grow in cheap substrate
- Minimal by-product formation
- Ease of recovery
- Freedom from antibiotic activity
- Non-pathogenic

10.13 ADVANTAGES OF MICROBIAL ENZYMES

The preference of microbial enzymes to enzymes from other sources stems from:

- Normally high specific activity per unit dry weight
- Independent of seasonal variability

- Enzymes of a wide range of properties and stability produced
- Microorganisms are easier to manipulate genetically for the improvement
- Due to short generation time, the productivity is comparatively high
- The downstream processing is comparatively simple
- Even whole cells can be used as an enzyme source, which is impossible in other cases
- Enzyme engineering in microorganisms is both possible and pragmatic
- Some special enzymes, e.g., reverse transcriptase, heat-stable DNA polymerase, DNA ligase, etc., can be obtained from microorganisms only.

10.14 GENERAL PRODUCTION AND PURIFICATION METHODS

The raw materials for industrial enzyme fermentations have normally been limited to substances that are readily available in large quantities at low cost, and are nutritionally safe. Some of the most commonly used substrates are starch hydrolysates, molasses, corn steep liquor, whey, and many cereals. Microbial enzymes are produced by two main methods, *viz*., (i) *solid substrate cultivation*, and (ii) *submerged cultivation*.

10.14.1 SOLID SUBSTRATE FERMENTATION

Solid substrate methods of producing fungal enzymes have long-standing historical applications, particularly in Japan and Far East countries.

In this method, the microbial growth and product formation occur on the surface of the solid substrate. The system is suitable for the production of extracellular enzymes, certain valuable chemicals, toxins, and fungal spores. Some of the enzymes produced commercially by solid substrate fermentation are given in Table 10.5.

Table 10.5 Enzymes produced by solid-state fermentation

Enzyme	Organism (examples)	Substrate	Function
Cellulase	*Trichoderma viride*	Wheat bran	Cellulolytic (in detergents, etc.)
Amylase	*Aspergillus oryzae*	Rice	Saccharification
Protease	*Aspergillus oryzae*	Wheat bran	Proteolysis

The media in solid-substrate fermentations for enzyme production are mainly based on wheat bran. This material is particularly suitable because of its high content of nutrients and large surface area. Other materials such as rice, soybeans, etc., can also be used but they must first be cracked to suitable size to allow profuse superficial growth of the relevant organism. The type of enzyme desired also dictates the choice of the material. The amount of water needed for moistening the substrates is in the range 30-70%. The process is used largely for molds or other mycelial bacteria, e.g., *Streptomyces* species. It must be noted here, the organisms used in solid substrate fermentations are highly aerobic and are good producers of extracellular enzymes. This has to be so because they have to simplify the complex substrate presented to them before the nutrients can be taken inside the cell for intracellular metabolism. In

industrial fermentation, effort is expended to accentuate this natural capability of the organism. Thus, the medium is often supplemented with some salts or other suitable components. The pH is adjusted with an acid. The medium is sterilized normally by autoclaving (with stirring). Fungal spores (or other cultures) are inoculated on the surface. Aeration is achieved by circulating conditioned air over the surface. The conditioned air helps maintain the humidity, reduces the temperature, supplies oxygen, and removes the CO_2 generated in the metabolic process. The temperature should be controlled within narrow limits. This calls for use of appropriate cooling systems in large fermenters.

The main advantage of solid-substrate process is the low investment required. Also, being low in moisture compared to submerged process, the enzyme concentration in the fermented medium is much higher. Of the limitations, the following three may be mentioned: (i) labor intensive, (ii) requires more space, (iii) greater risk factor.

As such, solid-substrate fermentations are difficult to scale up. Consequently, fermentation is carried out in small batches. The distribution of heat, air, and nutrient is also very difficult. Nevertheless, industries have come a long way using solid-substrate fermentation, which are mainly of two types, *viz*., (i) *Thin Layer Technique* (*tray process*), and (ii) *Deep Bed Process*.

10.14.1.1 The thin layer process (cabinet method)

The process works with substrate layer (2-4 cm thick) spread on wooden or metallic trays. After inoculation with spores, the trays are incubated in air-conditioned rooms or cabinets (hence *cabinet method*). The heat produced by the growing culture is removed by passing moistened, cool air over the surface of the culture. Cooling systems can also be provided. A typical flow sheet of tray method of enzyme fermentation is given in Fig. 10.12.

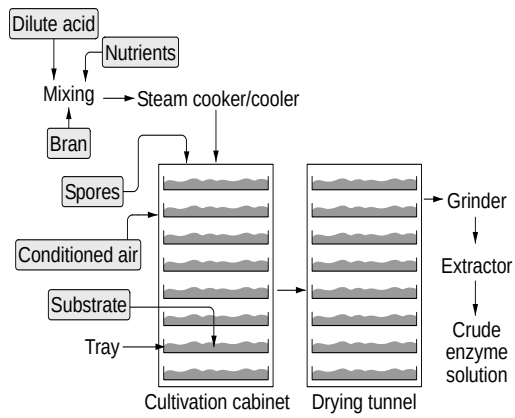


Fig. 10.12 Thin layer solid state fermentation

10.14.1.2 The deep bed process

The deep bed process is a modification of the traditional solid-substrate (= solid-state) fermentation. The process solves most of the problems associated with traditional processes. A typical deep bed process uses substrate layers of 2-6 feet. Deep bed plants are fully automated. The substrate is sterilized by first moistening with acidified water and then injecting steam to give 95°C for 15 min. Decontamination of the substrate, e.g., bran, can be carried out using bactericides such as formaldehyde. Inoculation of the sterilized medium is carried out with spores in a dry or suspended form. Cooling system coupled with passage of conditioned air is universal in solid-state fermentations.

10.14.2 SUBMERGED CULTURE

Submerged fermentations were not as widespread until recently. The fermentation on commercial scale is carried out in *continuous stirred tank bioreactor* (CSTBR; this is the same as STF mentioned elsewhere, page 93) of stainless steel with capacities ranging from 10-15 m^3. With enzyme fermentations, the formulation of the production medium and to a lesser extent, control of fermentation conditions, play major roles in the success of the process.

The production medium should basically contain an energy source, nitrogen source, and any special growth requirements (amino acids, vitamins, etc.). However, good growth is not enough to obtain a high enzyme yield. Inducers may have to be used. As inducers are rather expensive, it is preferable to use constitutive mutants, which do not require the inducer. Where catabolite repression is observed, slow feeding in *fed-batch* mode (= *extended* culture method) is desirable. Incremental feeding of slowly metabolizable sugars is also possible. The presence of certain surfactants in the production medium increases the yield of certain enzymes. Non-ionic detergents, e.g., *Tween-80*, are frequently used. Most enzyme fermentations are carried out at neutral pH. The change in pH is controlled by adding buffers. An alternative is to add certain compounds, which upon metabolism, bring about change in pH in the desired direction. See Fig. 10.13 for the generalized scheme of production of liquid microbial enzyme and Fig. 10.14 for generalized scheme for extraction and purification of enzymes.

10.15 GENERAL PROCESS OF ENZYME RECOVERY

The downstream processing of enzyme fermentation can be as simple as drying of substrate to as involved as chromatographic purification. The complication depends on: (i) whether crude or high-grade enzyme is to be produced, and (ii) whether the enzyme is intracellular or extracellular. Whatever the concentration/purification technique, the fermentation broth is usually cooled to about 4°C immediately after completion of the fermentation to arrest contamination and degradation of the enzyme.

The recovery of fungal enzymes is normally straightforward and usually involves centrifugation or filtration. Bacterial enzymes are more difficult to concentrate/purify. Typically, bacterial broths are treated with coagulating or

flocculating agents such as calcium phosphate to separate the bacterial cell and the colloids. The cells can later be filtered using 2-3% diatomaceous earth as the body feed.

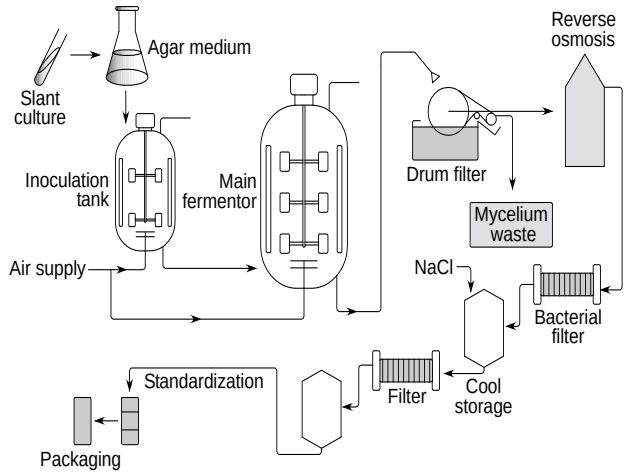


Fig. 10.13 Schematics of stages in the production of a liquid enzyme preparation

When the fermentation is of solid-substrate type (e.g., mold bran fermentation) and the enzymes are extracellular in nature, the extraction is basically a washing process. Countercurrent techniques of percolation are the most frequently used unit operation. In many cases, the mold bran is dried prior to extraction. The actual extraction may be done on demand. The solvent used for the extraction is universally water. Certain components, e.g., buffer, salt, etc., may be added to facilitate extraction or improve stability in solution.

The extent of purification is dictated by the intended use of the enzyme, *viz.*, industrial use, food use, and laboratory use. In the industrial category, for economic reasons of enzyme application, a concentration up to 10-fold is usually satisfactory. For example, enzyme products employed in detergents contain about 5-10% protease while amylase preparations for use in flour treatment contain only about 0.1% pure amylase. However, in applications where high purity enzymes are required, e.g., in enzymic hydrolysis, 1000-fold purification is quite common. In some applications, such as baking and dextrose manufacture, the presence of contaminating enzymes must be very low or rigidly controlled.

Crude preparations, although much easier to produce, suffer from the decreased stability. Since the trend in enzyme applications is towards use of liquid preparations, stabilization is a very important aspect.

10.15.1 RECOVERY OF EXTRACELLULAR ENZYMES

The general steps in sequence are *centrifugation* (to remove cells) at low temperature (alternatively, vacuum filtration with filter aid) and *purification*. Purification may involve any one of the following general techniques: *Membrane filtration, Gel filtration,*

Adsorption, Ion-exchange or Precipitation. A brief treatment of the different concentration/purification process is described in the following sub-sections.

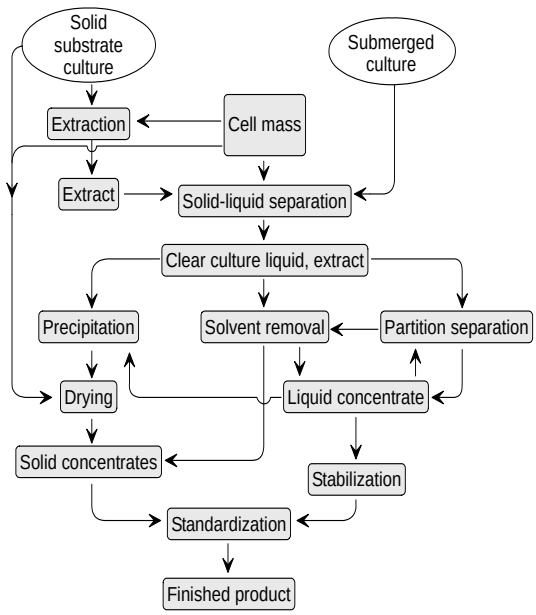


Fig. 10.14 Extraction and purification of an enzyme

10.15.1.1 Ultrafiltration

It is used for concentration and demineralization of solutions of proteins, sugars, and organic solutes. The method is typically a membrane separation technique. From normal filters it differs by the size range of particles to be separated (molecular cutoffs between 500 and 300,000).

10.15.1.2 Reverse osmosis

It is also a membrane separation technique. It is used for the recovery of dissolved proteins, ionic salts and small organic molecules. Unlike ultrafiltration, it can separate molecules of similar sizes. In particular, reverse osmosis allows only water to pass through.

10.15.1.3 Gel permeation or exclusion chromatography

This separates molecules on the basis of size. The separation is unique in that the molecules that happen to enter the channels in the gel body will be delayed in passing out while those that do not enter the same pass out fast. Consequently, large molecules come out faster than the small molecules.

10.15.1.4 Chromatography

This separation technique is based on ion exchange, hydrophily/lipophily, etc., and is carried out in columns. The partially purified solution is passed through the column that contains immobilized ion-exchange components. As the liquid percolates down, the relevant enzymes are selectively retained in the column due to interaction with the immobilized counterparts while the rest pass through the column unhindered. In the second phase, the bound enzymes are eluted from the complex with a suitable solvent. Once again the column becomes ready for receiving a new charge.

10.15.1.5 Precipitation

Separation by salting out (in electrolytes) is one of the oldest and yet the most important methods of enzyme concentration/purification. An additional treatment of the topic is therefore appropriate here.

The logarithm of decrease in protein solubility in concentrated electrolyte solutions is a linear function of increasing salt concentration (ionic strength), as described by the equation:

$$\log s = B' - K's\frac{\tau}{2}$$

Where s = the solubility of protein (g/L); τ = ionic strength (mol/L); K' = salting out constant (dependent on protein and salt type); B' = intercept constant (dependent on pH, temperature, and the nature of protein in solution).

The above equation implies that electrolyte concentration required for protein precipitation varies with protein concentration.

The influence of the most important precipitation parameters can be outlined as follows: Higher valency salts produce higher ionic strength than lower valency salts. At constant ion strength, protein solubility increases with increasing distance (in both directions) from its isoelectric point. As a result, lower ionic strength is required for precipitating when carried out at the isoelectric point of the protein.

The most commonly used salt for protein precipitation is ammonium sulfate. The reasons can be found in the high solubility of the salt, low price, non-toxic nature (to most enzymes), and enzyme-stabilizing property. Enzymes prepared by precipitation with ammonium sulfate are often stable for years when stored at low temperatures.

In very simple terms, the precipitation of enzymes/proteins with salt can be described as follows: Protein solubility tends to increase when salt is added to the solution. This phenomenon is called *salting in*. With further addition, however, the salt will begin to compete with the protein for water and at some point, force the protein to precipitate out. This phenomenon is called *salting out*. When ammonium sulfate is used for the precipitation, terms like 25% saturation, 50% saturation, 75% saturation, etc., are frequently encountered. These refer to the percentage by volume

of saturated ammonium sulfate used for the precipitation. For instance, 25% saturation means mixing of 1 volume of saturated ammonium sulfate with 3 volumes of enzyme/protein solution.

Precipitation of enzymes with solvents is less common for large-scale purification. The main reasons behind it are high cost of solvent, equipment and processing, risk of explosion, and tendency of enzymes to denature at processing temperatures above 4°C.

Solvent precipitation is based on the fact that the solubility of enzymes decreases with the decrease in *dielectric* constant (ε) of the solvent. The concentration required is lower the less hydrophilic the solvent is. Thus, an increasing precipitation effect can be achieved in the series methanol ($\varepsilon_{25} = 33$), ethanol ($\varepsilon_{25} = 24$), and isopropanol ($\varepsilon_{25} = 18$). Besides aliphatic alcohols, acetone ($\varepsilon_{25} = 20$) is often used as a precipitant.

In the solvent category, polyethylene glycol (mol wt: 6000) appears to be the best in that it does not bring about enzyme denaturation and is relatively independent of temperature and ionic strength. However, there is a strong dependence on hydrogen ion concentration. The best results are obtained at the isoelectric point of the enzyme to be precipitated. The hydrogen ion concentration can be easily adjusted with acid or alkali. As the solubility of a protein molecule is lowest at its isoelectric point, successive precipitation of different enzyme species can be affected from the same solution by altering the pH. The precipitated enzyme can now be easily separated by centrifugation.

10.15.2 RECOVERY FOR INTRACELLULAR ENZYMES

The separated cells are washed free from impurities and subjected to any one of the following cell disruption techniques: (i) *Chemical/Biochemical*, *viz.*, autolysis, or (ii) *Physical disruption*, *viz.*, homogenization (e.g., in Manton Gaulin homogenizer) or bead milling (e.g., Dynomill). The subsequent purification technique is the same as for extracellular enzymes. An additional step must be included for the removal or reduction of the contaminating nucleic acids and cellular debris.

10.16 CONVERSION TO STORAGE FORM

Enzymes are usually very unstable (due to denaturation, microbial degradation, photochemical effect, charge destabilization, etc.) in aqueous solutions. This calls for appropriate treatment of enzyme solution for storage. Some of the practical methods of treatment are:

- Use of highly concentrated solutions of salt and sugar (to repress microbial growth)
- Conformation- or charge stabilization and/or protection from dilution-dissociation by using buffers, glycerol, substrates, or inhibitors
- Protection of active site *thiol* via disulfide exchange by thiols, redox dyes, oxygen-binding agents, or chelating agents
- Inhibition or removal of proteolytic enzymes

Following the above treatment, it is imperative that the preparation be stored at low temperature and at suitable pH in appropriate packaging material.

10.17 AMYLASE PRODUCTION

Of the various enzymes, amylases play probably the most important part in food technology. The use ranges from production of alcoholic grain beverages (whiskey, beer, sake, etc.), non-alcoholic beverages (soft drinks, coffee, etc.), confection, corn syrup, to pharmaceutical products (digestive enzymes).

Amylase is the collective name given to a group of enzymes characterized by their ability to hydrolyze 1,4-glucosidic linkage in polysaccharides (e.g., starch and glycogen). There are two main subgroups of amylases, *viz.*, *a-amylase* and *β-amylase*. α-amylases are also known as *endoamylases*, keeping with their random hydrolytic action within the starch molecule. β-amylases (called *exoamylases* by analogy) liberate maltose units by hydrolyzing the starch molecule sequentially from the *non-reducing end*. Both the enzymes, however, are unable to hydrolyze glucose polymers of starch linked by an α-1,6-glucosidic bond (see Fig. 10.15).

Microbial amylases are extracellular enzymes. Depending on the source organism, the amylases exhibit differing properties especially with respect to mode of action, products of hydrolysis, pH and temperature optima, etc.

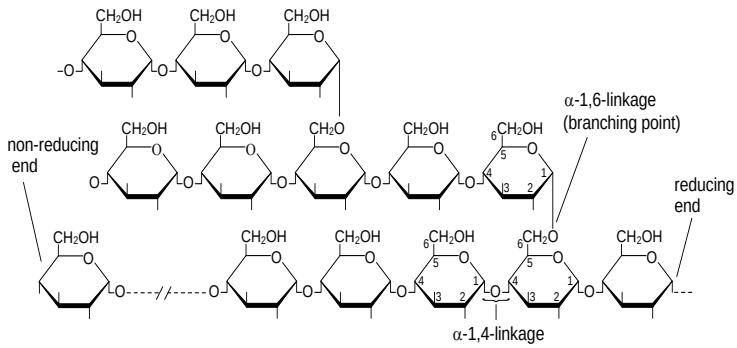


Fig. 10.15 Simplified structure of starch molecule

10.17.1 MICROBIAL PRODUCTION OF α-AMYLASE

The enzyme (α-1,4-glucan-glucanhydrolase, EC.3.2.1.1) is produced industrially from bacteria as well as fungi (mold).

10.17.1.1 Fungal a-amylase

Fungal α-amylase is produced industrially from *Aspergillus oryzae* and *Aspergillus niger* by either solid-state or submerged fermentation process.

Solid-state process

In the solid-state method, wheat bran serves as the basic component of the medium. The treatment of the medium, inoculation, and fermentation is not much different from a typical solid-state fermentation described elsewhere for molds. Fermentation is carried out at 30°C for 1-4 days. The recovery consists of either extraction in water or drying of the mold bran to produce crude enzyme. For reasons of consistency, the preparation must be carefully standardized for activity according to the intended use.

Submerged fermentation

Submerged fermentation exhibits marked rheological complexity (because of gradual mycelial growth) and consequent aeration problem. A typical fermentation medium is given in Table 10.6.

It is to be noted that glucose has not been included in the above medium. The basic reason for this is that glucose exerts catabolite repression thereby interfering with the enzyme yield. The pH is monitored with organic acids (citrate, gluconate, etc.) or alkalizing nitrogenous compounds (nitrates, urea, proteinaceous matter, etc.) but the shift in pH should be gradually towards alkalinity as the fermentation progresses. This is true of bacterial process as well. The basic reason for this is the tendency of α-amylase to denature at pHs below 6. When buffering is needed, $CaCO_3$ may be added.

Table 10.6 Medium composition for submerged fermentation of α-amylase

Component	Amount (g/L)
Corn starch	24
Na_2HPO_4	47
Cornsteep liquor	36
KCl	0.2
$CaCl_2$	1
$MgCl_2.6H_2O$	0.2

10.17.1.2 Bacterial a-amylase

Bacterial α-amylase (mol wt: ~ 50,000) is produced industrially from *Bacillus amyloliquefaciens* and *Bacillus licheniformis*. *Bacillus subtilis* is also a good candidate. The production is done in submerged mode. A typical composition of the main fermentation medium is given in Table 10.7.

A temperature in the range of 30-40°C is satisfactory. The pH should be near neutrality but not below 6. The production of amylase begins when the bacterial count reaches 10^9-10^{10} cells per ml after about 10-20 hrs, and continues for another 100-150 hrs.

Bacterial amylase is commonly produced with minimum purification. The enzyme is preserved in 20% NaCl. The most active liquid preparations contain 2% active amylase. The most active solid preparations contain 5% active amylase. Highly active and purified preparations are obtained by precipitation and/or adsorption techniques.

Table 10.7 Medium composition for bacterial α-amylase production

Component	Amount (%)
Ground soybean meal	1.85
Autolyzed brewer's yeast fraction	1.5
Distiller's dried solubles	0.76
Enzymic casein hydrolysate	0.65
Lactose	4.75
Antifoam	0.05
$MgSO_4.7H_2O$	0.04
Water	90.40

10.18 PROTEASES

Protease is a generic term for proteolytic enzymes that use proteins and peptides as the substrate. The microbial proteases which are of interest for application in the food industry are all of the endopeptidase type and are all extracellular enzymes. There are many different types of proteases produced by an extraordinarily large number of microorganisms, but in actual practice the enzymes prepared commercially are of very limited number and types and they are derived from very few organisms.

Proteases can be divided into two main groups, *viz*., (i) *acid proteases*, and (ii) *alkaline proteases*. The proteases are sometimes classified in a manner meaningful to each specific purpose, for example, *serine* proteases, *metalloproteases*, *thiol* proteinases, etc.

The industrial production of microbial protease is carried out on a large scale by a number of companies in Europe, Japan, and the United States. The microorganisms involved are species of *Bacillus* (*Bacillus subtilis*, *Bacillus licheniformis*) and some genera of molds (*Aspergillus oryzae*, *Aspergillus niger*, *Mucor miehei*, *Mucor pusillus*, etc.). The bulk of bacterial proteases go to the detergent industries, followed by leather tanning- and food industries while fungal proteases go to food- and pharmaceutical industries.

10.18.1 ALKALINE SERINE PROTEASE

Alkaline serine protease, called Subtilisin Carlsberg, is the most widely used alkaline serine protease. It is obtained from *Bacillus licheniformis* by submerged fermentation. Subtilisin Novo is another alkaline serine protease of commercial interest. Although it is distinct from Subtilisin Carlsberg, it possesses many similar properties.

A typical medium for the production of Subtilisin Carlsberg by submerged fermentation is given in Table 10.8.

Other media are also available for this purpose. The temperature of fermentation in the range of 30-40°C has been found to be satisfactory. The pH of the production medium is kept at 7.0, as low pHs markedly lower the yield. The production of enzyme begins when maximum cell growth is achieved after 10-20 hrs and this continues at an almost constant rate till the completion of fermentation.

Table 10.8 Medium composition for the submerged fermentation of Subtilisin Carlsberg

Component	Amount (g/L)
Starch hydrolysate	50
Soybean meal	20
Casein	20
Na_2HPO_4	3.3

At the end of the productive fermentation, protease is the only protein present in the production medium. The reason for this is the occurrence of hydrolysis of all proteins present in the medium by protease. The yield may be 10% of the initial protein content of the medium. The enzyme is marketed primarily in the form of dust-free granules (see later). The granules contain 1-5% enzyme. The enzyme remains stable in liquid preparations, which contain about 2% of the enzyme.

10.18.2 FUNGAL ALKALINE PROTEASE

Fungal alkaline protease is mainly produced from *Aspergillus* species, in both solid-substrate and deep tank fermentations. Solid substrate cultures, extensively used in Japan, are carried out with wheat or rice bran or whole grains as the basic substrate. The production is inhibited be NH_4^+ but promoted by nitrate- and sodium salts of aspartate and glutamate.

10.18.3 ACID PROTEASE

Acid proteases constitute the most interesting group of proteases with respect to use in food industry. They are characterized by maximum activity and stability at pH 2.0-5.0. The molecular weight is around 35,000. Acid proteases are low in basic amino acid content and have low isoelectric pH. They are sensitive to SH-reagents, metal chelators, and heavy metals, and are generally stable in the acid range (pH 2-6), but are rapidly inactivated at higher pH values.

Acid proteases of commercial importance are exclusively produced from fungal sources and are tentatively divided into two subgroups by their physiological characteristics: *pepsin-like* protease (from *Aspergillus niger* var *macropus* and some species of *Penicillium* and *Rhizopus*) and *rennin-like* protease (from *Mucor miehei*, *Mucor pusillus*, etc.).

10.18.3.1 Production method for acid protease

The enzyme can be produced by either semi-solid culture or submerged culture, depending on the fungal species employed. For example, *Mucor pusillus* is cultivated

on a semi-solid medium. The medium consists of 60% wheat bran with water. The optimum temperature of fermentation is 30°C. The fermentation lasts for 3 days. The yield is 3,200 *Soxhlet units* per gram of wheat bran. 1 Soxhlet uint is the amount of enzyme activity which can coagulate 1 ml of milk solution in 40 min. The yield of enzyme can be increased by addition of ammonium salts. Finally, the enzyme is extracted with water. See Table 10.9 for medium composition of the fermentation.

Table 10.9 Medium composition for the submerged fermentation of acid protease

Component	Amount (%)
Starch	4
Soybean meal	3
Ground barley	10
$CaCO_3$	0.5

The fermentation is carried out at 30°C for 7 days. The yield is about 3,500 Soxhlet units per ml of broth.

The enzyme preparations, which contain 0.2-0.3% active enzyme, are marketed at concentration of 10,000-1,50,000 Soxhlet units per ml.

10.8.4 RENNET PRODUCTION BY *Mucor miehei*

Many microorganisms are capable of producing rennet, the milk clotting enzyme used in cheese production. Microorganisms like *Rhizomucor miehei*, *Rhizomucor pusillus*, *Endothia parasitica*, *Aspergillus oryzae*, and *Irpex lactis* are extensively used for rennet production. The aspartyl protease from *Mucor miehei* is commonly used as a chymosin substitute in cheese making. This enzyme has a high ratio of MCA/PA (milk clotting activity/proteolytic activity).

Rennet production using *Mucor miehei* can be carried out in solid-state as well as submerged fermentation. The strain (e.g., *Mucor miehei* NRRL 3420) is maintained on Sabouraud agar slants at 15°C. To recover the spores, the culture is grown on Sabouraud agar plate or Raux bottles at 35°C for 72 hrs. Thereafter the spore suspension (about 10^6 spores/ml) is grown in sterile broth. Growth as well as fermentation occurs in a medium maintained at pH 6. For submerged fermentation, inclusion of cornsteep liquor (2.2 g/liter), casein (2-4 g/liter), and glucose (18 g/liter) in the medium appears to be optimum. Molasses and sucrose are not considered good carbon sources. The highest enzyme activity occurs after about 48 hrs of cultivation. The aeration rate is maintained at around 2 vol/vol/min (with agitation).

For solid-state fermentation, wheat bran is moistened with 0.3*N* HCl and sterilized in autoclave. Inclusion of casein (0.1-0.2%) and skim milk powder (5%) in the bran gives better result. The enzyme can be recovered by extraction with water and subsequent centrifugation.

10.19 IMMOBILIZED ENZYMES

The use of enzymes in a soluble or free form must be considered as very wasteful because the enzyme generally cannot be recovered at the end of the reaction. This is where immobilization technique comes in. Present applications of immobilized enzymes are confined mainly to industrial processes, e.g., production of L-amino acids, organic acids, and fructose syrup. The future potential for immobilized enzymes lies in novel applications and the development of new products rather than as an alternative to existing processes using free enzymes.

Enzyme immobilization may be defined as confining the enzyme molecules to a distinct phase from one in which the substrates and the products are present; this may be achieved by fixing the enzyme molecules to or within some suitable material. It is critical that the substrates and the products move freely in and out of the phase to which the enzyme molecules are bound (confined). Immobilization of enzymes does not necessarily render them immobile; in some methods of immobilization, e.g., *entrapment* and *membrane confinement*, the enzymes are freely mobile within their phase, while in cases of *adsorption* and *covalent bonding* they are, in fact, immobile.

10.19.1 GENERAL TYPES OF IMMOBILIZATION

In practice, both physical and chemical methods are routinely used for enzyme immobilization. Physically, enzymes may be adsorbed onto an insoluble matrix, entrapped within a gel, or encapsulated within a microcapsule or behind a semi-permeable membrane. Chemically, enzymes may be covalently attached to solid supports or cross-linked. Before a brief treatment on the available methods of immobilization, see Fig. 10.16 for the summary of techniques used for the same.

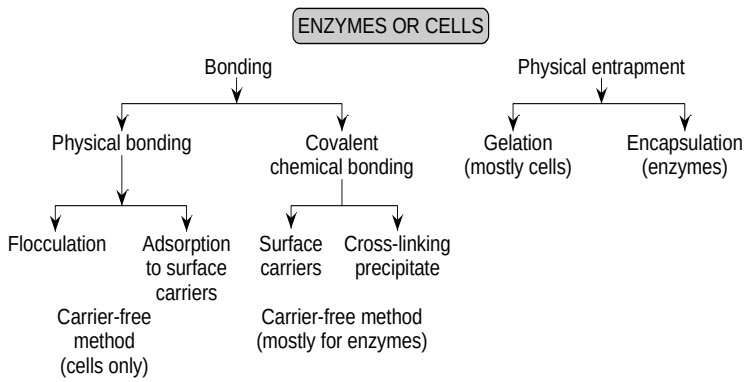


Fig. 10.16 Summary of cell and enzyme immobilization principles

10.19.1.1 Adsorption

The enzymes are adhered to the surface of carrier matrix (support) due to the combination of hydrophobic effect and formation of several *salt links* per enzyme molecule. The most widely used supports are *carboxymethyl cellulose* (CMC) and Diethylaminoethyl cellulose (DEAE cellulose). See Fig. 10.17 for the principle.

10.19.1.2 Covalent bonding

A large number of chemical reactions have been used for the covalent binding of enzymes by way of their non-essential functional groups to inorganic carriers such as ceramics, glass, iron, zirconium and titanium; to natural polymers such as sepharose and cellulose; and to synthetic polymers such as nylon, polyacrylamide and other vinyl polymers and copolymers possessing reactive chemical groups. In many of these procedures the covalent binding of enzymes to the carriers is non-specific, i.e., the binding of the enzyme to the carrier is by way of the enzyme's chemically active groups distributed at random. More recent studies have attempted to develop techniques of enzyme immobilization in which the enzyme binds to a carrier with high activity without affecting its catalytic activity.

Because of the covalent bond formed between the enzyme and the carrier molecule, the binding is very strong. The most commonly used matrices are agarose, cellulose, and polyacrylamide. The most useful amino acid residue (of the enzyme) in covalent bonding is that of lysine. Glutaraldehyde is another important support. An additional feature of glutaraldehyde is that it not only binds enzyme but also cross-links them (see Fig. 10.17).

10.19.1.3 Entrapment

The entrapment of enzymes in gel matrices is achieved by carrying out the polymerization or precipitation/coagulation reactions in the presence of the enzyme. Polyacrylamides, collagen, silica gel, etc., have all proved to be suitable matrices but the entrapment process is relatively difficult and results in low enzyme activity. During the polymerization, the enzyme molecules get entrapped within the gel or fiber of the carrier and it may or may not involve formation of covalent bonding. Cellulose triacetate, agar-agar, gelatin, alginate, etc., are some of the widely used support (see Fig. 10.17).

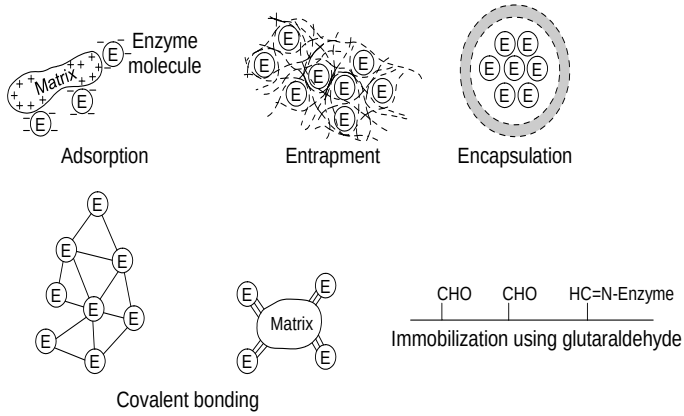


Fig. 10.17 Different immobilization methods

A very straightforward entrapment procedure usually carried out in laboratories is the entrapment of cells or enzymes in sodium alginate gel. A 3-4% aqueous solution of sodium alginate is prepared and the required amount of cell suspension is added (and mixed). With the help of a pipette or a syringe, the mixture is transferred drop-wise to a 2% aqueous solution of calcium chloride. Calcium chloride is a denaturant that causes hardening of the gel surface by exchanging sodium ions for calcium ions. The gel droplets change into porous, plastic beads. The beads can be taken out, washed with water or buffer, and used in place of enzyme. They can also be stored for future use.

10.19.1.4 Membrane confinement

In this method, the enzyme molecules are confined within a semi-permeable membrane. *Encapsulation* is one of the most widely used methods of membrane confinement. Nylon, liposomes, polyacetic acid, etc., may be used for the encapsulation (see Fig. 10.17).

It must be noted that not all immobilization techniques yield equally efficient enzymes. Some of the differences, especially with respect to advantages and limitations, among the immobilization techniques appear in Table 10.10.

Table 10.10 Comparison of different types of immobilization techniques

Method	Advantages	Disadvantages
Covalent attachment	Not affected by pH, ionic strength of the medium or substrate concentration	Active site may be modified; costly process
Covalent cross-linking	Enzyme strongly bound, thus unlikely to be lost	Loss of enzyme activity during preparation; not effective for macromolecular substrates; regeneration of carrier not possible
Adsorption	Simple with no modification of enzyme; regeneration of carrier possible; cheap technique	Changes in ionic strength may cause desorption; enzyme subject to microbial or proteolytic enzyme attack
Entrapment	No chemical modification of enzyme; enzyme not subject to microbial or proteolytic action	Diffusion of substrate to, and product from the active site; preparation difficult and often results in enzyme inactivation; continuous loss of enzyme due to distribution of pore size; not effective for macromolecular substrates

Immobilized whole cells are becoming increasingly utilized and tend to eliminate the tedious, time-consuming and expensive enzyme purification steps. Immobilization of whole cells is normally achieved by the same methods as for cell-free enzymes. The greatest potential for immobilized cell system lies in replacing complex fermentations such as secondary product formation (i.e., semi-synthetic antibiotics),

in the continuous monitoring of chemical processes (via enzyme electrodes), water analysis and waste treatment, continuous malting processes, nitrogen fixation, and synthesis of steroids and other valuable medical products.

The advantages of immobilized system over normal biocatalysts (cells or enzymes) are:

- Permits the reuse of the component enzyme(s)
- Ideal for continuous operation
- Product is enzyme-free
- Permits more accurate control of catalytic process
- Improves stability of enzymes
- Allows development of a multi-enzyme reaction system
- Offers considerable potential in industrial and medical use
- Reduces effluent disposal problems
- Allows savings in downstream processing
- The reaction is specific and faster
- Certain stereochemical reaction are impractical with chemical methods

Immobilized systems also have their associated limitations, the important ones of which are:

- The cost of enzyme is high
- The activity is sometimes inferior to free enzymes because of undesirable conformational changes resulting from immobilization
- Not all enzymes can be immobilized by general methods. Suitable protocols must be developed in such cases.

It is also possible to make a comparison between immobilized cells and immobilized enzymes. Immobilized enzymes offer the advantage of minimum byproduct formation and a high product yield (because the substrate is not converted to biomass). On the other hand, enzymes can be less stable. Besides, it is difficult to run an enzymatic process that requires multienzyme complex. At this point, immobilized cells have tremendous advantage over purely enzymatic process. A whole cell can in fact be considered a bag of enzymes and therefore has a far greater potential.

10.19.2 USE OF IMMOBILIZED ENZYMES IN INDUSTRIAL PROCESSES

In industrial practice, immobilized enzymes or immobilized whole cells are generally utilized within the confines of bioreactors. Bioreactor systems can have many forms depending on the type of reaction and stability of the enzyme. Some of the important types of enzyme reactors are (i) *stirred tank reactor*, (ii) *membrane reactor*, and (iii) *continuous flow reactor* (e.g., *packed bed reactor*, *continuous flow stirred tank reactor*, and *fluidized bed reactor*). A schematic sketch of each of the above reactors is given in Fig. 10.18.

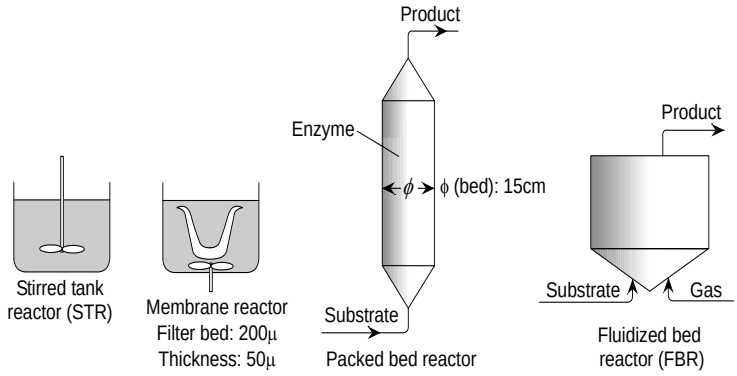


Fig. 10.18 Different types of enzyme reactors

10.19.2.1 Production of high fructose corn syrup (HFCS)

D-glucose is only 70% as sweet as sucrose and is comparatively less soluble in water. Fructose is 30% as sweet as sucrose and is twice soluble in water. Therefore glucose syrup is treated with glucose isomerase to produce high fructose syrup (42-55% fructose). The enzyme is obtained from *Actinoplanes missouriensis*, *Bacillus coagulans*, and *Streptomyces* species. The enzyme is immobilized by cross-linking with glutaraldehyde and the process of isomerization carried out in continuous packed bed reactor (PBR). The enzyme is used continuously for about 150 days, which is equal to 3 half-lives of the enzyme. 1 kg of enzyme yields 10-11 MT of 42% fructose syrup. See Fig. 10.19 for the flow diagram of production of high-fructose syrup.

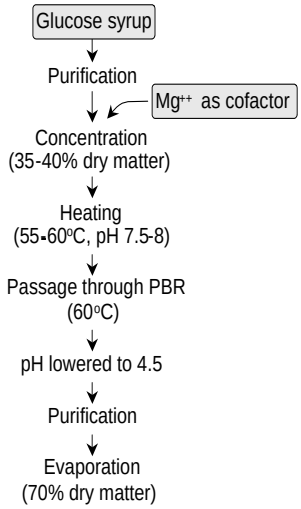
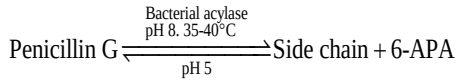


Fig. 10.19 Production of high fructose syrup by enzymatic method

10.19.2.2 Production of semi-synthetic antibiotics

Semi-synthetic penicillins are produced by a combination of microbial and enzymatic or chemical process. 6-aminopenicillanic acid (6-APA) is the principal intermediary in the manufacture of semi-synthetic penicillins. This must first be obtained in sufficient amounts. Under suitable conditions, *Penicillium chrysogenum* produces large amounts of 6-APA by *interrupted* biosynthesis. Interrupted biosynthesis is now no longer economical. Once the 6-APA is obtained, it can be joined by chemical means with any other desired side chain. The trade production of 6-APA starts from either penicillin G or V. The side chains in these natural penicillins are first removed and the nucleus recovered for the chemical fusion with the desired side chain. The hydrolysis of penicillin G and V can be carried out with the help of an enzyme called *penicillin acylase* or *amidase*. Acylases are produced by yeasts, bacteria as well as molds but the commercially used penicillin acylase probably comes from molds and bacteria (*E. coli*). Microbial hydrolysis of natural penicillins is favored at high temperature and alkaline pH. Bacterial acylase is more specific for the hydrolysis of penicillin G. Mold acylase is specific for penicillin V. The reaction can be reversed when the pH is reversed.



Semi-synthetic penicillins have been developed to overcome the shortcomings in the natural penicillins. Today, thousands of such semi-synthetic penicillins have been prepared and a number of them have been found to be superior to natural penicillins in oral absorption, acid stability, and resistance to penicillin-inactivating enzymes. See Fig. 10.20 for some examples of some semisynthetic penicillins.

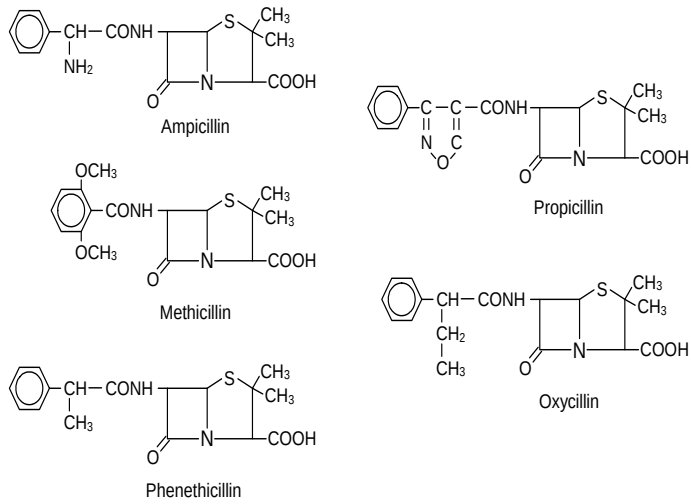


Fig. 10.20 Some examples of semisynthetic penicillins

The enzymatic hydrolysis of penicillin V or G can be carried out using immobilized acylase. There are several patented- and industrial methods of acylase immobilization. A typical method used by Hindustan antibiotics involve covalent

linking of the enzyme in cyanogen bromide-activated sephadex 200 (see Fig. 10.21). Either batch or semi-continuous method can be used. The reactors can range from STR to PBR. In a packed bed reactor (Fig. 10.18 for an idea about PBR), the enzyme has been used for 100 days yielding 2 MT of 6-APA per kg of immobilized enzyme.

In essence, the process of 6-APA production is as follows:

Penicillin substrate is dissolved in methanol and passed through the reactor for the hydrolysis to take place at about 36°C. A pH of about 8 is maintained with 4*N* NaOH during the reaction. When the enzymatic hydrolysis is over, the released side chains are extracted with organic solvent and the 6-APA isolated by precipitation at pH 4.2 (adjusted with 6*N* HCl). The precipitate is filtered, washed with methanol, and dried under vacuum. In general, the purity of the product is more than 90% and the yield is about 86%.

At least 3500 MT of 6-APA are produced each year, requiring about 30 MT of the enzyme.

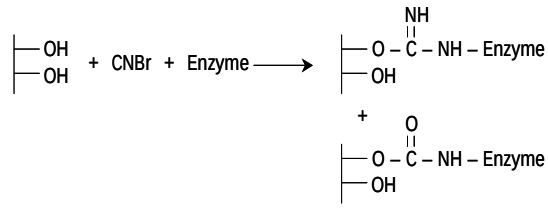


Fig. 10.21 Immobilization of penicillin acylase

Before bringing the 6-APA and the side chains together for condensation, the side chains must be activated. These days, the activation of 6-APA is done by silylation (replacement of an acidic hydrogen on the compound with an alkylsilyl group, for example, N-trimethylsilylacetamide and other silicon hydride derivatives). The process entails mixing of 1 equivalent of 6-APA with 1-2 equivalents of silylating agent in an inert solvent, allowing the reaction, bringing the mixture to isoelectric pH for precipitation, and recovery by filtration.

A method described in a patent for the production of ampicillin trihydrate is as follows:

52.5 g of N-trimethylsilylacetamide was added to a suspension of 6-APA in 350 ml of methylene chloride under agitation. The reaction was carried out at 40°C for 2 hrs and then cooled to -25°C. Abundant precipitation occurred during the cooling stage. 43.3 g of α-phenylglycyl-chloride-HCl (an acyl halide) was added at -25°C and the temperature was brought to -5°C. After holding for 1.5 hrs, 450 ml of water was added to the mixture. pH was adjusted to 4.5 with dilute NH_4OH. The mixture was then agitated for 1 hr at 10°C. The precipitate was filtered, washed 2 times with 75 ml portions of water, 3 times with 125 ml portions of acetone, and dried at 35°C. The yield was about 81%.

10.19.3 USE OF IMMOBILIZED ENZYMES IN BIOSENSORS

Biosensors are group of sensors that possess a biological sensing layer, comprising a receptor and an antibody or enzyme intimately associated with a transducer. These produce a signal (electrochemical, optical or thermal) which is suitably calibrated. Glucose biosensor is an example. An Indian version of this biosensor uses glucose oxidase that is immobilized on an electrode surface. It can assay more than 100 samples per run, is stable for several weeks and is sensitive to 0-15 millimoles. The enzyme is trapped in a *liposomal* bag. Biosensors hold an immense potential in fields as diverse as diagnosis, agriculture, environmental monitoring and defense. See Fig. 10.22 for component parts and working principle of a biosensor.

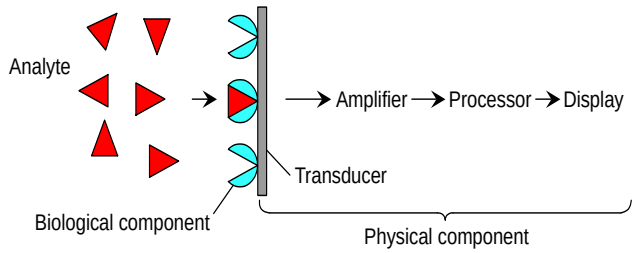


Fig. 10.22 Component parts of a biosensor

10.20 ENZYME ENGINEERING

Improvement in the activity and usefulness of an existing enzyme or creation of a new enzyme by making suitable changes in its amino acid sequence is called *enzyme engineering*. Enzyme engineering is a part of the larger activity of protein engineering. Enzyme engineering utilizes recombinant technology to introduce the desired changes in the amino acid sequences of enzymes. It must be understood that genetic engineering in which whole genes are transferred from one organism to another is not enzyme engineering. Enzyme engineering rests on modification of amino acid sequence of the concerned enzyme.

The chief objective of enzyme engineering is to produce an enzyme that is more useful for industrial and/or other applications. The various properties of an enzyme that may be modified to achieve this objective are as follows:

- Improved kinetic properties
- Elimination of allosteric regulation
- Enhanced substrate reaction specificity
- Increased thermostability
- Alteration in optimal pH
- Suitability for use in organic solvents, and
- Increased/decreased optimal temperature, etc.

10.20.1 PRINCIPLES OF ENZYME ENGINEERING

The structure and function of an enzyme molecule, or for that matter any protein molecule, are chiefly determined by its amino acid sequence, i.e., its *primary structure*. Therefore any change in the properties of an enzyme is always reflected in its primary structure. Conversely, a change in the amino acid sequence should alter the properties of the enzymes. But this is not always the case because the enzymatic properties, etc., are changed only when amino acid changes are introduced in certain *critical* regions of the proteins. Therefore it is of great importance to know the critical regions for the various functions of an enzyme, and to be able to predict the effect of specific amino acid changes in these areas on the various functions. However, the knowledge of the relationships between amino acid sequence and three-dimensional structure and properties of enzymes, obtained from a larger database, is only partially operative. It allows an explanation of the changes in structure and function on the basis of the changes in amino acid sequence, but it does not allow a dependable prediction of the influence of genetic amino acid changes on the structure and function of an enzyme.

It may, however, be reasonable to anticipate that as more elaborate databases and improved softwares become available, it should become possible to predict with far greater confidence the structural and functional changes in enzymes produced by the specified changes in their amino acid sequences. The effectiveness of enzyme engineering will be greatly enhanced then, and this activity may have a tremendous influence on enzyme technology.

CHAPTER 11

YEAST TECHNOLOGY

11.1 INTRODUCTION

Yeasts are fungi, which in a stage in their life cycle, occur as single cells, reproducing commonly by budding or less frequently by fission. They lack chlorophyll. Hence they must live a saprophytic or in some cases parasitic lifestyle. They have rather rigid, thick cell walls, have a well-organized nucleus with a nuclear membrane, and have no motile stages. With some exceptions, they do not form mycelial structures. They are typically 4-5 μm in size.

11.2 TAXONOMIC CONSIDERATION

In the currently accepted classification, yeasts are arranged hierarchically into subdivisions, families, subfamilies, genera and species. At the highest level, identification is based on fundamental aspects of *yeast sexuality* (Ascomycotina or Basidiomycotina), or the lack of it (Deuteromycotina). Ascomycotina is a subdivision that includes ascosporogenous yeasts. By analogy, Basidiomycotina includes basidiomycetous yeasts. Deuteromycotina includes those that do not produce sexual spore.

The above classification scheme, however, does not necessarily imply sporulation as the dominant mode of reproduction. Out of the four main subdivisions of the fungal kingdom Mycetae, the abovementioned three subdivisions contain yeasts but the main domain is Ascomycotina. This subdivision contains the more familiar yeasts such as *Saccharomyces, Schizosaccharomyces, Hansenula*, *Debaryomyces*, *Endomycopsis, Kluyveromyces*, etc. The Deuteromycetes or "fungi imperfect" includes *Brettanomyces, Bullera, Candida, Cryptococcus, Kloeckera, Rhodotorula, Torulopsis* and a number of important molds such as *Aspergillus* and *Penicillium.* Basidiomycotina is lesser known to contain yeast. *Leucosporidium, Filobasidium, Rhodosporidium,* etc., are present in this subdivision. Molds such as *Rhizopus* and *Mucor* are included in the fourth subdivision Zygomycotina. See Fig. 11.1 for a recapitulative diagram.

As of now, there are 86 genera of yeasts consisting of 597 species. Genera are primarily distinguished on the basis of morphological characteristics and sporing details. Speciation, on the other hand, relies heavily on nutritional requirements.

11.3 REPRODUCTION IN YEASTS

In general, yeast can reproduce sexually as well as asexually. Asexual mode consists of (i) budding, (ii) pseudomycelia formation, (iii) fission, (iv) bud-fission, (v) budding and fission, and (vi) clamp connection.

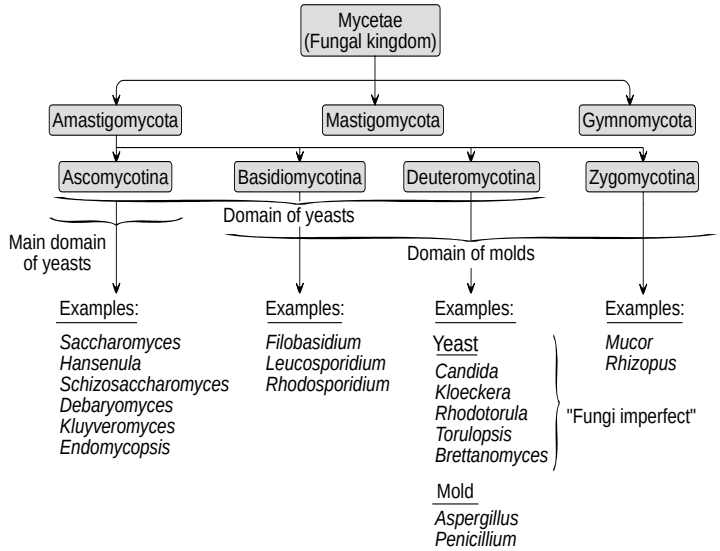


Fig. 11.1 Recapitulative diagram for classification of yeast

The sexual mode of reproduction in yeasts consists of sporulation in which yeast sexuality can be homo- or heterothallic. *Parasexuality*, in which yeast alternates between haploid and diploid phases without the formation of sexual spores, is also observed. Of the asexual modes, budding and fission are by far the most important ones.

11.3.1 BUDDING

The development of a new bud begins with a localized weakening of the mother cell wall (because of weakening of sulfhydryl bridges in the wall protein). The bud continues to grow and after the nucleus copy has migrated to the budding cell, septum wall is formed. The pattern of budding is variable. In *S. cerevisiae* and some other ascomycetes it is multilateral and can occur at any point except at a *previous* budding site. Other yeasts have bipolar or even tripolar budding pattern in which budding occurs repeatedly at the same point. Such variations in budding often give characteristic morphology to yeasts. See Fig. 11.2a for some typical budding patterns and the resulting cell morphologies.

Laboratory *Saccharomyces* yeast is produced by cross-breeding. The yeast has a sexual life cycle. Haploid cells have one set of chromosomes (n = 16) and are of either mating Type *a* or mating Type α. Such haploid cells cannot sporulate. When mixed together, *a*-cells can mate with α-cells, forming diploids (2n = 32) zygotes containing a double set of chromosomes. Both haploid and diploid cells can multiply asexually by budding. Under certain starvation conditions, the diploid cells can undergo sporulation, resulting in the formation of asci containing 4 spores. These spores contain the haploid (n = 16) number of chromosomes and can germinate to give rise to two *a*-, and two α-cell cultures. Lager brewing yeast strains are genetically more complicated, being species hybrids carrying the tetraploid (4n = 64) number of

chromosomes. Furthermore, they are heterozygous (carrying more than one type of certain gene). Sporulation and subsequent intercrossing of the spore clones may form new combinations of genes, resulting in yeast strains with altered characteristics, some of which may be attractive to the brewer. See Fig. 11.2b for an idea about the mechanism of cross-breeding.

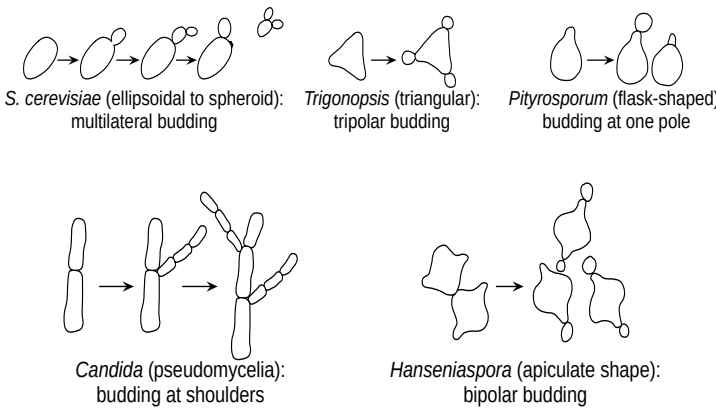


Fig. 11.2a Budding pattern and resulting morphology of yeast cells

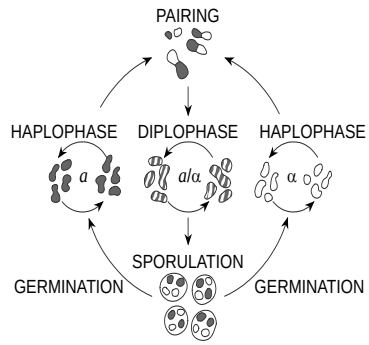


Fig. 11.2b Cross-breeding mechanism in yeast

11.4 INDUSTRIAL CATEGORIZATION OF YEASTS

Yeasts can be categorized in several ways. In industries, yeasts are generally categorized as:

1. Culture yeast versus *Wild yeast*

The yeast used in an industry for a particular process is called *culture* yeast. The rest becomes *wild* yeast for that particular process. As an example, brewer's yeast from *San Miguel* can be a wild yeast for *Tuborg* beer.

2. Top yeast versus *Bottom yeast*

Top yeasts rise to the top of the fermentation broth after the fermentation has completed. By analogy, *bottom* yeasts settle down after the fermentation has completed. *S. cerevisiae* var *cerevisiae* is the representative yeast for top yeast. *S. cerevisiae* var *uvarum* (*carlsbergensis*) is an example of bottom yeast. There is one very important biochemical difference between top- and the bottom yeast. Top yeasts cannot utilize *melibiose*, (a disaccharide) while bottom yeasts can. This is because bottom yeast possesses a gene called *MEL*, which is responsible for the production of an enzyme called *melibiase* (α-galactosidase) needed for the breakdown of melibiose. Bottom yeasts can also utilize raffinose, a trisaccharide. Top yeast does not have *MEL* genes. It yeast can utilize only 1/3 of raffinose (see Fig. 11.3 also). Another fundamental difference between top yeast and bottom yeast is the ability to produce alcohol. Top yeasts generally produce more alcohol (up to or above 18% *abv*) than the bottom yeast (8% *abv*). The notation *abv* is used here for "alcohol by volume".

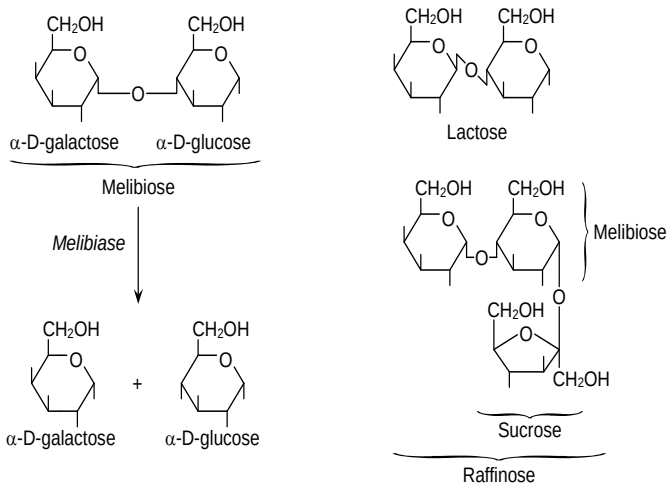


Fig. 11.3 Melibiase activity in bottom yeast

3. Flocculent versus *Powdery yeast (non-flocculent)*

Flocculent yeasts are those that flocculate after maturation or completion of fermentation, e.g., top and bottom yeast. *Powdery* yeast derives its name from its non-flocculent nature. The cells remain suspended in the broth to give a turbid impression (like powder). Flocculation refers to reversible aggregation of cells to form a fluffy mass (floc). Flocculation has great importance in fermentation in that it helps in the removal of cells from the fermentation broth.

11.4.1 SOME IMPORTANT USES OF YEASTS

Yeasts are probably the oldest of microorganisms used (and cultivated) by man. Man has used them for bread-making and alcohol fermentation since pre-historic times.

Today, yeasts have much more diverse uses. Some of the more important uses of yeasts are:

- Production of Single Cell Protein (food and fodder)
- Production of enzymes (lactase, invertase, lipase)
- Microbiological assay
- Production of leavened breads
- Production of alcoholic beverages (beer, wine, etc.)
- Production of vitamins (riboflavin)
- Production of flavors
- Genetic engineering studies

11.5 FOOD YEAST

Food yeasts come in three important forms: (i) active cells, (ii) killed cells, and (iii) processed products.

11.5.1 DRIED YEAST

Dried Yeasts are nutritional concentrates employed as a source of protein and vitamin B-complex to enrich food and feed. Dried Yeasts are heat-killed yeasts obtained either by *primary fermentation* or separated and recovered as cell concentrates from brewing and distilling residues. Baker's yeast, *Candida*, *Rhodotorula*, and *Cryptococcus* (torula) can also be used for the same. Details of production of the source yeast are similar to those of bakers yeast production.

To qualify as food yeast, Dried Yeast must be of acceptable type in terms of flavor, color, microbial purity, chemical composition and vitamin content. Additionally, the yeast must conform to the specifications covered by three recognized definitions of Dried Yeast: (i) The International Union for Pure and Applied Chemistry (IUPAC), (ii) National Formulary (NF-XII) of American Pharmaceutical Association, and (iii) FDA. See Table 11.1 for the IUPAC Standard.

Table 11.1 IUPAC Standard for Dried Yeast (food)

Parameter	Value
Moisture % (max)	10
Ash% (max)	10
Live bacteria count (max)	7500/g
Mold count (max)	50/g
Nitrogen % (min)	7.2 (≈ 45% protein)
Thiamine µg/g (min)	10
Riboflavin µg/g (min)	30
Niacin µg/g (min)	300
Lead and arsenic µg/g (max)	5
Starch and bacteria of the genus *Salmonella*	Nil

- In fabricated foods such as baked goods, baby foods, geriatric foods, and as extenders. The concentration varies between a few tenths to about 2%.
- In health food industries as solid tablets or dry powders. The preparation is usually fortified with water-soluble vitamins.

11.5.2 YEAST AUTOLYSATES

Yeast autolysates (self digests) are produced by the induced action of intracellular enzymes (principally proteases) on polymeric proteins of yeast cells. Autolysis is not limited to proteins: carbohydrates and nucleoproteins are also degraded by their respective hydrolytic enzymes.

The production of yeast autolysates is carried out at temperatures which kill the yeast cells but do not inactivate the hydrolases (that is, at a temperature between 40 and 55°C). It is advantageous to initiate autolysis by the addition of plasmolyzing agents, which may also serve as antiseptics to suppress the growth of thermophilic bacteria. Some of the more common antiseptics are chloroform, toluene, thymol, phenol, ether, ethyl acetate, and formaldehyde.

Autolysis is carried out at a slightly acidic pH. It may proceed for 12-36 hrs depending on the degree of hydrolysis required. Customarily, proteolysis is carried out to a point where 25-50% of the nitrogen of the cell is present as α-amino nitrogen. The whole is then pasteurized at 80-90°C, cooled, and filtered with diatomaceous earth. The filtrate can now be concentrated under vacuum to a paste of 80% solids. Frequently, salt is added to the autolysate before spray-drying since the material is generally used as a condiment. A typical composition of the paste is given in Table 11.2.

The starting material can be brewers yeast (either bitter or debittered) or bakers yeast. The composition of commercially available autolysates varies greatly with the source, the processing conditions, and the addition of MSG, salt, and 5'-ribonucleoside. Most commercial products are indeed extracts since autolysis has been followed by filtration to remove the insoluble cell wall debris. The extracts are completely water-soluble and form clear solutions whose colors vary between amber and brown.

Table 11.2 Typical composition of yeast autolysates paste

Parameters	Percentage
Total solids	80
Salt	15
Ash (other than added salt)	6
Total nitrogen	6.5-7.5
α-amino nitrogen	2-4
pH	5-6

11.5.2.1 Uses of yeast autolysate

Due to pleasant meat-like flavor and aroma, it finds use in soups, gravies, meat dishes, pet foods, and generally as condiments. In Australia and New Zealand, and to some extent in Great Britain, autolysate in paste form is used as bread-spread. Yeast autolysate finds use in food fermentation industries as fermentation nutrient. It is also an indispensable component of routine media used in microbiology laboratory

11.5.2 FEED YEAST

In the West, use of yeast as an ingredient in feed and feed concentrate is well established. Indeed, a major fraction of the brewers yeast and *Candida utilis* is used in feed. A special application is the use of active dry yeast or compressed yeast (see later) in the preparation of 'yeast culture'. This feed supplement is prepared by seeding a cereal grain mash with yeast cells, incubating, and then drying the seeded mash under controlled condition so that live yeast cells, enzymes, and other heat-sensitive nutritional factors are preserved.

For the most part, however, feed yeast is produced as Dried Yeast which can be categorized as *Primary Dried Yeast*, *Brewers Dried Yeast*, *Distillers Dried Yeast*, *Torula Dried Yeast*, etc. To qualify as feed supplement, Dried Yeast must be non-fermentative, and contain not less than 40% protein. A typical composition of Brewers Dried Yeast is given in Table 11.3.

Recently, *Candida utilis* (also known as feed- or fodder yeast) has received considerable attention. Unlike bakers yeast, *Candida utilis* does not have stringent cultural and environmental requirements. The respiratory capacity of *Candida utilis* is higher than that of bakers yeast. It is immune to "glucose effect" (see later, page 154) and can utilize a wide range of cheap substrates. Additionally, since the organism is versatile, very little alcohol is generated, which means much of the carbon source is diverted to storage polysaccharides. See Fig. 11.4 for the metabolic route in *Candida utilis*.

Table 11.3 Typical composition of Brewers Dried Yeast

Parameter	Value
Protein (%)	> 45
Fat (%)	≈ 2
Fiber (%)	≈ 2
Ash (%)	7-8
Thiamine	Adequate amounts
Riboflavin	Adequate amounts
Niacin	Adequate amounts
Pantothenic acid	Adequate amounts

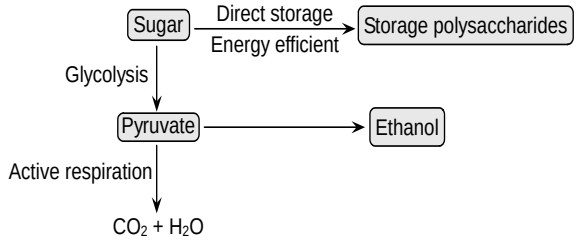


Fig. 11.4: Metabolic route in *Candida utilis*

Yeasts are commercially grown for feed yeast production only if the process is economically justifiable, e.g., as in the case of *Candida utilis*. In other cases, yeast is obtained from other sources such as brewery, bakers yeast industry, distillery, etc. When yeast is grown *primarily* for feed yeast production, it is commonly referred to as Primary Grown Yeast. See Fig. 11.5 for generalization of the description made above.

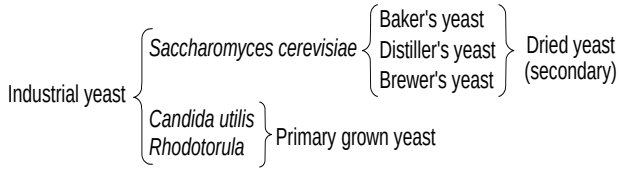


Fig. 11.5 Use of yeast for feed production

11.5.2.1 Production outline of fodder (feed) yeast

The method of fodder yeast production from sugar sources is similar to that of bakers yeast production, except for the utilization of continuous culture techniques. The functional property of bakers yeast is not a requirement here because the cells are used as a protein source only.

The fermentation vessel is sized according to the desired production level. The specific growth rate, μ, of microorganisms is generally limited to a defined region for optimum cellular protein level and biomass-to-substrate yield. Fermenters of 1500 to 2000 m^3 are not uncommon. The aeration systems used for bakers yeast production do not work in fermenters of sizes above 200 m^3 and so the aeration system becomes more sophisticated. Two types of aeration systems seem appropriate here. They are *airlift* design and the *Rumanian plant aeration* system. In particular, ICI (Imperial Chemical Industries)-airlift fermenters have no mechanical parts for agitation. The forced air passing through the fermenter is responsible for the required degree of mixing. The operating region most suitable (industrially) is a dilution rate of 0.1 to 0.2 h^{-1}. Under this arrangement, a combination of high biomass yield, high cell protein content, and economically practicable oxygen supply is possible.

11.6 BAKERS YEAST

Bakers yeast belongs to *Saccharomyces cerevisiae*. There are many different strains in this group, all of them having somewhat different qualities. The yeast manufacturer therefore takes great pain to find out strain most suited to various local conditions.

Improved strains are chosen not only on the basis of yield, growth rate, genetic stability, etc., but also for the following properties:

- Gas generation rate
- Stability on storage
- Ability to withstand drying
- Osmotolerance
- Low alcohol generation
- Ability to disperse readily in water
- Mild flavor
- Ability to propagate readily

11.6.1 NUTRITION FOR BAKERS YEAST

As with all organisms, yeast needs energy source, carbon source, electron source, nitrogen, minerals, growth factors, water, and oxygen for growth and reproduction. The description made in the following paragraphs relates to nutritional requirements of bakers yeast.

11.6.1.1 Carbon source

In bakers yeast production, the most important carbon source is the sugar present in molasses. Molasses can be either from beet or sugarcane. Usually, the two molasses are mixed in a suitable ratio for the mutual supplementation of nutrients and growth factors. Some of the important physicochemical properties of cane and beet molasses are given in Table 11.4.

Table 11.4 Physico-chemical properties of cane and beet molasses

Description	Beet	Cane
Dry matter (%)	74-78	75
Fermentable sugar (%)	45-47	46-52
Invert sugar (%)	0.2-1.2	15-20
Nitrogen compounds (%)	6-8	2-3
Betaine* (%)	3-4	--
Glutamic acid (%)	2-3	--
Ash (%)	10-12	10-15
Potassium (%)	2-7	1.5-5
Vitamins		
Biotin (ppm)	0.04-0.13	1.2-3.2
pH	7-9	5-6

* Betaine, $(CH_3)_3N^+CH_2COO^-$ (= trimethyl amino acetate) is a nitrogenous compound found only in beet molasses. It is not utilized by yeast. Betaine increases BOD of the effluent.

Molasses treatment

Molasses as it comes in tank cars is of about 80° brix with a fermentable sugar of about 45-50%. It first undergoes an initial treatment, the purpose of which is to remove colloids, firm particles, and to kill unwanted microorganisms. In the normal procedure, molasses is diluted to 35-45° brix and pH adjusted to 5 by adding H_2SO_4. Clarification is done by centrifugation or filtration. The clarified molasses is heated to boiling point and kept at this temperature for a couple of hours after which the liquid is drawn out from the settled sediments. The molasses is now cooled to either 75°C or 20°C and stored until required. See Fig. 11.6 for the protocol for molasses treatment.

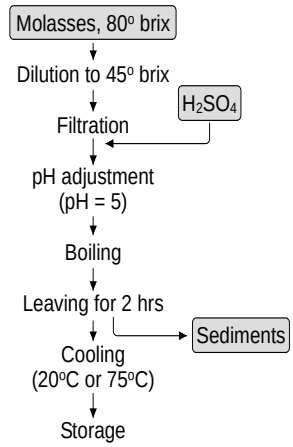


Fig. 11.6 Treatment of molasses before using as an the medium

Alternatively, HTST treatment (140°C for 4 sec) can be done using live steam. The use of extreme storage temperatures ensures that the contaminants that might gain entry during the course of storage do not multiply.

11.6.1.2 Nitrogen source

Amino acids in beet molasses comprise about one-third of total nitrogenous compounds present in it. All of these amino acids (present mainly as glutamic acid) are quickly utilized by the yeasts. Rest of the requirement must be met by external addition of nitrogen source, usually ammonia, ammonium salts (ammonium sulfate, ammonium phosphates, ammonium nitrate, ammonium chloride), and urea. If cane molasses is used, the entire requirement of nitrogen must be met by external supply.

11.6.1.3 Minerals

Yeast needs a wide range of minerals. The more important ones are phosphorus, sulfur, potassium, magnesium, calcium, sodium, barium, zinc, iron, and manganese. In bakers yeast production, the only mineral supplied is phosphorus. Other minerals are automatically inclusive in the medium because of the complex nature of molasses. For trade fermentation, phosphorus is supplied in the form of H_3PO_4,

KH_2PO_4, Na_2HPO_4, $NH_4H_2PO_4$, etc., because it is the inorganic phosphate (and not the elemental phosphorus) that is assimilated by yeasts. Sulfur, if required, may be added as K_2SO_4 or $Na_2S_2O_3$, although the yeast can also utilize sulfur-containing amino acids.

11.6.1.4 Growth factors

Growth factor requirement varies amongst species and strain, as does the actual concentration. A typical data for the most common strain of *S. cerevisiae* is given in Table 11.5.

Table 11.5 Growth factor requirement for *S. cerevisiae*

Growth factor	Requirement (mg/L)
Inositol	125
Ca-pantothenate	6.25
Pryridoxine-HCl	6.25
Thiamine-HCl	5.0
Nicotinic acid	5.0
Biotin	1.0

Folic acid, *p*-aminobenzoic acid, and riboflavin are occasionally added to the medium although normally the yeast can synthesize them all. Biotin requirement in particular is very critical in bakers yeast production using molasses. Beet molasses, being low in biotin, must be blended with cane molasses (10-20% cane molasses) to achieve the balance. The mixing of cane and beet molasses is desirable for other reasons also, namely, for balancing nitrogen and reducing sugar levels.

11.6.1.5 Anaerobic growth factors

Yeast requires *ergosterol* and *unsaturated fatty acids* for the synthesis of its cell membrane components. This requirement vanishes when the yeast is supplied with oxygen: oxygen is utilized by the yeast, among other things, for the synthesis of ergosterol and unsaturated fatty acids. This fact implies that ergosterol and unsaturated fatty acids are *anaerobic* growth factors for yeasts.

11.6.1.6 Water

Commercial production of bakers yeast requires water to be potable. Contextually, the term 'potable' implies 'fit for drinking purpose'. Stated differently, water should be acceptable in terms of all physicochemical qualities. See Table 11.7 for other requirements.

Table 11.6 Water standard for bakers yeast production

Parameter	Requirements
Oxygen demand (ppm)	10
Total hardness (ppm $CaCO_3$)	150 (max)
Iron (pm)	0.1 (max)
Manganese (ppm)	0.05 (max)
Total count	
At 21°C	100/ml (max)
At 37°C	10/ml (max)
Coliforms and *Clostridium. perfringens*	0/100 ml

11.6.1.7 Oxygen requirement

Oxygen is required for the synthesis of cell components as well as respiratory metabolism. A quantitative reflection of the oxygen requirement can be shown by the following stoichiometry:

$$\left.\begin{array}{l}200\text{ g sucrose}\\+\\10.32\text{ g }NH_3\\+\\100.44\text{ g }O_2\end{array}\right\} \longrightarrow \left\{\begin{array}{l}100\text{ g yeast solids}\\+\\140.14\text{ g }CO_2\\+\\78.12\text{ g }H_2O\end{array}\right.$$

Thus, theoretically, 1 g of O_2 is required for every gram of dried yeast produced. Under practical condition, however, the supply of oxygen should exceed the theoretical value by several fold. This is because oxygen is supplied in the form of air and partly because the supplied air is vented off to facilitate agitation. The cells thus consume only about 20% of the oxygen supplied, the rest being vented away.

11.6.2 CULTURAL ENVIRONMENT FOR BAKERS YEAST

Besides supplying a well-balanced medium, attention must also be paid on various requirements such as pH, aeration rate, and temperature of fermentation. Yeasts exhibit a wide range of pH and temperature optima for growth. The pH may range from 2.8-6.5 and temperature 0-47°C. However, the majority of the commercially important yeasts grow best at pHs between 3.5 and 5 and temperatures between 10 and 33°C. It must be noted, the pH and temperature optima for growth may not be the same as those for metabolite- or product formation. For bakers yeast, the optimum pH is 4.5-5 and the optimum temperature is around 30°C.

11.6.3 OUTLINE OF BAKERS YEAST PRODUCTION

The principal sources of carbon and energy for bakers yeast production are the fermentable sugars from cane- and beet molasses. Whenever possible, a judicious mixture of these two is used as the substrate. The fermentation medium is

supplemented with ammonia, ammonium salts, or urea for nitrogen. For phosphorus, orthophosphates are used. The medium must also contain other minerals and growth factors in adequate amounts.

Customarily, the production is carried out in *multiple stages*. The inoculum is built up separately in a series of propagators before the final pitching. The final trade fermentation is carried out under highly aerobic condition, and with incremental feeding (fed-batch mode) of the sterilized molasses wort of 45° brix. The feeding and the aeration rates are controlled such that the reducing sugar content of the medium at any instant is about 0.2 g/lit, the specific growth rate (μ) about 0.2, and the respiratory quotient (RQ) about 1. The controlled feeding is required to shift the process from *fermentative* mode to *respiratory* mode. The optimum pH and temperature are 4-6 and 30°C respectively. Normally, the duration of fermentation is about 8-20 hrs. The yeast increases by five to tenfold (~ 3 generations) and the concentration of yeast solids reaches 4-6% at the end of the fermentation period. The fermentation is terminated with half an hour of *maturation* in which the feeding is stopped but aeration is continued. The nominal capacity of the main fermenter is 200 m^3. About 20 MT of *sales yeast* (yeast mass with 27% dry matter) can be obtained per batch.

After maturation, the yeast cells are recovered using a battery of centrifuges. The step produces yeast cream of about 15-18% dry matter. The cream is stored under refrigeration for future use or can be passed through filter press for further concentration (27-28% yeast solids). Alternatively, concentration may also be achieved by a rotary vacuum filter (Fig. 17.10a and 17.10b). The resulting *compressed-yeast* is blended with suitable emulsifiers and plasticizers, and extruded in the form of semi-plastic rectangular blocks. Packaging is done in wax paper. Storage of packaged yeast in cold room marks the final stage of processing. The compressed yeast has a storage life of 10 days at 5-8°C.

The productivity is ~ 3 g/lit/hr and under optimum condition, the practical yield is 0.50 kg yeast of solids per kg of fermentable sugars utilized. A recapitulative diagram of bakers yeast production (multistage type) is shown in Fig. 11.7.

11.6.4 PRODUCTION DETAIL OF BAKERS YEAST

11.6.4.1 Preservation and maintenance of culture

Although "low risk" methods are highly desirable, simple and rapid methods such as "active transfer" are more widely used. Selected strains of *S. cerevisiae* are prepared conventionally on agar stabs or as slant cultures and stored under refrigeration at 4°C. Transfer to fresh slant (subculturing) is done every 3-6 months. Malt Yeast extract Glucose Peptone Agar *(MYGP)* or other commercial media may be used for the subculturing.

11.6.4.2 Inoculum build-up

In practice, bakers yeast is not produced by direct pitching in the main fermenter. Rather, it is preceded by a sequence of smaller fermentations (and therefore

multistage) in which pitching yeast is grown. Typically, yeast is first grown in laboratory in a medium containing 5-7.5% sugar. This is then taken to the first propagating plant. Under light aeration and positive pressure, it is left for 20 hrs. The whole is transferred aseptically to an even bigger second stage propagator. Again, light aeration takes place and after 16 hrs, the content is again transferred *in toto* to yet another large propagator. The propagating vessels are kept in a separate room so that the entire work can be carried out aseptically. The final propagator needs about 26 hrs. This vessel can be of closed or open type but the propagators preceding it are necessarily closed vessels.

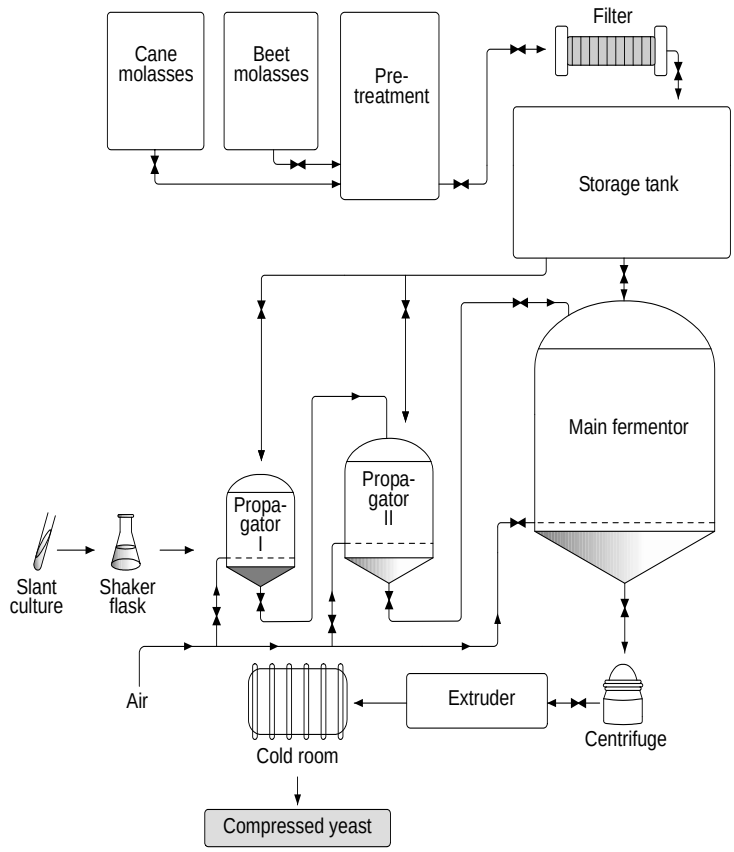


Fig. 11.7 Recapitulative flow diagram of bakers yeast production (multistage)

Aeration maintains the positive pressure, which is later used also for the aseptic pressure-transfer of inoculum. A protocol for inoculum- and seed yeast build-up for a 200 m^3 fermenter is shown in Table 11.7.

From Table 11.7 it is clear that alcohol is generated only in steps A and B. Here, the sugar concentration of the medium is relatively high (because of batch nature). The fermentation is midway between fermentative and respiratory metabolism. Step C is carried out in a manner similar to the main fermentation to be carried out later on. The vessel capacity of step C (and also the configuration) need not be different from

that of the final fermenter. Step C, like the main fermentation, proceeds in a fed-batch mode. The medium is fed in incremental manner, closely corresponding to the actual carbon requirement for the respiratory growth of participating yeast cells. The vigorously growing yeast cells quickly assimilate alcohol formed in steps A and B. The final propagator (vessel of step C) has a capacity of about 200 m^3, which yields about 20 MT (27% solids) of yeast. The yeast cells are collected by centrifugation. The resulting biomass, called 'yeast cream' can be now used for pitching 6 main fermenters each of 200 m^3 capacity. Each main fermenter will in turn yield 20 MT of sales yeast. If the seed yeast is not to be used immediately, it can be stored for a week under refrigeration.

Table 11.7 Scheme for inoculum build-up for a 200 m^3 fermenter

Stage	Molasses consumed (kg)	*Yeast produced (kg)	Alcohol generated (L)	Duration (hr)
Lab.	1	0.1-0.2	--	--
Step A	40	9	9	20
Step B	1000	200	250	16
Step C	22000	20000	0	26

* Yeast produced in terms of 27% solids (also called *sales yeast*)

11.6.4.3 Pitching

The addition of inoculum to the main fermenter is called pitching. A basic difference between bakers yeast and other fermentations is that the former uses yeast cream (solid, called *seed yeast*) instead of liquid inoculum.

Into vats that have been sterilized with steam and cooled, are pumped adequate amounts of potable water, various chemicals (phosphates, ammonium salts) and a small amount of prepared medium. Seed yeast is then added at a rate of 3 MT per 200 m^3 fermenter. The yeast cream from the propagator is usually stored in cooled tanks (0-4°C) for 1-2 weeks before actual use in the final fermenter. Sometimes, a short acid treatment is also given (pH ≈ 2) to seed yeast before use. This will reduce the level of microbial contaminants without affecting the seed yeast. The air supply is immediately opened and the incremental feeding of nutrient (prepared molasses) started.

11.6.4.4 Main fermentation

The main fermentation is carried out in fed-batch mode. Bakers yeast fermentations are not carried out under conditions of exponential growth. Since the fermentation is carried out in fed-batch mode, the constant feeding (without simultaneous removal facilities) does not permit exponential growth but can only provide a constantly diminishing rate. The fermentation must be complete by the time the medium reaches the predetermined level. The duration of fermentation for a typical fed-batch production for bakers yeast is 8-20 hrs.

Successful bakers yeast production requires monitoring of several process variables, such as aeration, temperature, feed rate, etc. Brief descriptions of some these important factors are given in the following paragraphs.

Feed rate control

The requirement of the feed rate control arises from the fact that bakers yeast exhibits *glucose effect* (*Crabtree effect*) at hexose concentrations above 5% in broth. Glucose effect refers to the shifting of metabolism from respiratory mode to fermentative mode. This occurs because the organism tends to attain cell economy. That is, when adequate amounts of readily assimilable substrates are available, the organism does not use the *Tricarboxylic Acid Cycle* and *Electron Transport Chain* which are energy intensive: the organisms meet their energy requirement from fermentation alone. Since fermentation by *S. cerevisiae* leads to ethanol production, this is an undesirable aspect: it leads to substrate loss. Bakers yeast production therefore requires that feed rate be controlled so that the nutrients are instantly utilized for the respiratory metabolism only. The shift from fermentative to respiratory metabolism is called *Pasteur Effect*. It may be noted, the fundamental difference between bakers yeast and ethanol production is that the former entails Pasteur effect while the latter, glucose effect. In bakers yeast production, the feed rate is maintained such that glucose concentration of the medium at any instant is below 0.2 g/L.

Aeration

Bakers yeast production is a highly aerobic process, requiring (theoretically) 1 g O_2 per gram of bakers yeast (dry) produced. Aeration systems used in bakers yeast production vary considerably (for instance, agitated-, stationary-, and proprietary systems) but the most common is the stationary aeration system. In the common type, the bottom is covered by a horizontal, diametrically placed main pipe containing 24 side tubes provided with a total of 30,000 holes each of 1.5 mm diameter. The air is sparged as fine bubbles, the movement of which also aids in agitating the medium (see Fig. 11.8).

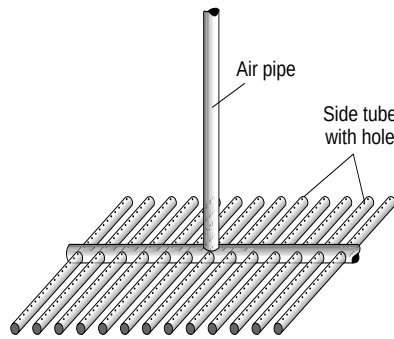


Fig. 11.8 Stationary aeration system

Customarily, filtered air containing 21-30% O_2 is used for aeration. Since only 20% of the supplied O_2 is consumed before finally being vented out, the supply of air far exceeds the theoretical requirement. The aeration is typically controlled at 0.8-1.4

vol/vol/min. Concentrated O_2 gas mixture is not preferred as this leads to disturbance in yeast metabolism: yeast's ability to respire and ferment becomes impaired.

Cooling

Cooling can be carried out with internal cooling coils. Bakers yeast fermentation is carried out at 28-30°C. High temperature is undesirable because this leads to contamination problem. The performance of the yeast is also affected. Cooling requirement is great in bakers yeast production because the heat liberated during aerobic growth is very high: it is about 3.5 kcal/g of yeast solids produced.

Defoaming

Considerable foaming occurs in bakers yeast production. This is why about 1/3 space of the fermenter is left as headspace. Foam can be controlled by adding antifoams like silicone oil, edible oil, surface-active agents, etc.

Growth rate, generation time, and generation

In bakers yeast production, extreme growth rates are not desirable. Feed rate and aeration are adjusted such that the growth rate always remains very near to 0.2. Control of aeration serves double purpose: it maintains *Respiratory Quotient* (RQ = mole of CO_2 generated per mole of O_2 consumed) at an optimum of 1.0 and growth rate at around 0.2. High level of aeration increases growth rate, liberates more CO_2, disturbs RQ balance, and eventually leads to "respiratory" fermentation.

The growth is expressed as an average. It does not remain constant throughout the fermentation period. The growth rate decreases with the corresponding generation time: 3 hr $\rightarrow$ 5 hr $\rightarrow$ 7 hr. Therefore, for a 15-hr fermentation, 3 generations and an 8-fold multiplication can be expected.

11.6.4.5 Final stage of fermentation

Maturation of yeast cells marks the final stage of the fermentation. Maturation is required to impart yeast cells the ability to withstand future adverse conditions (e.g., extruding, drying, etc.). The maturity is expressed as percentage of budding. A low percentage of budding reflects maturation and the resultant improved stability on storage.

Maturation is achieved by sharply reducing the feed rate towards the end of the fermentation. Even after terminating the feeding, aeration is continued for another half an hour.

11.6.4.6 Yield and productivity

During fermentation, growth of bakers yeast decreases rapidly at concentrations exceeding 3-4% yeast solids in the broth. For practical reasons, commercial bakers yeast fermentation is terminated at solids concentration of 4-6%.

The average productivity of a typical fermentation is 3 g/lit/hr. In practice, ethanol formation is minimized and kept below 0.1%. Using an ordinary beet molasses with addition of necessary growth factors and nutrients, approximately 0.5 kg yeast solids can be obtained per kg of fermentable sugars. This compares well with a number of theoretical views.

11.6.4.7 Harvesting

The final broth is separated for yeast cells in a battery of vertical, nozzle-type, continuous centrifuges which can develop a *G of 4000 to 5000 (Fig. 11.9). This is sufficient to affect the separation of cells which have a water content of 62 g/100 g cells and a density of 1.133 g/cm^3. The first pass (along with washings) through such a centrifuge triples the yeast concentration. Additional passes produce yeast cream with 18-20% solids. The cream can be stored at 1-4°C for several days.

* G refers to velocity of settling of particles due to gravity. It depends on viscosity (η) of the liquid, diameter (D) of the particle, acceleration (g) due to gravity, and density (ρ) of the particle as follows:

$$\text{Velocity of settling of particles due to gravity} = \frac{D^2 \rho g}{18\eta}$$

Velocity of settling due to centrifugation depends on radius (r) and angular velocity (ω) of the centrifuge in place of acceleration due to gravity (g). The relation is as follows:

$$\text{Velocity of settling of particles due to centrifugation} = \frac{D^2 \rho \omega^2 r}{18\eta}$$

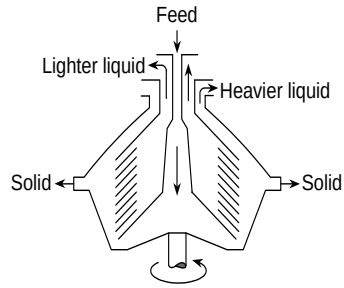


Fig. 11.9 Nozzle-type vertical centrifuge

11.6.4.8 Filtration/Compression

Yeast cream is further concentrated by filtration (by pressing, which accounts for the term *compressed* or *pressed* yeast). Filter aids must not be used as they contaminate the yeast. Plate and frame filter press is normally used but rotary continuous vacuum filters are now getting increasingly popular. In the latter type, the drums are precoated with edible starch and a small amount of salt before filtration. Salt is added to reduce the moisture content of the yeast mass. After filtration the salt can be removed by spraying water. The press cake, called *sales yeast*, contains 27-28% yeast solids. Bakers yeast obtained by vacuum filtration has slightly higher solids content (30-33%). Salt works as a dehydrating agent. Theoretically, it is roughly 11 times as effective as sucrose. The equation showing the dehydration effect is: $\pi V = nRT$, where π is the osmotic pressure and n is the number of solute particles. In the case of Na^+Cl^-, there are two particles per mole (58.5 g). On the other hand, sucrose (342 g) contains only one particle.

11.6.4.9 Extrusion and packaging

The yeast cake is mixed in a blender with small amounts of emulsifier and cutting oil (soybean or cottonseed oil). These additives, which may be added at the rate of 0.1-0.2%, facilitate subsequent extrusion and provide better, lighter appearance. Mono- or diglycerides, sorbitan esters, and lecithin are the commonly used emulsifiers.

The blended cake is extruded through nozzles in the form of continuous, thick ribbons with a rectangular cross-section (open-throated nozzles). This is cut into appropriate lengths to form the well-known shape of packaged bakers yeast (~ 500 g). The packs are immediately cooled in refrigeration chamber with a vigorous air circulation. Generally, a cooling period of 24-48 hrs is needed.

11.6.4.10 Storage stability

The storage stability of compressed yeast at 5-8°C is quite good (about 10 days). A loss of 3.5% gassing activity may occur. To some extent, the stability also depends on processing conditions, maturity, etc. Cells with nitrogen contents between 6-7% show excellent stability. Temperatures above 10°C are detrimental to yeast quality. The storage stability of compressed yeast can be checked by determining the effect of storage on viability (by *plate count* method).

11.6.4.11 Contaminants

The most numerous are lactic acid bacteria of the genera *Lactobacillus* and *Leuconostoc*. The counts are normally between 10^4 and 10^9/g.

11.6.4.12 Wastewater

Typically effluent from bakers yeast industry contains 0.1-0.3 kg BOD/kg yeast produced. Beet molasses contribute to more BOD than cane molasses because of betaine. The general method of reducing BOD (by ~ 80%) is anaerobic purification.

The organic compounds are decomposed to CO_2 and methane. Methane may be used in factory boilers.

11.6.4.13 Quality control

The quality of bakers yeast is judged by three important categories of tests, namely, (i) Chemical, (ii) Microbiological, and (iii) Physical (see Table 11.8).

Table 11.8 Test of bakers yeast

Test category	Test	Description
Chemical	Dry matter	
	Nitrogen	
	Phosphorus	
	Trehalose	Disaccharide of glucose with no reducing property
Microbiological	Total count of living cells	
	E. coli count	
	Streptococcus fecalis	
	Wild yeasts and molds	
Physiological	Raising power	
	Keeping quality	Leavening power after storage at 30°C for 2 and 4 days, 20°C for 7 days, and 5°C for 4 weeks.
	Autolysis	No. of days at 35°C before the yeast becomes soft.

11.6.5 ACTIVE DRY YEAST

The production of *Active Dry Yeast* (ADY) begins with the selection of bakers yeast which will yield the desired characteristics on drying. In order to produce an ADY with acceptable leavening activity, viability and storage stability, following factors should be taken into account during drying:

- Drying temperature
- Drying rate
- Final moisture content

At present, the only method used on commercial basis starts from press cake. The cake is subdivided into thin strands or fine particles. Drying is carried out with currents of air at temperatures which keep the temperature of the yeast itself below 40°C. The yeast chosen for drying should be mature enough to withstand the harshness of drying. The common methods of drying are:

- Continuous belt tunnel dryers

- Rotolouver dryer
- Through circulation dryers
- Air-lift (fluidized-bed) dryer
- Spray dryer

Air-lift dryer is probably the most popular of dryers. It can be used either as a batch- or a continuous process. For batch operation, the extruded yeast strands are fed into a drying chamber with a metal screen or perforated plate at the bottom. Heated air is blown from the bottom through the yeast particles in a fluidized bed. Emulsifiers and swelling agents are often added to the yeast preparation (suspension) prior to drying. The drying time may vary between 10 min and 4 hrs. For rapid drying (between 10 and 30 min) an inlet air temperature of 100-150°C can be used at the beginning, while keeping the temperature of the yeast itself at 24-40°C. Temperature of the yeast particles up to 50°C is not detrimental at the end of the drying period. A multichamber continuous air-lift dryer patented by Pressindustria (1977) uses the conditions given in Table 11.9.

Table 11.9 Yeast drying protocol used by Pressindustria (1977)

Description	Measurement
Air velocity	4000m/h
Air flow volume	4000m^3/h
Air temperature	
1st chamber	46°C
2nd chamber	36°C
3rd chamber	32°C
4th chamber	30°C
Retention time	3h
Yeast productivity	160-350kg/h

Using the protocol given in Table 11.9, the final moisture content of the ADY can be brought down to 7%. In general, the survival rate of yeast in fluidized-bed dryer is about 85%, which is not possible using other drying methods.

In commercial practice, sorbitan monostearate is used in ADY at concentrations between 0.5 and 2 % based on dry weight of yeast cells. The addition improves dehydration characteristics of ADY. *Butylated Hydroxy Anisole* (BHA) may be used at the rate of 0.1% for the protection of low-moisture ADY against oxidative rancidity. Such ADYs are available under the trade name *protected ADY*.

Generally, ADY is dried to moisture content of 7.5-8%. This level represents a compromise between the demand for good quality ADY (good bake activity), which is higher at higher moisture levels, and good stability, which is better at lower moisture level.

11.6.5.1 Packaging

The ADY is normally given a protective packaging in an atmosphere with less than 2% O_2, or under vacuum. Yeast for consumer trade is usually packed in aluminum foil-laminated, heat sealable plastic pouches. The inert gas for smaller packages is usually N_2.

11.6.5.2 Stability

ADY loses about 7% of its bake activity per month at ambient temperatures and has a useful life of 1-2 months. If packaged in an inert atmosphere or vacuum, it loses only 1% of activity per month, with an annual loss not exceeding 10%. Under good packaging, a good quality ADY will have a shelf-life of 1 to 2 years. The stability of ADY is also related to its chemical composition (Table 11.10). Nitrogen content of about 7% is thought to be optimum.

Table 11.10 Composition of ADY

Parameter	Range
Moisture, %	7.5-8.5
Nitrogen, %	7 (based on yeast solids)
Phosphorus (as P_2O_5), %	1/3 × nitrogen content

11.6.5.3 Rehydration

Rehydration of ADY is done in warm water (40°C), and it takes about 5 min. If cold water is used, a loss of 20-25% yeast solids can occur by leaching. Among the materials that may leach during rehydration, glutathione (a tripeptide of γ-glutamyl-L-cysteinylglycine) has an important bearing on the bake quality of bakers yeast. This compound activates proteolytic enzymes in the flour, which in turn affect gluten membrane in the dough. The slackened protein membranes can no longer hold back CO_2 and the loaf becomes flat.

11.6.6 INSTANT ACTIVE DRY YEAST (IADY)

This preparation can be directly used with the flour (without rehydration). The fundamental difference between ADY and IADY is the processing. IADY uses threads as small as 0.5 mm in diameter for drying. Emulsifiers such as sorbitan monostearate, citric acid esters are used. The rest is similar to ADY production.

CHAPTER 12

BREWING TECHNOLOGY

12.1 INTRODUCTION

Beer is one of the most important alcoholic beverages. The term is generic and implies undistilled alcoholic drinks made from malted barley and adjuncts, and are flavored and preserved with hops. The art and science of brewing is called *zymurgy*.

12.1.1 TYPES OF BEER

There are two major types of beers, which are distinguished mainly on the basis of fermentation differences. They are (i) *Lagers* and (ii) *Ales*.

12.1.1.1 Lagers

Most bottom-fermented beers are called lager beers, or simply, lagers. This term was derived from the German verb *lagern*, which means to *lay down*, or *store*. Historically, storing for long periods was an integral part of lager beer production: it served to mature and clarify beer. This significance no longer exists in modern lager beers. Lager beers are produced employing the yeast *Saccharomyces carlsbergensis* (synonym: *Saccharomyces uvarum*), a bottom-fermenting yeast.

12.1.1.2 Ales

Most top-fermented beers are called ales. The yeast employed is *Saccharomyces cerevisiae*, a top-fermenting yeast. Usually, ale fermentation occurs at a temperature higher than that for lager. The duration of fermentation, however, is shorter. Another fundamental difference between lager and ale is in the method of mashing. Lagers are produced by *decoction* mashing (see later, page 183) while the ales are produced by *infusion* mashing.

Both lagers and ales can be further classified on the basis of *color* and *body*, into *light* and *dark*. It has to be remembered that there are several other beers, which do not neatly fall in the aforementioned categories. *Lambic* beers, for example, are produced by spontaneous fermentation.

The fundamental differences between light and dark beers are shown in Table 12.1. Some examples of beer and recapitulative representation of classification of beers are shown in Fig. 12.1.

Table 12.1 Fundamental differences between Light and Dark beers

Characteristics	Beer type	
	Light	Dark
Color	Light	Dark
Body (gravity)	Weak	Heavy
Palatability	Dry	Sweeter
Attenuation*	Full	Not full
Hops level	Medium to high	Low

**Attenuation refers to weakening of beer in terms of nutrient content. A well-fermented beer is supposed to be attenuated because the yeast has utilized almost all of the substrate.*

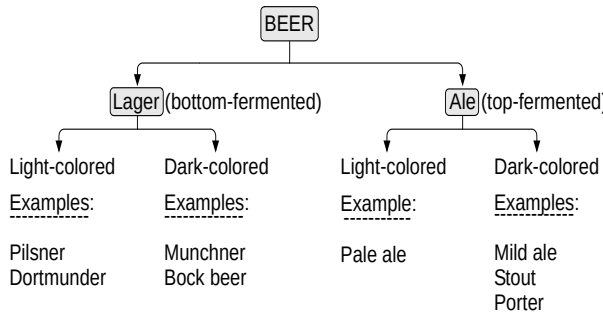


Fig. 12.1 Classification of beer

12.1.2 COMPOSITION OF BEER

Beer contains water, alcohol, carbohydrates, CO_2, organic acids, resins and essential oils, proteins and amino acids, higher alcohols, minerals, and congeners. These constituents come from the raw materials, yeast, and fermentation. A typical composition of beer is given in Table 12.2.

Table 12.2 Normal composition of beer

Components	Amount
Water, %	88-92
Alcohol, %	4-5
Acidity, %	0.15-0.5 (as lactic acid)
CO_2, %	0.4-0.5
Nitrogenous substances, %	0.58-0.74
Sugar, %	0.9-2.6 (as maltose)

There are several exceptions, though. For instance, Switzerland produces one of the world's strongest beers (14% *abv*). Switzerland is also the first country to market *alcohol-free* beer.

12.1.3 ALCOHOL-FREE BEER

These beers were developed in view of concern over the effects of alcohol consumption on health and partly in an attempt to provide an acceptable social drink for motorists.

The most common means of production involves post-brewing removal of alcohol from beer. Thermal evaporation under vacuum is currently used. Such equipment is typically capable of producing an end product with an alcohol content of about 0.03%. The beer retains the *brewed* taste. There has been considerable interest in the development of yeast strain also, which produces the brewed flavor but is unable to complete the alcoholic fermentation.

12.1.4 THE STORY OF CARLSBERG BEER

The best known of all Scandinavian (i.e., Denmark, Norway, Sweden, and Iceland) breweries is the Carlsberg of Denmark, which dates from 1847. The story runs thus: Christien Jacobson of Denmark visited Munich several times. He studied the bottom-fermenting technique and brought back the yeast from there in 1846 to start his own brewery the following year. He named the brewery after his son *Carl* and the fact that the brewery was on a hill, *berg*. In 1883, Carlsberg brewers identified and isolated a single cell yeast culture which has left the name in the annals of lager brewing as *carlsbergensis*. Tuborg was a rival of Carlsberg but these companies merged in 1970.

12.2 MATERIALS REQUIRED FOR BEER MAKING

The principal materials required for beer making are: (i) Yeast, (ii) Malt, (iii) Hops, (iv) Adjuncts, and (v) Water. A brief discussion on each of these materials is given in the following paragraphs.

12.2.1 BREWERS YEAST AND ITS MANAGEMENT

Depending on the type of beer, either S. *cerevisiae* or *S. uvarum* is used. Whichever the yeast type, it must possess certain properties in order to make a good beer. Some of the most important properties required of these yeasts are:

1. Rapid growth rate (but without excessive yeast growth)
2. Efficient utilization of maltose and maltotriose with good conversion to ethanol
3. Ability to withstand stress imposed by alcohol concentrations and osmotic pressure encountered in breweries
4. Reproducible production of the correct levels of flavor and aroma compounds
5. Ideal flocculation characteristic for the process employed.
6. Good *handling* characteristics (e.g., retention of viability during storage, genetic stability, DOG resistance, killer strain resistance, etc.).

The selection of mutants resistant to glucose analog, 2-deoxyglucose (= DOG), has proved extremely useful. These mutants have the ability to utilize glucose and maltose simultaneously: they are insensitive to catabolite repression.

The most widely used method for stock-culturing in breweries is active transfer, which may be either broth culturing or slant/slope culturing.

12.2.1.1 Broth culture

The yeast is inoculated and incubated at 28°C for 2-4 days in MYGP broth. Thereafter it is stored at 2-4°C. Subculturing is done every 2-3 months.

12.2.1.2 Slant/slope culture

Either MYGP agar or *Universal Beer Agar* (UBA) slopes are inoculated and incubated at 28°C for 2-4 days and stored at 4°C. Subculturing is done every 6 months. If stored under paraffin oil, the culture is stable for a year.

12.2.1.3 Quality control

Several tests are available for the quality control of yeasts. To ensure that the contamination and mutant levels are low (whether stock-culture or recycled yeast) following general tests may be carried out.

- *Microscopic examination:* Screening can be done for unusual size, shape, and morphology for the evidence of wild yeasts. The examiner must be familiar with the diagnostic morphological characteristics of the culture yeast, though.
- *Cycloheximide in UBA:* Cycloheximide added at the rate of 2-4 µg/ml in the medium selectively inhibits the culture yeast. All yeasts capable growing in such a medium are therefore wild.
- *Lysine/Crystal violet/Fuchsin sulfite media*: These three media are used to detect wild yeasts in the presence of culture yeasts. Culture yeasts cannot utilize lysine as the sole source of carbon and are inhibited by crystal violet and fuchsin sulfite.
- *Test for heat resistance:* The yeast culture is heated at 53°C for 10 min. Wild yeasts survive this treatment but the culture yeasts are rapidly destroyed.
- *TTC overlay method:* Upon prolonged use, some culture yeasts mutate to Respiratory Deficient Mutants (RDMs). These mutants cannot oxidize glucose and their number exceeding 10% of total yeast cells is considered unsatisfactory. Triphenyl tetrazolium chloride (TTC, a colorless salt) is used to detect the presence of (and also enumerate) RDMs. In essence, the method involves aerobic growth of yeast culture in general growth medium at 20-30°C and then overlaying the colonies with 20 ml of molten TTC overlay agar. After about 3 hrs at room temperature, colonies begin to differentiate. Pink to red colonies are respiratory sufficient while the colorless colonies are RDMs.

12.2.1.4 Yeast purity

Pure yeast strains are prerequisites for good brewing performance and product uniformity. The purity of brewers yeast is most precisely analyzed by DNA fingerprints. Using this technique, the yeast chromosomes are first separated according to size by gel electrophoresis. Chromosomal DNA bands fluoresce in UV light when stained with ethidium bromide. Each of the analyzed strains exhibits a unique chromosomal DNA pattern (Fig. 12.2).

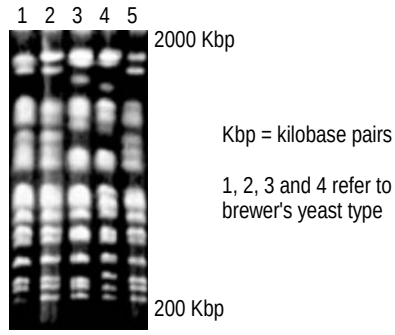


Fig. 12.2 Electrophoretic analysis of yeast chromosome

Wild yeasts in beer

Wild yeasts in brewing are undesirable for a host of reasons, the more important of which are:

1. Poor flocculation characteristic in wild yeasts produce haze and turbidity in beer
2. Wild yeasts may form film or pellicle on the surface of the wort/beer, which is objectionable
3. Super-attenuation may result due to utilization of maltodextrins by wild yeasts. This affects the body, taste, and alcohol content of the beer.
4. Off-flavors may be produced

12.2.2 MALT

Malt is cereal, usually barley, which has been allowed to germinate for a limited period of time and the growth terminated by drying. Malt is used for following reasons: (i) as an enzyme source, (ii) as a substrate, and (iii) for flavor and color.

European beers use two-row variety barley for malting. The US beers use six-row varieties for the same. The latter has lower malting quality but has higher level of enzyme activity and hence suitable when adjuncts (see later) are used.

Before a new barley variety can be accepted for the production of malt, it is tested in micro-, pilot-, and production brewing trials. Some of the varieties accepted in many

European countries are Alexis, Barke, Prestige, and Scarlet, all of which are 2-row variety.

Recently, newer varieties of barley, collectively called *proanthocyanidin-free barley* (e.g., *Chamant*, *Prominant*, etc.) are being used for preparing beer with better haze (turbidity that appears when beer is cooled) stability and greater brilliancy.

12.2.2.1 Safe storage of barley

Barley should be stored at a low temperature and moisture content below 12% to avoid fungal problems and reduction in seed vigor (see Table 12.3). The molds most implicated are species of *Aspergillus*, *Penicillium*, and *Fusarium*. These molds, *Fusarium* in particular, produce metabolites (not yet characterized) that have been implicated for *gushing* in beer, where the beer spontaneously gushes from the bottle on opening. Gushing in beer is a very severe quality defect.

Table 12.3 The influence of moisture and storage temperature on seed vigor

	Seed moisture				
Temp. °C	10%	12%	14%	16%	18%
0	16 years	6 years	2 years	1 year	190 days
6	9 years	3 years	1.3 years	210 days	105 days
12	5 years	1.6 years	240 days	110 days	55 days
18	2.3 years	290 days	115 days	50 days	25 days
24	1 year	130 days	55 days	25 days	12 days
30	210 days	55 days	22 days	10 days	5 days

12.2.2.2 Testing for barley vigor

Fast and even germination during malting is an essential quality parameter of malting barley. Prior to malting, all barley lots are analyzed for both germination percentage and germination rate, measured as *germination index*.

Just after harvest, especially after a cold summer, the barley may exhibit dormancy, preventing germination. Dormant barley has to be stored for a certain span of time until it has developed full germination capacity. In order to analyze the germination capacity of a barley lot just after harvest, the dormancy has to be broken. Carlsberg Research Laboratory has developed a method for breaking this dormancy, in which the barley is treated with H_2O_2.

12.2.2.3 Selection of barley grain for malting

Barley selected for use in the malting industry must meet special quality specifications. Accepted malting barleys have to *modify* (see later) evenly and produce a finished malt whose properties lie within the brewer's specifications. The malt

quality of a given barley variety is determined by its genetic background and the physical conditions during growth, harvest, and storage.

Malting quality has to be tested in micro-, pilot- and industrial malting trials. Thereafter, brewing trials are done in pilot and production scale.

The physical condition of the barley must meet specifications concerning:

- Germination: min. 97% after 3 days
- Germination index: min. of 6.0
- Moisture content: 12%, max.13%
- Protein: > 9% and < 11.5%
- Grading: 90% > 2.5mm
- β-glucan: max. 4%
- Variety purity: min. 99%

12.2.2.4 Malting

The key physiological events of malting, which determine the quality of the final malt, include rapid and uniform germination, the synthesis of hydrolytic enzymes in the scutellum and aleurone tissues surrounding the endosperm (Fig. 12.3) and finally the degradation of endosperm cell walls, described as *modification*. Gibberellin, which is a plant hormone that regulates the physiological events, is produced by the germinating embryo.

During germination, enzymatic hydrolysis of endosperm mobilizes the nutrients and energy reserves of the grain for the growing barley plant. Growth of germ is an unwanted incidental to the making of malt, because it leads to respiration and growth of new parts. After germination to a desired extent, the growth of the embryo is therefore terminated by drying. It is customary to further dry the malt in kilns for long-time storage. In summary, malting process involves:

- Collection of suitable stocks of barley
- Storage of the cereal until required
- Steeping in water
- Germination of the grain
- Drying and curing in the kiln

The selected malt grains are steeped in water at 10-16°C to raise the moisture content of the grains to 42-46% whereupon germination begins. Steeping is done to provide correct environment for the synthesis of hydrolytic enzymes and their controlled action on the cell wall of the reserve protein, hordein. The distinction between steeping and germination, however, is artificial. A steeping time of 70-72 hrs is not uncommon, and usually, steep water is changed every 4-10 hrs. Water enters the grain via the embryo, and after 24 hrs, the first visible sign of germination is the appearance of the root as a white "chit". See Fig. 12.3 for the cross-section of barley grain.

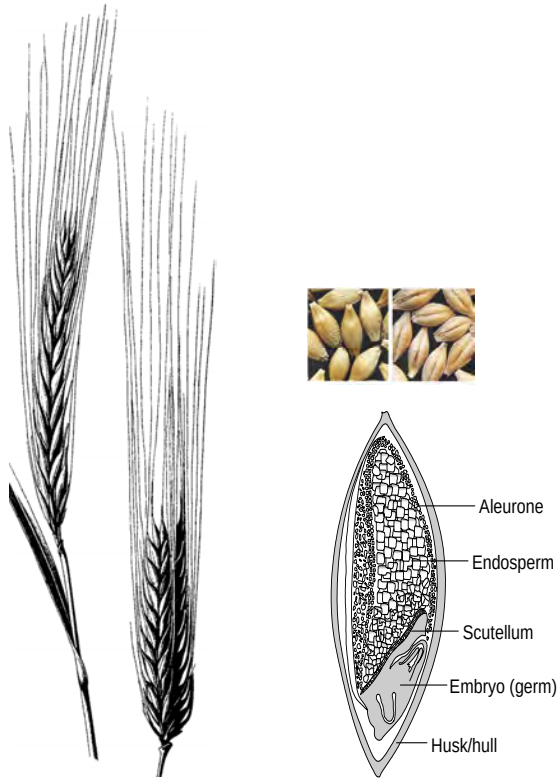


Fig. 12.3 The barley grain

Germination can be carried out by various methods, e.g., on concrete floor, in drum germinators, compartments, and pneumatic germinators.

Modern malting employs combined steeping-germinating technique. The equipment consists of a cylindroconical tank of a short height. The grain is transferred to make a shallow grain bed. Every 4-6 hrs, water is changed and the barley is intensively aerated from the bottom following each drainage. Air can also be introduced in the steep-water. Aeration is essential because germination cannot occur in absence of O_2. CO_2 that accumulates during germination is drawn out for 10-15 min every hour or so. Germination can be accelerated and regulated by post-steeping application of gibberellic acid (a plant growth hormone) to initiate enzyme production by the aleurone layer. It is possible to enhance the passage of gibberellic acid into the aleurone layer by mechanically abrading the husk and underlying layer. This process is used commercially to accelerate malting or to assist the malting of poor quality barley. The application of gibberellic should be as early as possible. Typically, 0.03-0.08 g of this hormone is needed per 1000 kg of barley.

In modern practice, condition during germination is controlled by passing humidified, temperature-controlled air through the germinating grain. Continued growth of embryo, with the appearance of rootlets and acrospires, can lead to root entangling. The grain bed is regularly turned with a rotating screw to prevent grain matting together. It is desirable to reduce malting loss by limiting growth and

respiration and this may be achieved by resteeping. A final steep at 40°C may also be employed to achieve root killing. The whole process may take 8-9 days. Tropical grains may require temperatures above 24°C for malting.

Barley is preferred over other grains for the preparation of malt. This is because of following reasons:

- The grain retains husk, which affords protection during transportation and storage
- Coarse husk particles aid in wort filtration
- Comparatively low germination temperature of the barley
- Comparatively high β-amylase content of the malt.

Assessment of the completion of malting is carried out organoleptically, by chewing and rubbing. The loss due to respiration and growth is about 6-10% on dry basis.

Modification

Proteolysis of aleurone protein is the first hydrolytic reaction to occur. The enzymes then move on to the endosperm. Endospermic starch and storage proteins are enclosed in the cell walls composed mainly of β-glucans. The endospermic hydrolysis is therefore preceded by hydrolysis of β-glucans by glucanases. Once the cell walls are removed, starch and proteins are hydrolyzed to varying degrees by their corresponding enzymes. α- and β-amylases are the most important carbohydrases from brewery point of view. The sugars and amino acids thus produced are mobilized for the nutrition of the growing barley plantlet.

β amylase is already present in the grain but increases during the initial phase of germination. α amylase is undetectable in the ungerminated barley but is formed during the latter phase of germination.

A term frequently used by malsters is *modification*. The term refers to alteration of structure of three main categories of polymers in the barley endosperm, namely:

- Cell wall polysaccharides
- Reserve protein, hordein
- Starch granules

A *well-modified* malt is one in which all the cell walls have been eliminated by hydrolysis. *Overmodification* means extensive damage (corrosion) of starch granules. The cell walls of barley endosperm primarily contain β-glucan and pentosans. β-glucans must be degraded as completely as possible because they are highly viscous. They complicate recovery of malt extract, affect filtration, and impede wort run off.

The development of the acrospires and the progress of modification of the kernel run approximately parallel to one another.

12.2.2.5 Drying and curing in kilns

The degree of modification required depends on the type of beer to be brewed and when this has been achieved, malting is stopped by drying.

These days, several types of highly efficient kilns are available for drying and curing of germinated barley. Modern kiln types include single floor circular kilns, two floor circular kilns, high performance kiln with loader and unloader, and vertical kilns. The duration of kilning is 18-20 hrs. After drying and curing, the rootlet fraction (called "culms", and it constitutes 3-4% of malt) is removed in a malt deculming screw. Before dispatch, the grain is subjected to mechanically operated brushes to give the kernels an attractive, polished appearance.

Drying can be carried out in 2-3 stages. A typical three-stage method involves:

- *Initial stage:* 50-60°C until 25% moisture content is achieved
- *Second stage:* Temperature slowly increased to 71°C. Moisture content of malt is lowered to 12%
- *Final stage:* This stage is also called *curing* or *kilning*. The malt is dried at 71-92°C (sometimes even higher temperatures are used) until the moisture content of barley drops to 4-5%

There are, of course, considerable variations in the time-temperature combination. For instance, a final temperature of 95-105°C is used for the production of *dark* malt. Such malts are not primarily used as enzyme sources. They only impart malt favor and color.

The grains are immediately cooled to 38°C or lower. The final stage eliminates the green, grainy taste and supplies the characteristic malt flavor. Higher temperatures increase acidity. The color will also darken due to *Maillard* reaction.

Purpose of kilning

- Removal of water to obtain keeping quality of the highly perishable unkilned *green* malt
- Interruption of germination, i.e., inhibition of metabolism by drying
- Formation of aroma, taste, and color by curing

Effects of curing on endogenous enzymes

- α- and β-amylases are only slightly decreased during drying. During curing, β-amylase is inhibited to a greater extent compared to α-amylase.
- Proteolytic enzymes are destroyed noticeably only at curing temperatures above 100°C
- Catalase activity is strongly diminished during drying, and curing temperatures completely inactivate it
- Polyphenolase activity is hardly affected
- Lipase activity is partially inactivated

The kilned-and-cooled malt is dropped into collecting hoppers, sprouts severed over sprout cleaner (deculming device), and then stored for 3-6 weeks to several months before blending and shipping. Before shipping, malts from different lots are blended to meet the specification. This is essential because it is seldom possible to produce malt of the same quality (despite utmost care in malting). Malt with moisture content higher than the specification is called *slack*.

12.2.2.6 Malt milling

Malt milling is carried out in the brewery. There are two types of malt milling processes, viz., (i) dry milling, and (ii) wet milling. However, there are several variations within each milling type.

Dry milling

Dry milling can be carried out in roller mills or hammer mills. There are several types of roller mills and may contain 2 to 6 rollers. The six-roller mill (see Fig. 12.4 for the principle) is the best and most frequently used type of grist mill. Its three pairs of rollers are called (a) primary crushing roller pair, (b) husk roller pair, and (c) grits roller pair.

There is a vibrating screen unit with two different mesh sizes suspended between each pair of rollers. These screens sort the milled material from the preceding pair of rollers into three fractions, viz., (i) coarse components (husks with attached grits), (ii) middle components (grits), and (iii) fine components (fine grits and flour). The flour portion is led directly into the grist case since it is not milled any more. The husks are crushed, with as little damage to the husks as possible, by the second pair of rollers. The grits are ground by the third pair of rollers to the extent desired.

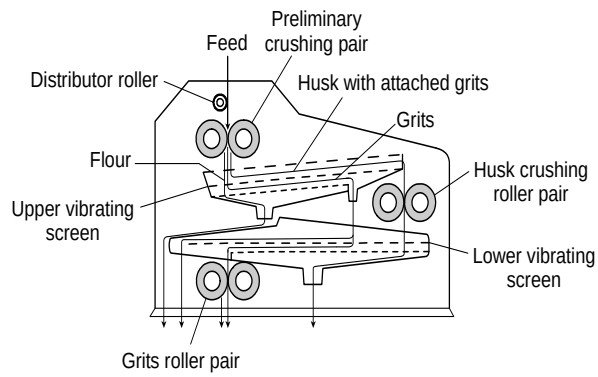


Fig. 12.4: Principle of six-roller malt milling

The bulk density of the malt thus milled is about 20.8 lb/ft^3. It is important to note that husks are not discarded. A typical composition of malt that goes into mashing is: husk 15%, coarse grits 23%, fine grits 34%, and flour 28%. The main objective of adding husk is to facilitate filtration. Husks also provide some flavor components to beer. Dry milling produces good extract but the wort run-off time is longer.

In modern type mash filters, filtration is performed by means of small pore polypropylene filter cloths with a spent-grain layer thickness of only 4 cm. In such cases husks are not needed as filter material and the malt can be ground finely. Fine milling is done using a hammer- or pin mill. In a hammer mill, swinging hammerheads are attached to a rotor that rotates at high speed inside a hardened casing. The principle is illustrated in Fig. 12.5. The material is crushed and pulverized between the hammers and the casing and remains in the mill until it is fine enough to pass through a screen which forms the bottom of the casing.

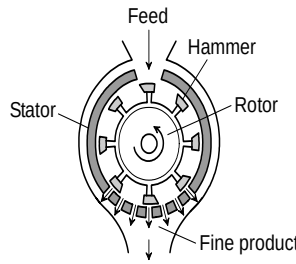


Fig. 12.5 Principle of hammer milling

Wet milling

Wet milling of malt can be carried out by several methods. Today, the conventional wet mills are no longer manufactured. A variant of wet milling process called "steep conditioning" is widely used. A steep conditioning operates as follows (Fig. 12.6):

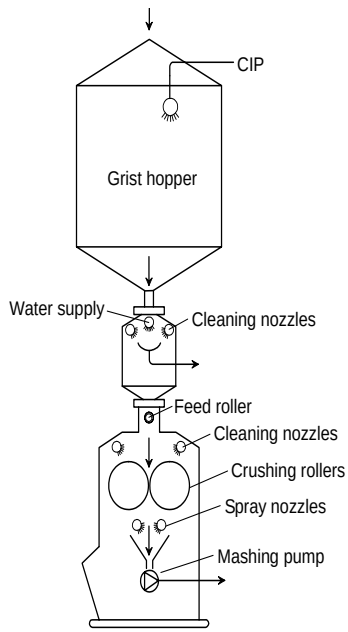


Fig. 12.6 Schematic of steep conditioning

In the grist hopper the weighed amount of grist is stored and is supplied with hot water in the conditioning stage for about 60 s. The water temperature is kept at around 60-70°C. The rapid uptake of water by the kernel makes the husk rubbery. The grain contents are then comminuted by a pair of rollers. The milled grist is intimately mixed with water (by means of spray nozzles) at mashing-in temperature and transported by means of a pump from below into the mash vessel. Some oxygen uptake occurs during milling because of the longer grist mashing-in time and this is undesirable. These days, there are systems that inject CO_2 during milling to minimize the entry of oxygen.

12.2.3 ADJUNCTS

Adjuncts are defined as non-malted carbohydrate materials of suitable composition and properties that beneficially complement (go well with, often contrasting characteristics) and supplement (add to make complete) barley malt. Adjuncts are used primarily as a cost-saving initiative but other advantages can also be achieved, for example, it produces beer:

- With lighter color
- That is less satiating and has thinner taste
- With greater brilliancy
- With enhanced stability
- With superior *chillproofing* quality (see page 209)

Adjuncts can be classified into two groups, viz.:

1. *Starchy materials:* cassava, maize, corn, rice, etc.
2. *Sugar and sugar syrup:* Today's liquid adjuncts are clear, colorless, non-crystallizing liquids consisting of carefully controlled mixtures of glucose, maltose, maltotriose, and maltodextrins. Liquid adjuncts have following advantages:
    - Better wort run off
    - Clearer wort
    - Better control over kettle operation
    - Increased productivity (more hectoliters per brew)

12.2.4 HOPS

Hops as used in brewing are the dried blossoms of the female hop plant (*Humulus lupulus*, Fig. 12.7) or their preparations (extracts, powders, pellets, etc). Hops are primarily employed in the brewing process for imparting a bitter flavor to beer. Since hops also contain many unique components, the flavor they impart to beer is of unique hop character. Hops have other beneficial functions also, *viz.*:

- Contribute to palatefulness, colloidal stability, and *head retention* (page 208)
- Have antiseptic properties
- Tannins present in hops aid in precipitation of undesirable nitrogenous substances of the wort by forming *protein-phlobaphene* complex (page 188)

Fig. 12.7 A female hop cone

12.2.4.1 Hop varieties

There are three species of hops, viz., (i) *Humulus lupulus* (common hop) (ii) *Humulus japonicus* (Asian hop), and (iii) *Humulus yunnanensis* (Yunnan hop). From brewing point of view, hops are of two main types: (i) bitter hops, and (ii) aroma hops. Bitter hops are used universally. Some examples of bitter hop varieties are: Brewers gold, Northern brewer, Galena, Target, etc.

12.2.4.2 Hop components and chemistry

A good beer needs a good aroma, an acceptable palate, and a great deal of flavor that is provided by hops. It has been said: "Malt is the soul of beer and yeast gives it life but the kiss of the hops is the consummation of that life".

Hop constituents of importance in brewing are hop resins (14-21%), essential oils (0.5-1.5%), polyphenols (2-5%, about 80% of which are anthocyanidins), proteins (20%), and minerals (8%). Essential oils are responsible for hop aroma. The main components (80-90% contribution) of essential oils are terpene hydrocarbons (myrcene, humulene, and caryophyllene). There are about 200 compounds in the oil fraction of the hops. Resins are responsible for the pleasant bitterness in beer. The total hop resin can be classified as shown in Fig. 12.8.

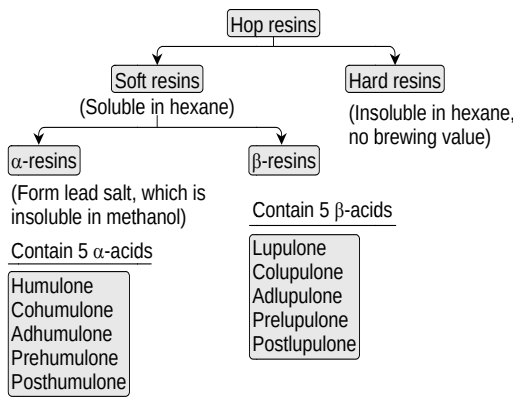


Fig. 12.8 Hop components

Alpha-acids (α-acids) are of prime importance in brewery (Fig. 12.9). Breweries therefore use α-acid content as a criterion in purchasing hops. Normally, hops contain 6-8% α-acids and 3-4% beta acids (β-acids). In the improved varieties, however, the α-acid content can reach as high as 19%, or even more.

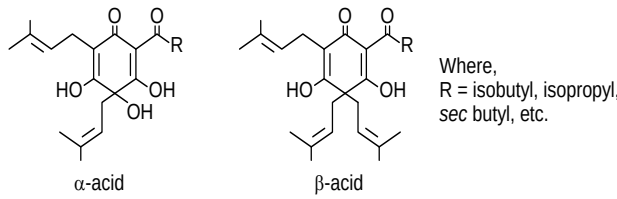


Fig. 12.9 General structure of α- and β-acid

12.2.4.3 Hop processing

Only female hop cones are used (Fig. 12.7). The picked hops contain 75-80% water. Drying is performed on belt driers or in small firms, in batches in kilns. The hops are carefully dried at a maximum temperature of 60°C to a water content of 8-12%.

The dried hops are then cooled and packed into rectangular bales of 50×80×40 cm^3, typically weighing about 90 kg.

Because hop components are very unstable, stabilization of the dried hops is essential. For this, the sacks of loosely packed hops are emptied into sulfur treatment chamber at the packaging plant. Sulfur is burnt beneath this chamber and the sulfur dioxide produced acts as a disinfectant and antioxidant. The sulfur dioxide is then driven off by fresh air.

The hops thus prepared can be used as such or processed further into different forms. A brief description of processed hops is given in the sub-sections to follow.

12.2.4.4 Hop storage

Hops are unstable at higher temperatures and in environment having access to air. They are generally stored at 2-4°C. However, this does not fully arrest the changes: they still lose their bittering potential and the α-acid content (at a slower rate, though). The decrease in α-acid content is greater than the bittering potential because the oxidation products of α-acids also have some residual bitterness. β-acids also contribute to bitterness but it is about 9 times less bitter than α-acids. Bitterness value is therefore expressed as:

$$\text{Bitterness value} = \alpha\text{-acids} + \frac{\beta\text{-fraction}}{9}$$

12.2.4.5 Some commercial hop products

Of the commercial hop products, *hop pellets* (and their variants), *hop extracts* and *iso-a-acid extract* are the most important.

Hop pellets

A very effective way of preserving the contents of hops is pelletization. In this process the dried hops are milled to a powder and then compressed into pellets. Three types hop pellets are available commercially, viz., (i) pellets type 90, (ii) enriched pellets, and (iii) isomerized pellets.

In the production of type 90 pellets, 90 kg of powder, containing all the important contents of the original hops, are produced from 100 kg of intact hop cones. Hop pellets are prepared by hammer-milling of dried hops to powder followed by pressure-pelletizing in dies. The dense pellets, which are roughly 4.5 mm × 4-6 mm in size, are vacuum-packed and stored under refrigeration. Instead of vacuum packaging, CO_2 or nitrogen can also be used as a protectant.

Manufacture of enriched pellets is much more complicated. All the resins and the oils are present in the lupulin gland of the hops. Since the glands have a natural particle size of ~ 0.15 mm the task of removing them from the cone is not easy. For this, gentle milling and sieving at low temperature (-35°C) is done in a special machine. The finely milled material contains lupulin gland and about 50% of the dried cone mass. For the production of enriched pellets, the lupulin glands must be intact and not crushed.

Enriched pellets give about 10% more bittering yield compared to cone hops. The packaging of enriched pellets is similar to that of pellet 90.

Isomerized pellets are produced by adding magnesium oxide (as a catalyst for isomerization of α-acid to iso-α-acid) to the milled hops before pelletizing. The pellets are produced as usual, packed, and then placed in a warm chamber at 50°C until isomerization is complete.

Hop extracts

Hop extracts are concentrations of α-acids. Hop extracts have several advantages over pellets, powders, and cones. Improved hop utilization is the most important factor contributing to cost saving. In conventional brewing in which hops are boiled, bitter principles are lost to the extent of 75% during boiling. The loss figure when hop extract is used is 5-20%. Depending on the type of hop extract, it is also possible to add the extract at various stages of wort boiling.

Some of the important advantages of hop extracts are:

- Improved hop utilization
- More consistent bitterness between successive brews

- Improved stability on long-term storage
- Reduced transportation, storage and handling costs

Hop extracts are prepared by extraction with liquid CO_2 or ethanol, the former being superior. In order to be able to use a gas such as CO_2 as an extractant it must have a density similar to that of a liquid. This high density in the region 0.9-1.0 kg/liter is obtained by compressing the CO_2 gas.

In principle, CO_2 exists in two states which can be used for extraction. The critical pressure of CO_2 is 73 bar and the critical temperature 31°C. Above this pressure but below this temperature CO_2 is liquid but its solvent properties are very limited. Above the critical points the terms used are *supercritical CO_2* or *fluid CO_2*.

For hop extraction purposes, useful solubility properties are obtained with supercritical CO_2 pressures of 120 bar and above. Worldwide, hop extraction is nowadays performed with supercritical CO_2 at pressures of 150-300 bar and temperatures of 32-100°C.

The hops to be extracted are put, as pellets, into the extraction vessel and the latter are brought to extraction pressure (Fig. 12.10). Liquid CO_2 at 60-70 bar is drawn from a working tank and compressed to extraction pressure. The heat exchanger is set to produce the extraction temperature and the CO_2 is pumped through the extraction vessel. The bittering and aroma substances are thereby dissolved in the CO_2. The enriched CO_2 passes into the separation tank. Before this, the pressure is reduced to 60-70 bar in the expansion valve and the CO_2 evaporated in the heat exchanger. As a result the CO_2 loses its ability to act as a solvent and the extract is separated in a container. The gaseous CO_2 is liquefied in the condenser and returned again to the extraction circuit.

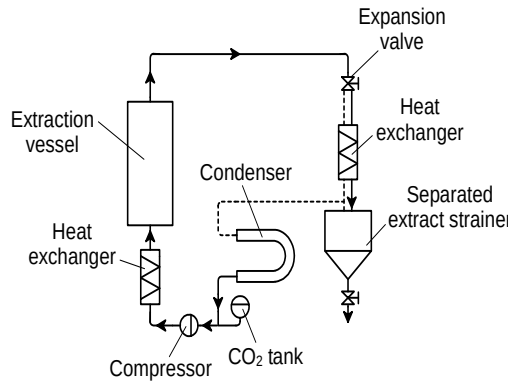


Fig. 12.10 Schematic of liquid CO_2 extraction

Extraction with liquid CO_2 is the best method available. This solvent is highly selective: essential oils and α-acids are extracted sequentially. The final product is free from solvent and is stable at room temperature

Iso-α-acid extract

α-acids themselves are not bitter. It is the iso-α-acid (Fig. 12.11), produced during wort boiling, which is responsible for the bitterness. Thus, boiling of hop extract is obligatory. *Preisomerized α-acids* are now available. They can be directly metered into the final beer. The preparation begins with the extraction process. The hop extract is then isomerized by various means. One method is to treat the extract with aqueous sodium- or potassium carbonate to obtain respective salts of iso-α-acids.

CO_2 extract is used to produce isomerized extract. The extract is heated and emulsified with degassed water. This emulsion is heated further and isomerized by the addition of a basic catalyst (Mg^{++}). This isomerization must be controlled. After the isomerization, hop waxes and uncharacterized soft resins, which are of no use in the further processing, are removed. The solution is then cooled and brought to a pH of 7-8. This results in precipitation of the β-acids and they are removed. As a result of a further pH adjustment to 5-6 the unisomerized α-acids are precipitated and they are then removed by a separator.

Free iso α-acid is now precipitated by the pH reduction to 2 and stored in this form until final packing. Only shortly before delivery to brewery is the iso-α-acid converted to the potassium salt and then brought to the sales concentration and filled into containers.

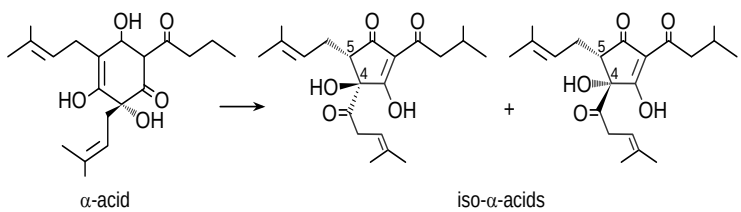


Fig. 12.11 Conversion of α-acid into iso-α-acid

Reduced, isomerized α-acid extract

This special type of isomerized hop extract is obtained by simultaneously reducing and isomerizing α-acids by treating with borohydride. This extract permits the use of clear bottles for packaging without the development of *sunstruck* flavor (page 202).

Hop extract powder

It is prepared by spraying hop extracts onto silica gel. In order to make the hop extract powder pour easily, at least 30-40% silica gel is required. Instead of silica gel, hop powder or hop pellets can also be used.

12.2.5 BREWING WATER

Brewing water must meet not only the general requirements for potable water but also other specific requirements in order to:

- Ensure proper mash pH
- Ensure efficient hop extraction
- Produce good kettle break
- Allow sound fermentation
- Produce acceptable color and flavor in the finished beer

In almost all breweries, the incoming water is passed through activated carbon and in some cases, also through ion-exchange resin. Since the mineral composition of natural water is very much subject to variation, most brewers consider it advantageous to control the composition by external manipulation. The water may be rendered soft and salt mixtures such as gypsum ($CaSO_4$) added in controlled amounts to give known hardness. Addition of $CaSO_4$ to make it similar to the natural water of *Burton Upon Trent* (of United Kingdom) for brewing of beer is called *burtonization*. For lager beers, soft water is desirable at 200-300 ppm hardness. Hardness, particularly due to calcium, has many important functions, especially during mashing. For a good brew, it is normal to maintain 60-70 ppm Ca^{++} content. Alkalinity is also important, and is maintained at 25 ppm.

12.2.5.1 Functions of Ca^{++}

- Protects α-amylase from heat destruction
- Stimulates activity of proteases and amylases
- Increases the yield of fermentable extracts
- Controls the mash pH. Ca^{++} precipitates out calcium phosphate and decreases pH to 5.4 from an initial pH of 6.
- Aids in flocculation of proteinaceous materials and yeast cells
- Aids in oxalate removal. Oxalate is poisonous.

12.3 OUTLINE OF BEER PRODUCTION

The brewing process proper starts with the *mashing* of barley malt and adjuncts. Mashing entails *cooking* of properly ground malt and malt adjuncts (for example, in the ratio 97:3) at a combination of time-temperature so that substrate constituents are degraded to forms readily assimilable by yeasts. Mashing is followed by separation of the solids, which can be carried out either in *mash filters* or *lauter tuns*. The liquor, called *wort*, is then boiled. Hops are added at a rate of about 0.4% and boiling carried out for a total period of about 1.5-2.5 hrs. Hop residues are then strained off, *trub* (the precipitate) removed in a *whirlpool separator*, and the hopped wort cooled to a *pitching* temperature of about 10°C. The main fermentation consists of adding vigorously growing yeast inoculum (~ 10 million cells per ml of wort) in the cooled, pre-oxygenated wort at pH 5 and fermentable sugar concentration around 12%, and allowing anaerobic fermentation at: 8-12°C (about 14 days) for *lager*s, and 12-18°C (about 8 days) for *ales*. Fermentation is carried out in cylindroconical vessels, allowing 25% headspace. Considerable amounts of foam and heat may develop. Foaming can be controlled using antifoams like sorbitan esters, alcohols, etc. The heat (and therefore the fermentation rate) can be controlled by internal cooling coils. The CO_2 evolved is collected and purified for future use. During the course of fermentation, the pH drops to 4, the yeasts utilize 90% of the

fermentable carbohydrates and nitrogen, and the cell population increases by 4-6 folds. The settled (or floating) yeast cells can be removed after cooling to 0°C by sedimentation (or skimming) respectively. The *green beer* (also called *ruh* beer) is pumped to a storage tank for *fassing* while the yeast is recycled. Fassing may take place for several weeks to several months. Depending on the process followed, fassing may be called *lagering*, *aging*, or *maturing*. The process may or may not involve secondary fermentation, called *kraüsening*. A sizeable fraction of yeast *biomass* and *haze* settle down during the fassing period. The process can be hastened by immediate centrifugation of the green beer. The beer is then passed through kieselguhr filter and a polishing filter, CO_2 is amended to 0.5%, and packed in containers. Pasteurization can be done before or after packing by chemical- or physical (heat, filtration) means. See Fig. 12.12 for the flowsheet of beer production.

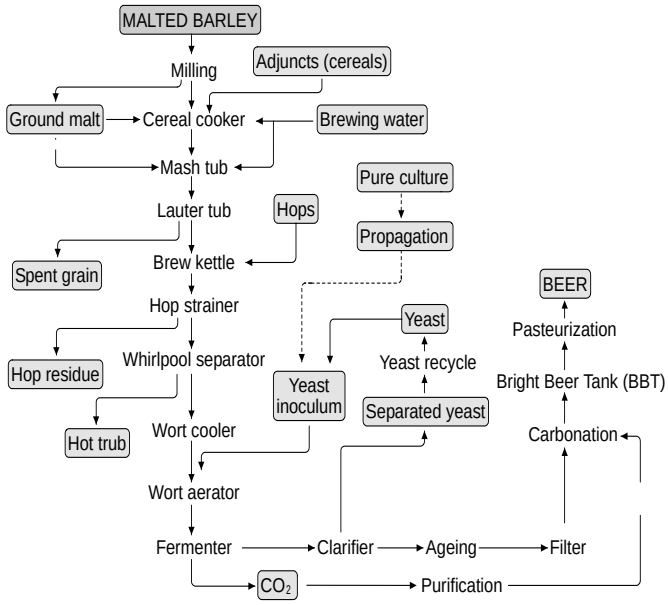


Fig. 12.12 Outline of beer production

The production steps include:

1. Mashing
2. Wort boiling
3. Wort cooling
4. Wort oxygenation and pitching
5. Fermentation
6. Yeast and particle removal
7. Aging and final processing
8. Carbonation, pasteurization, and packaging

12.4 PRODUCTION DETAIL

12.4.1 MASHING

Mashing is a process whereby a combination of time and temperature is used to cook malt and/or adjuncts so that malt enzymes are activated to hydrolyze the substrates and produce as much of valuable soluble portions of malt and/or adjuncts as possible. These soluble fractions are needed for yeast nutrition and metabolism. The objectives of mashing can be summarized as:

- To dissolve as much of readily soluble fractions of the ingredients as possible. This fraction constitutes 10-15%
- To render soluble (through enzymatic actions) substances which are insoluble in their natural state
- To change the chemical structures through simultaneous enzymatic action, of some constituent substances in a planned and predictable manner.

From unit-operation point of view, mashing entails (i) mixing, (ii) enzymatic reactions, (iii) liquid-solid separation, and (iv) elution. The major enzymes and their reactions are:

$$\text{Starch} \xrightarrow{\alpha\text{-and } \beta\text{-amylases}} \text{Dextrins} + \text{maltose}$$

$$\beta\text{-glucans} \xrightarrow{\beta\text{-glucanases}} \text{Low molecular weight carbohydrates}$$

$$\text{Proteins} \xrightarrow{\text{proteases}} \text{Peptides and amino acids}$$

Other enzymes of importance are limit dextrinase, xylanase, and α-glucosidase.

The malt starch is degraded to the extent of 90-95%. The undegraded part consists of dextrins. Malt protein is degraded to the extent of about 40%. Phosphatase enzymes also help in releasing phosphate, which in turn is utilized by the yeasts.

Mashing begins with doughing in, which is simply mixing of ground malt and/or adjuncts with brewing water. This is then heated in vessels called mashing vessels (also called mash converter, mash tun, mash tub, and mash cooker: see Fig. 12.13). The amount of water to be used is variable but in general is 1.6-3.2 times the weight of grist for *infusion* mashing and 3.2-5.4 times for *decoction* mashing. If the adjuncts (see later) consist of cereals, they are given an additional preliminary mashing in *cereal cooker* (vessel similar to mash converter but smaller in size) before adding them to the main mash. Some amount of malt (~10%, but this may be omitted if the adjunct is less than 20% of the grist) is also added in cereal cooker. Of the cereal adjuncts, rice is the most difficult to mash because the starch granules are very small and firmly embedded. The granules swell only slowly in water.

The temperature of water to be added for the doughing in (called *striking heat*) is also very critical and its calculation is quite involved.

The mashing vessel is similar to the brew kettle. The schematic of a modern mash converter with provisions for agitation, heating arrangement and CIP is shown in Fig. 12.13. The mash heating coils consist of semicircular welded-on pipes. The mixing is normally achieved in a premasher (grist hydrator) built into the mashing-in pipe (Fig. 12.14) before the actual mashing begins. Premasher is a mechanical unit that prepares the grist in the slurry form for mashing.

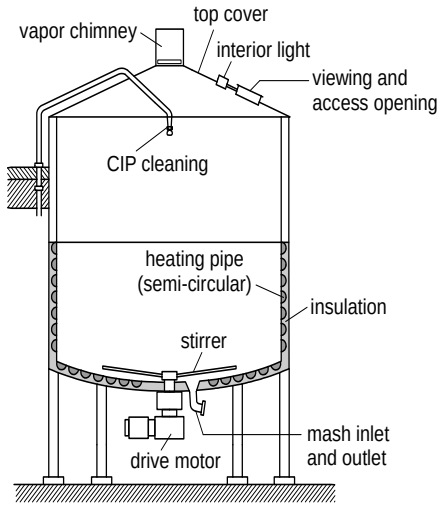


Fig. 12.13 Schematic of a modern mash converter

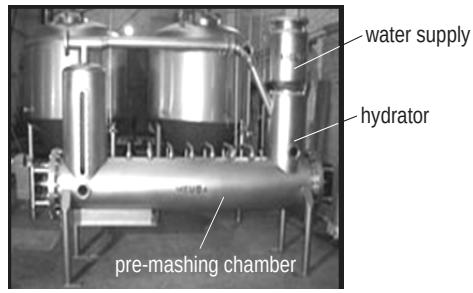


Fig. 12.14 Premashing equipment

During mashing, the stirrer (present at the bottom of the vessel) must be run at a controlled, slow speed for good contact and reaction with malt enzymes.

Basically, there are two main methods of mashing, *viz*., (i) infusion mashing, and (ii) decoction mashing. But there also exist a number of variations. Since the efficiency of mashing is dependent on pH, the mash pH is maintained at around 5 with food-grade acids like lactic acid.

The time-temperature combination during mashing is very important. For instance, prolonged mashing may liberate too much nutrients, which will consequently

encourage the growth of contaminating organisms, or even overgrowth of culture yeasts. This will significantly affect the organoleptic quality of the final beer.

Whatever the mashing technique, mashing entails following four common, obligatory events:

1. Mashing in: Mixing of malt and water
2. Protein pause (rest): Release of peptides and amino acids
3. Sugar pause: Release of maltose and maltodextrins
4. Mashing off: Degradation of residual starch, and inactivation of enzymes

12.4.1.1 Infusion mashing

There are two variations in this technique, *viz.*, (a) *temperature programmed infusion*, and (b) *single temperature infusion*, the former being the industry standard. Infusion techniques are used for the production of ales. The temperature-programmed infusion technique uses two sets of temperatures, *viz.*, (i) 38-50°C for about 1 hr, and (ii) 65-70°C for a few minutes. Both the sets are obligatory.

The lower temperature favors the activity of proteolytic enzymes and the mashing period is called *protein-rest period.* The higher temperature favors starch saccharification and dextrinization. The heat is provided by internal heating coils or welded-on pipes (Fig. 12.13). The mashing may follow in either direction. When the mashing is carried out in the direction (i)→(ii) it is called *upward infusion.* By analogy, the variation (ii)→(i) is called *downward infusion.* Whatever the method followed, mashing is terminated by raising the temperature to 75°C, or a little above. This increase in temperature is sufficient to destroy enzymes. The mash is filtered at this temperature. See Fig. 12.15 for the different enzymatic reactions taking place during mashing.

12.4.1.2 Decoction mashing

This is used for the production of lager beers. In general, slightly less modified malts are used here. Water is added at the rate of 3.2-5.4 HL/100 kg grist and the temperature maintained at 40°C after mixing. The temperature is raised in steps until about 75°C. The temperature increment is achieved in an interesting manner. A portion of mash (about 1/3rd) is withdrawn and boiled for a short period of time and returned to the main mash. Upon mixing, the heated mash raises the temperature of the entire mash bulk. The mixture is left as such for 20-30 min and the next portion is taken out again for the boiling. The enzymes in the boiled portions have been destroyed, but the cell walls of the grain are softened and starch is liquefied. Diastatic action is facilitated in this manner. The process may be called single-, two-, three-, etc.-, mash depending on the number of times portions are removed for heating. The single-mash method is essentially an infusion mashing technique. Usually, the decoction mashing employs up to three-mash level.

During the rest period, the mash separates into two fractions, viz., (i) the "thin mash" that occupies the upper portion, and (ii) the "thick mash" that occupies the

bottom portion. Thick mash consists of undissolved mash components. During decoction mashing, it is the thick mash that is taken for incremental boiling.

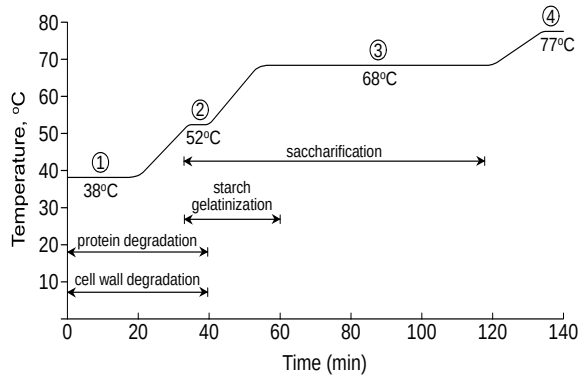


Fig. 12.15 Relation of enzyme action with mashing time-temperature regime

During mashing, the rest period is controlled carefully. Prolonged rest period leads to excess break down of the mash components, which is not desirable. Excessively hydrolyzed mash promotes growth of contaminating microorganisms and also leads to poor foam properties of the beer.

12.4.2 SEPARATION AND WASHING OF MASH

At the end of the mashing process the mash consists of a watery mixture of dissolved and undissolved substances. The aqueous solution of the extract is called the *wort*; the insoluble part is referred to as the *spent grains*. The process of separation of the soluble portion from the spent grains is called *lautering* (or mash separation).

Several types of mashing- and mash separation systems are available today. Great innovations have taken place in the mash filter designs. There are two basic types of mash separators, viz. (i) lauter tun (= lauter tub), and (ii) mash filters.

12.4.2.1 Mash filter

Mash filters are basically variants of membrane-assisted plate and frame filter press. They have provisions for compression and CIP also. The device can handle 12-14 brews per day. The amount of sparging water needed is about 2.5 HL per 100 kg malt. A typical mash filter assembly is given in Fig. 12.16. See Fig. 12.17 for the structural units of plate and frame.

Fig. 12.18 shows the principle of mash filtration in a modern mash filter designed by Meura S. A., Belgium. The system has filter cloth as well as membrane. During filtration, the wort passes through the filter cloth while the grains accumulate in the frame. After the mash supply finishes, the filter is blown with compressed air to recover the entrained wort. Next, sparging water is passed through another opening to elute the residual extract. Finally, the filter is once again blown with air to recover as much of the extract as possible. The filter needs to be cleaned and disinfected

after the frames become full with the spent grain cake. The cake can be removed by dismantling the assembly from the movable end. The filter assembly must be cleaned by CIP (cleaning-in-place) technique every one week or so.

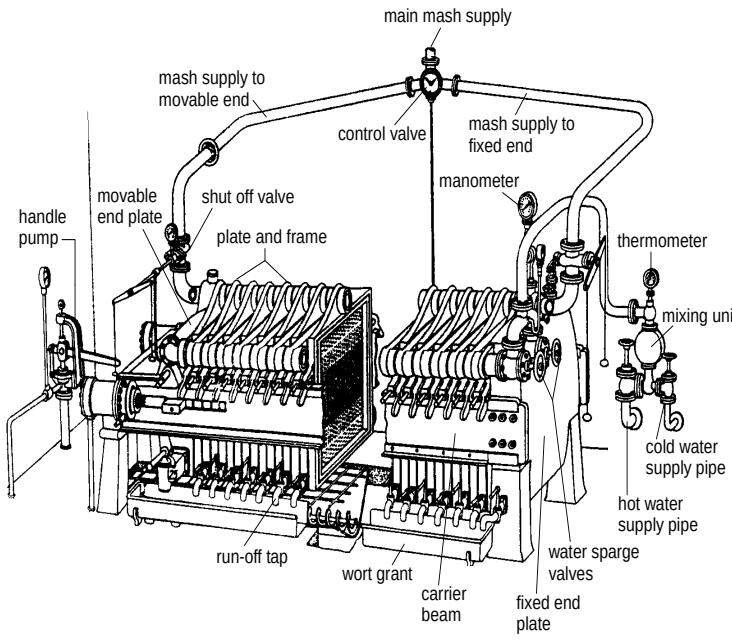


Fig. 12.16 Mash filter

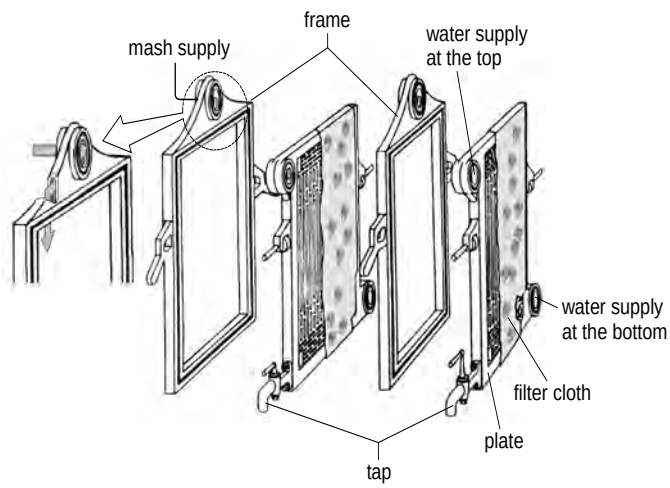


Fig. 12.17 Arrangement of plate, frame and filter cloth in mash filter

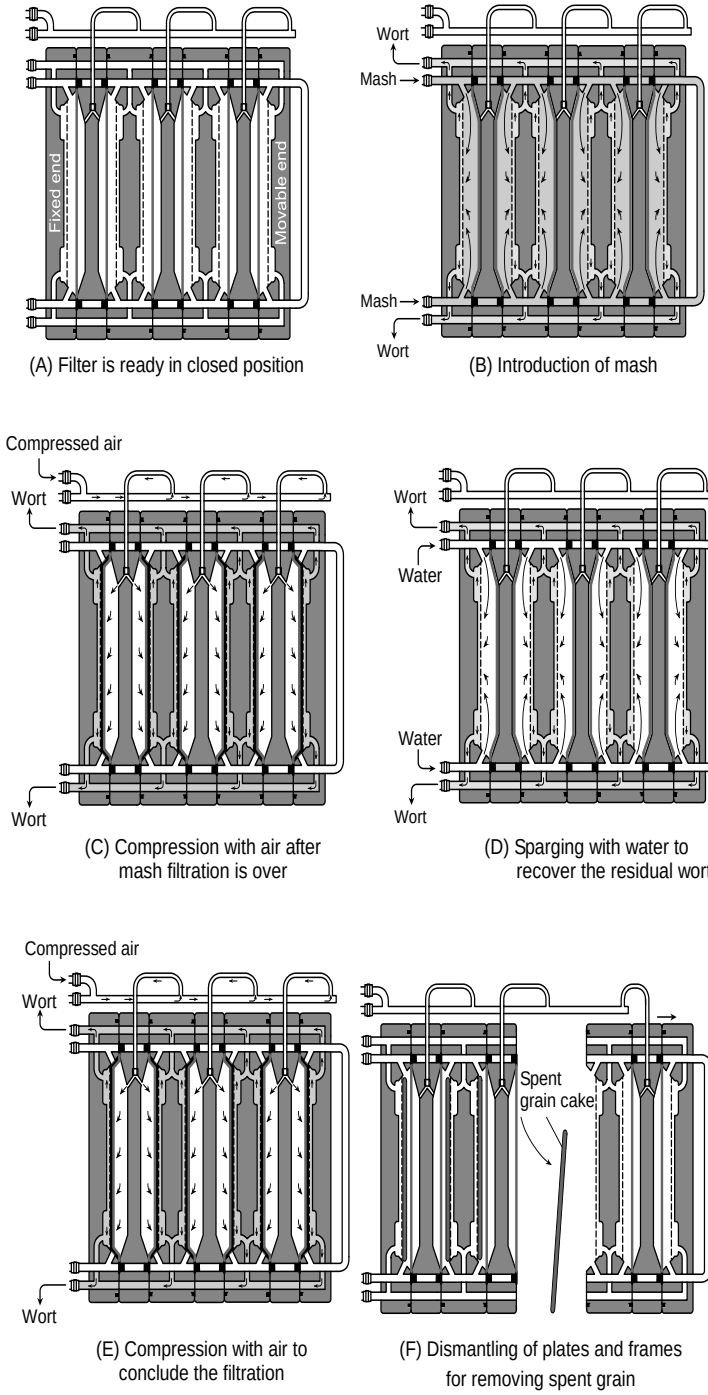


Fig. 12.18 Mash filtration mechanism

12.4.2.2 Lauter tub

Lautering machines are becoming increasingly popular these days. The system, however, is more complex than the mash filters. Lauter tub (also called lauter tun) consists of a cylindrical vessel with false bottom, a raking unit, and several ancillary parts (Fig. 12.19). The tank is first filled with recycled liquor (from the previous lot) to cover the bottom. The mashed grain is introduced on the false bottom to form a bed. The grain bed is agitated with the help of raking unit to facilitate extraction of the wort. The "sweet wort" that is leached out finds its way through the false bottom, ending up in a collecting vessel called "grant". The first run of the wort is recycled until the required degree of clarity is obtained. The residual soluble fractions are eluted by sparging with hot water (75°C) through a spray manifold at the top until the level of soluble fractions in the wash drops down to less than 1%. The run-off time is 2-3 hrs and 98-99% recovery is possible. The spent grains can be removed and dried for animal feed. After the extraction has finished, the vessel and the pipelines are cleaned by CIP process.

There are several variations of the lauter tub. A design by GEA-Huppmann is given in Fig. 12.19. See Fig. 12.20 for the schematic of the same design.

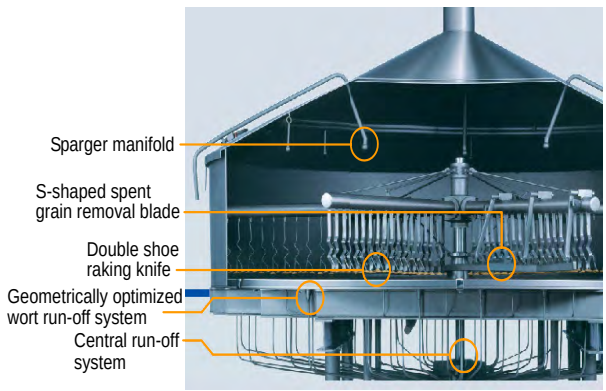


Fig. 12.19 Cut-away view of a modern lauter tub

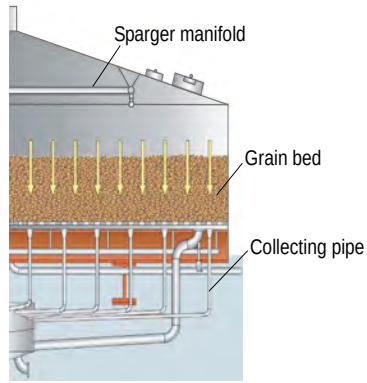


Fig. 12.20 Principle of lautering in lauter tub

12.4.3 WORT BOILING

The sweet wort is taken to a vessel called *brew kettle* or *copper* (as it is usually made of copper, see Fig. 12.22) and boiling started. The heating is done using internal heating coils to achieve vigorous *ebullition*. Requisite amounts of hops are added at this stage and boiling carried out for 1.5-2 hrs. The boiling fulfills various objectives:

- Isomerizes α-acids and extracts hop components
- Precipitates unwanted nitrogenous materials (*hot trub*)
- Removes undesirable volatile compounds
- Sterilizes the wort
- Concentrates the wort (about 15% volume is lost by evaporation)

Many complex reactions occur during wort boiling. All enzymes are destroyed. Some proteins are coagulated and some, together with simple nitrogenous compounds, interact with carbohydrates and/or tannins to give visible precipitates called *hot break* or *hot trub*. The hopping rate is adjusted to roughly 8 g α-acid/HL of wort (or to some defined final bitterness, e.g., 25 bitterness unit).

During kettle operation, the wort becomes slightly more acidic since the melanoidins formed on boiling are acidic and the hops also contribute some acid. The color of wort also becomes darker for the same reason.

There are many variations of the wort kettle. A modern wort kettle with internal boiling is shown schematically in Fig. 12.21.

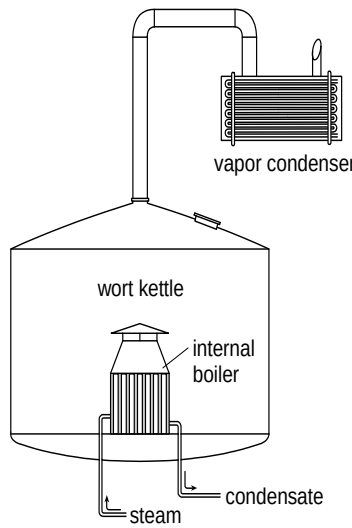


Fig. 12.21 Schematic of wort kettle (brew kettle) with internal heating

Fig. 12.22 The exterior of brew kettle

The boiling protocol for the kettle design shown in Fig 12.21 is as follows:

1. Heating up to 100°C in about 15 min
2. Initial boiling at 100°C for about 10 min
3. Heating up to 102°C in about 10-15 min
4. Boiling under pressure at 104°C for about 15 min
5. Final boiling at 100°C for about 10 min

This design reduces the boiling time by 50% in comparison to conventional boiling.

At the end of the prescribed boil period, which may be based on time, evaporation rate, or a combination of two, the wort is pumped through strainers (*hop strainers*) to remove hop residues. The hot trub and any hop particles remaining in the copper must be removed for subsequent processing of the wort. Whirlpool separation is the most elegant method for break removal and is the least costly alternative of all trub removal methods. In modern breweries, centrifuges are also used for the same.

A whirlpool is a vertical cylindrical vessel with no internal fittings, into which wort is pumped tangentially. This produces a sustained rotational flow in the vessel, which causes the hot break to settle in the shape of a cone in the middle of the vessel. The clear wort can be drawn off at the side of the bottom. A vessel with a conical recess in the middle has been shown to retain the trub better. See Fig. 12.23 for a typical design of the whirlpool.

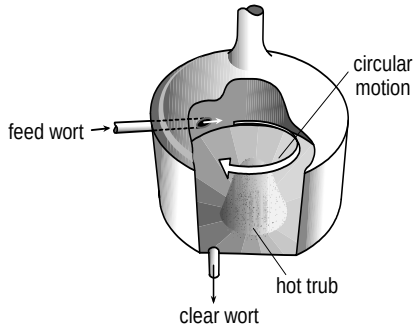


Fig. 12.23 Schematic of whirlpool separation

The way in which the wort is pumped into the whirlpool is particularly important. The wort inflow velocity should not exceed 5 m/s. The clear wort is removed from the whirlpool after a rest period of 20-40 min. In some variations, however, the wort is drawn even whilst the sedimentation is taking place in the lower part.

12.4.4 WORT COOLING

After trub separation the wort is cooled in tubular or plate-heat exchanger. Additional trub, called *cold trub*, can be removed in this stage. Cooling fulfils several objectives:

- Reduces the wort temperature to pitching temperature
- Aids in the removal of cold trub
- Reduces temperature to microbiologically safe zone

12.4.5 WORT OXYGENATION/AERATION

Brewery fermentation differs from most fermentations in that *oxygen is supplied only once*. The purpose of wort aeration is not the promotion of yeast growth as such, but the promotion of biosynthesis of lipids required for yeast growth. The oxygen supplied is used by the yeast for the synthesis of *ergosterol* and unsaturated fatty acids, which are integral components of cell membrane. Supply of these preformed components obviates the need of aeration. Wort oxygenation is achieved, usually with the incoming wort, by passing sterile air to a level of 8-10 ppm.

12.4.6 YEAST HANDLING AND PITCHING

Under normal conditions yeast cells grown during one fermentation cycle are used as inoculum in subsequent cycles. It is necessary to maintain yeast in a satisfactory physiological condition before reuse and, in this context, glycogen level within the cell is of prime importance. Yeast is usually stored in beer or water. Handling procedures should avoid the inclusion of air and the yeast should be rapidly cooled to 4-6°C. A larger inoculum size should be used if the yeast has low glycogen content.

Glycogen is an energy reserve for yeasts. During the first 6 hrs after pitching, the yeast depends solely in the intracellular glycogen for the synthesis of lipids and sterols needed for growth.

Two tests, the *acidification power*, and *specific O_2 uptake rate* have been developed, both of which correlate well with fermentation performance. Either test may be used to determine optimum pitching rate or to reject yeast of unsatisfactory performance.

Pitching yeast is now recognized as the major source of bacterial contamination, especially *Lactobacillus*, *Pediococcus*, and *Obesumbacterium*. These organisms are involved in beer spoilage. Therefore, if recycling is desired, the yeast is purified before pitching using any one of the following methods:

1. Repeated washing with sterile water
2. Treatment with dilute acids (pH 2.2-2.4) for 1-1.5 hrs. Acids such as tartaric H_3PO_4, or H_2SO_4 can be used
3. Treatment with 0.75% acidified Na- or ammonium persulfate. The temperature should be maintained below 5°C. Additional treatment with antibiotics such as nisin, polymixin, penicillin and neomycin has also been proposed.

It is advisable to acid-wash yeast only after 7-8 repitching (not at every repitching). Reculturing from stock culture (pure culture) is done after every 20-30 repitching.

Typically, the pitching rate (final) is about 2 g pressed yeast per liter. This works out to be a quarter crop from previous fermentation or 1 million cells/ml/°Plato. The term °Plato (degree Plato, °P for short) is a hydrometric measure, where 1% sucrose is equivalent to 1°Plato.

When top yeast is to be recycled, the first skimming, being most contaminated, is discarded. Only the middle skimming is used. In the bottom yeast also, the middle portion is desirable. The upper portion of crop tends to be of non-flocculating type while the bottom portion of the crop tends to be early flocculating type, both of which are undesirable in brewing.

12.4.6.1 Determination of yeast cell concentration

The correct number of yeasts cells in each batch is very important. Yeast number representing 1 million cells/ml of wort/°Plato is considered the correct pitching rate. Yeast cells are counted using hemocytometer and adjustment made accordingly. The counting may be done on the crop or on wort after pitching. A brief description of the cell counting process is given in the following paragraphs.

Using a clean, dry flask, take a small sample from the fermenter. Swirl the sample around to help break up any clumps of yeast and to make sure it is properly mixed. The swirling also helps remove any gas in solution. The hemocytometer and the cover slip should be clean and dry. Place the cover slip over the counting areas. Next, affix the pipette into the pipette pump. Pull out ~ 2 ml of beer into the pipette, and then purge out 2-3 drops to clear the tip of any differentiation that may

occur. Immediately place the tip of the pipette on the V-shaped groove (see Fig. 12.24) and gently fill the counting area under the cover slip without disturbing the cover slip; only a drop or so is needed. The counting chamber must be completely filled, but not overfilled. The sample should not run out into the canals or bulge at the edges.

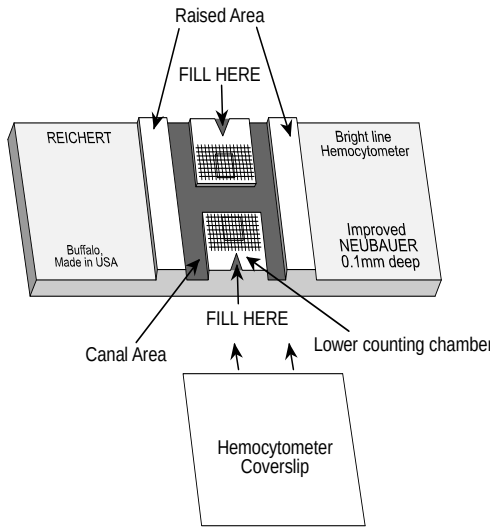


Fig. 12.24 Hemocytometer showing counting chambers and fill points

The sample on the hemocytometer is now ready to be viewed under the microscope. Without spilling any of the sample, carefully place the hemocytometer on the microscope stage. Using 10× objective lens, frame up one of the counting chambers. You should see a grid with 25 large squares, each of which contains 16 smaller squares. You can count all of the cells within the 25 squares, or, when there is a large number of cells, you can count the cells in the four corner squares and in the center square (Fig. 12.25) and multiply the total by 5. To count cells within an area, switch to 10× eyepiece with 40× objective lens, frame up the counting area of one of the 25 large squares, and take a count.

To eliminate the chance of counting a square twice or a cell twice, it is important to use a standardized counting procedure. Always count in one direction (left to right, top to bottom, for example). Cells touching the top or right-hand boundaries are not counted. Cells touching the bottom or left-hand boundaries are counted (Fig. 12.26). Cells that are budding are counted as one cell, unless the daughter cell is equal to or greater than one-half the size of the mother cell, in which case the daughter cell is counted as a separate cell (Fig. 12.27).

If there are more than ~ 50 cells per large square (that is, per square that contains 16 smaller squares) dilute the sample to be counted. The dilution factor must be used to back-calculate the final cell count. It is important to dilute and count samples of yeast slurry as soon as possible after sampling to prevent inaccurate counts due to

cell multiplication. Because there can be a high degree of inaccuracy inherent in this procedure due to human error, perform a second count to confirm the first count.

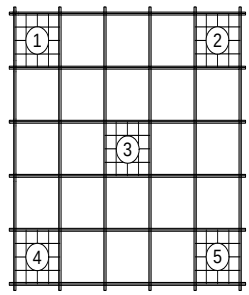


Fig. 12.25 Counting area grid on a hemocytometer counting chamber

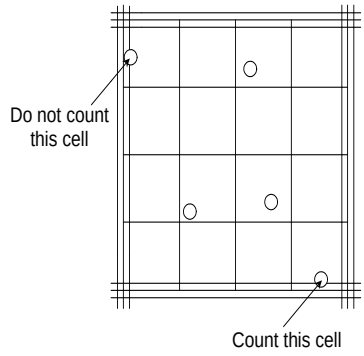


Fig. 12.26 Close up view of a counting square showing counting protocol

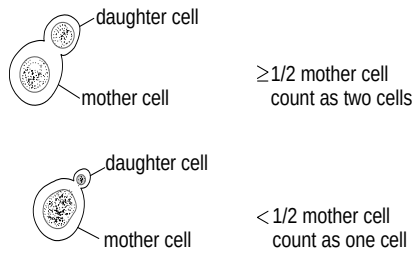


Fig. 12.27 Decision for counting yeast buds based on size

The total number of cells per ml of sample is calculated as follows:

$$\text{Cell count per ml} = N \times 5 \times F \times 10^4$$

Where, N = number of cells counted in 5 large squares; F = fold of dilution made.

For example, if you counted a total of 300 cells in your 5 large squares (without dilution of sample), the total cell count per ml of sample is given by:

$$300 \times 5 \times 10^4 = 1.5 \times 10^7 \text{ cells per ml}$$

12.4.7 YEAST PROPAGATION

When pure culture is to be used, a stepwise propagation is used. There are three distinct stages in pure yeast propagation, viz., (i) isolation of suitable yeast cells, (ii) growth in the laboratory, and (iii) growth in the plant.

For industrial use, authentic yeast cultures are used. Thus, it should be less general to frequently isolate yeast for the industrial production of beer. Nevertheless, if desired, yeasts can be isolated by micromanipulator technique from the actively fermenting beer. The following discussion relates, typically, to the propagation of commercially available brewers yeast.

12.4.7.1 Yeast propagation in the laboratory

The primary stock culture (which may be in vials, slants, or other storage forms) is initially cultured in 5 ml of sterile wort. After incubation for a couple of days, the culture is transferred to a flask with 50 ml of sterile wort and incubated again for a couple of days. Next, the contents are transferred to 500 ml of sterile wort. Further multiplication of the yeast is carried out until 20 liters of green beer in the vigorously fermenting stage (the high foam head stage) is obtained.

Vessels larger than 10 liters are made of chrome-nickel steel and are called Carlsberg flasks (Fig. 12.28). A small Carlsberg flask has 8-10 liters capacity while a large Carlsberg flask may have 20-25 liters capacity. The yeast needs to grow aerobically and for this, the Carlsberg flask is equipped with sterile air filter. It also has all the provisions for aseptic propagation of the yeast cells.

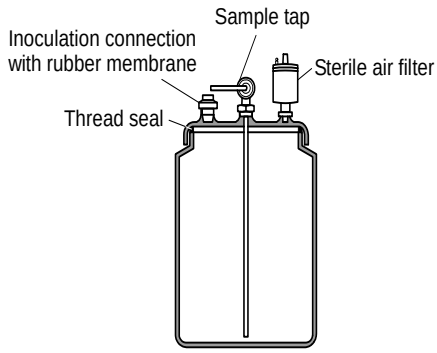


Fig. 12.28 The Carlsberg flask

12.4.7.2 Yeast propagation in plants

Further multiplication of the yeast occurs in the brewery in yeast propagation plants (closed type) or open growth vessels. There are several methods of yeast propagation but all of them use sterile wort. Small breweries carry out propagation by "milk churn process" in which 40-liter milk cans can be used for the vessel. Large breweries use cylindroconical vessels (CCV) for the propagation. The vessels have

facilities for aeration, agitation, CIP, and temperature monitoring. The propagation occurs in a batch process at about 22-25°C. Several variations are used with the CCV system, notable among which are Conti-Prop system, Prof Back system, and Wackerbauer single vessel system.

In the Conti-Prop system, the culture is grown in CCVs to a vigorous growth phase (called "high krausen" in brewery) for 24-36 hrs and the total contents of the vessel are pumped aseptically into the next largest vessel and topped up with sterile aerated wort. This process continues until the required amount of yeast is obtained.

In the Wackerbauer single vessel procedure (Fig. 12.29), the contents (10 liters) of the Carlsberg flask (at high krausen) are pitched aseptically into 25 HL of sterile wort medium previously placed in a CCV of about 50 HL capacity. The CCV is equipped with heating/cooling jacket, aeration lance, and CIP system. The culture is propagated for 36-48 hrs at 20°C with aeration at intervals (1 min aeration every 15 min for the first 24 hrs, and 1 min aeration every 5 min for the second 24 hrs). This "high krausen" culture is pitched into 500 HL of wort for fermentation.

12.4.8 FERMENTATION

Brewery fermentations have traditionally been considered to be of two distinct types: (i) top fermentation and (ii) bottom fermentation. Traditionally, ales were fermented in relatively shallow, circular or rectangular vessels while the lager fermentations were carried out in similar but deeper vessels. As of now almost all breweries use cylindroconical vessels (see Fig. 12.30).

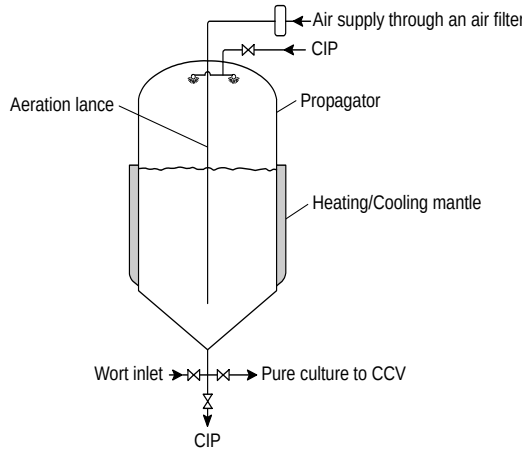


Fig. 12.29 Wackerbauer propagator

At least 25% of the vessel capacity is used as headspace to allow for foams that generate during the high krausen. The charging and discharging of wort, beer, and yeast are done from the bottom.

Customarily, lager beers are fermented at 6-12°C for 8-14 days and ales at 12-20°C for 4-7 days. Fermentation proceeds most rapidly at higher temperatures but the risk of flavor defect is greater and the temperature is a compromise between these factors.

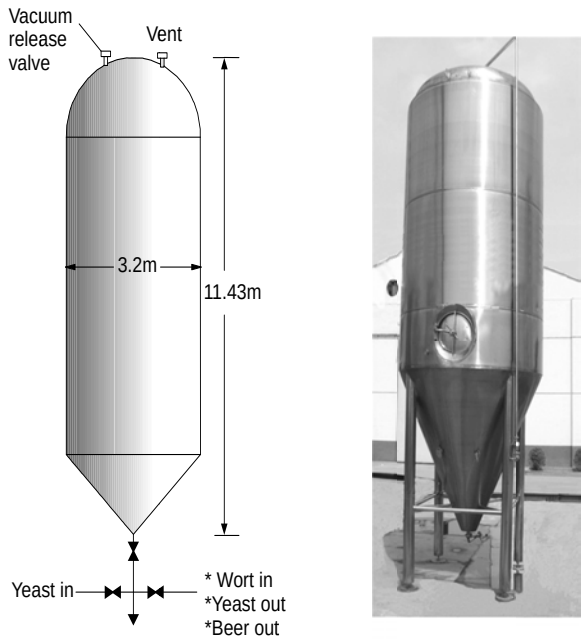


Fig. 12.30 Beer fermenting vessel

The most serious situation faced by the brewer is a *stuck fermentation*. The main medium contains roughly 12% sugar (measured as °Plato). Five to 6 hrs after pitching, nutrient uptake commences. Twenty four to 48 hrs after pitching, clumps of foam called *krausen* appear on the surface due to rapid evolution of CO_2: a depth of up to 1 m may be formed. This period of vigorous fermentation is called *high krausen* and corresponds to logarithmic phase of yeast growth. Yeast growth during the fermentation amounts to 3-5 times the original number. As the fermentation proceeds, the specific gravity decreases from an initial of about 1.048 down to 1.01-1.016. The process is referred to as *attenuation*. Fully attenuated beers have very low levels of carbohydrates and amino acids. Fig. 12.31 is representative of typical physicochemical changes occurring during fermentation.

The temperature is maintained by internal cooling coils. Temperature control is very important for controlling the fermentation rate. Excessive foaming can be controlled using antifoams such as neutral fats and oils, sorbitan esters, alcohol, polyethers, or silicone oils. In brewing industry, there are a number of advantages to controlling foam, some of which are:

- The fermenter capacity can be increased by up to 20%
- Lesser protein denaturation

- Improved foam stability
- Improved hop utilization (less bitter principles lost)

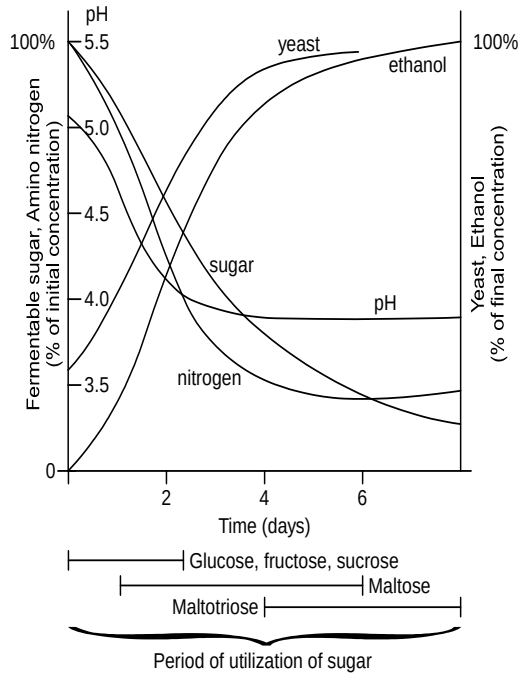


Fig. 12.31 Changes during beer fermentation

CO_2 generated during the fermentation is recovered, purified, and stored (page 212) for adjusting CO_2 level during finishing. The fermentation also lowers the pH to 4.0 and 3-5% alcohol by volume is produced. The dissolved CO_2 content in the final broth amounts to 0.3%. The main fermentation, called *primary fermentation*, produces a *young*- or *green* beer (also termed *ruh* beer), which has varying fates.

If the broth is not fully attenuated, it is subjected to *secondary fermentation*. Secondary fermentation is simply an extension of primary fermentation but at a very much reduced rate because of lower temperature and lesser concentration of both yeast and fermentable sugars. In order to invigorate the yeast and accelerate the secondary fermentation, *priming sugars* or syrups may be added. The fermentation occurs in closed tanks so that the beer becomes fully attenuated during the process.

Fully attenuated beer can also be subjected to secondary fermentation. In this case, about 10-20% of the vigorously growing yeast from the fresh batch is added to the attenuated beer. This addition of young beer to initiate a secondary fermentation is called *krausening*.

The most important objective of the secondary fermentation is flavor maturation of green beer. The unique quality the beer attains in the process is simply unmatchable. The flavor maturation is due to:

- Reduction in the concentration of H_2S.
- Reduction in the concentration of acetaldehyde
- Reduction in the concentration of diacetyl (= vicinyl diketone or VDK)
- Formation of esters that contribute to flavor

Other advantages of secondary fermentation are as follows:

- Clarifies beer
- Produces self-carbonated beer
- Chillproofs (page 209) and stabilizes beer

Krausening produces high quality beer but the primary fermentation yeast should be removed as completely as possible otherwise it spoils the beer.

The secondary fermentation proceeds at 5-6°C for a week or until it reaches the attenuation limit. Yeast is removed every 2-4 days, the last removal naturally being before filtration. An important indication to maturation is the reduction in diacetyl level. The maturation period is therefore also called *diacetyl rest period* during which the diacetyl level drops to about 0.1 ppm. Only after diacetyl removal is the beer cooled to the lagering temperature of -1°C and the cold lagering phase is continued for a week.

The removal of diacetyl during maturation is considered to be a decisive criterion. Consequently, most breweries begin cold lagering only after the diacetyl content has been reduced to less than 0.1 ppm.

Diacety is the most important immature beer aroma. Above the threshold value it gives beer undesirable slipperiness in the mouth and an unclean, sweetish to revolting taste, which in higher concentration is responsible for the aroma of butter. Because pentanedione also acts in a similar way, although with a substantially higher taste threshold, these substances are considered together. They are referred to as vicinal diketone (VDK) because both compounds are diketones. The breakdown of these vicinal diketones occurs parallel to other maturation reactions during the beer conditioning process and is therefore nowadays regarded as the essential criterion for the state of maturation of a beer.

The formation and removal of vicinal diketones occurs in three stages. The starting point for vicinal diketone synthesis is the pyruvate formed as an intermediate during respiration and fermentation. Next, the yeast converts pyruvic acid to acetohydroxy acids, which in turn are excreted out of the cell. The acetohydroxy acids give rise to vicinal diketones by oxidative decarboxylation outside, independent of yeast cell.

The diacetyl formed can only be removed again by the yeast cells and the removal occurs by reduction reactions. The yeast's ability to remove diacetyl is about ten times as great as its rate of formation during fermentation. Diacetyl removal is very temperature dependent and increases greatly with increasing temperature. Consequently, storage of beer at 18°C for about 15 days after the secondary fermentation is not uncommon.

Spontaneous clarification during krausen storage is due to the positive pressure developed by CO_2. At least 10-15 fold reduction in turbidity is possible. Clarification of beer during maturation is hastened by adding *fining agents* such as bentonite, isinglass, Irish moss (carrageenan), and silica gel at the onset of aging.

12.4.9 YEAST AND PARTICLE REMOVAL

A major portion of yeast is removed by sedimentation or skimming before maturation. Yeast collection in CCV system is different from conventional system. In the CCV system, for the bottom-fermented beers, the yeast is removed from the cone before the beer is transferred (for top-fermented beer, the yeast removal occurs after the beer has been drawn). Yeast is collected several times at short intervals. Basically, the yeast should be used again for repitching as soon as possible. To activate its metabolic processes it should be aerated for 2-3 hrs before pitching.

Washing and sieving weaken the yeast and also introduce the risk of microbiological infection. Therefore these should be avoided if possible. If the yeast is stored for only 2-3 hrs, cooling is not necessary, but the longer it is stored the more important cooling becomes. In pauses between brews, yeast should be stored at 0°C under beer containing residual extract or under water. For longer storage, however, it must be pressed and stored cold.

The young beer is further clarified either by secondary fermentation or by mechanical means such as centrifuges and filters. Centrifuges (Fig. 11.9) are primarily used (but is not obligatory) for removing gross impurities before carrying out kieselguhr filtration (discussed shortly). Today, a large number of filter types are available, e.g., diatomaceous earth filter, sheet filter, pulp filter, candle filter, leaf filter, cartridge filter, etc. The classification of these filters are rather contextual.

12.4.9.1 Plate and frame filter (diatomaceous earth filter)

Plate and frame filtration is classified under powder filtration, cross-flow filtration, etc., depending on the context. Powder filtration connotes filtration with kieselguhr or perlite as the filter aid. Kieselguhr is the term used for fossils of diatoms of which there are more than 15000 types in the sea. Kieselguhr usage can vary between 80 and 200 g/HL. Perlite is a material of volcanic origin and consists principally of aluminum silicate.

Kieselguhr filtration is widely used for the preliminary plate and frame filtration of beer. It is performed using a fine wire mesh, having a mesh gap of 70-100 μm, or other filters having a much larger pore size than the guhr particles (2-4 μm). This means that the guhr can pass unhindered through the mesh or filter (thereby making the beer even more turbid than it was before) unless some special techniques are used.

To obtain a perfect filtration effect a filter cake of filter aid is applied in three coating layers, viz., (i) base, primary, or precoat layer, (ii) second coat or safety layer, and (iii) continuous dosing. See Fig. 12.32 for the principle of kieselguhr filtration.

For the base layer, degassed water or filtered beer containing a concentrated suspension of a coarse guhr is circulated at an overpressure of 2-3 bar throughout the filter. A pressure-stable primary layer is thereby built up which will prevent the finest filter aid entering the filtrate. The primary layer forms the most important element for the further build-up of the cake and for filtration itself. The particles of this primary layer bear against one another and mutually prevent each other from flowing any further (through the wire opening or plate pore).

For primary coating, guhr is used at the rate of about 700 to 800 g/m². This is 70% of that used in the total precoat.

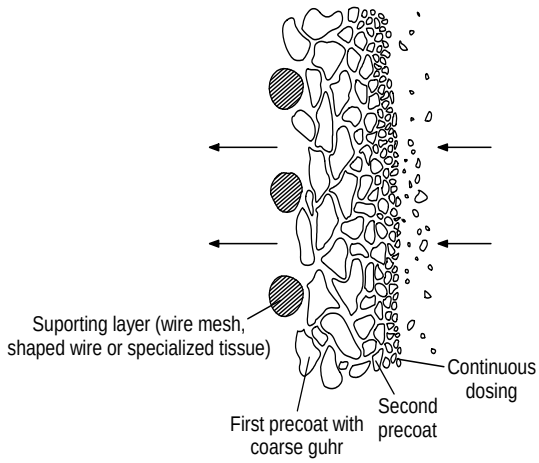


Fig. 12.32 Principle of kieselguhr filtration

The second coat ensures that even the first filtrate after precoating runs clear. This layer is again applied with degassed water or filtered beer but a finer, more effective kieselguhr mixture is used. This is adequate to retain the haze and prevent blocking of the filter. It is very important to have a very uniform distribution of the precoat over the entire filter surface. Thinner regions or edges in the precoat cause unevenness in flow and possibly also allow haze to pass through.

Continuous dosing serves mainly to maintain the permeability of the precoat after the change over to filtration with a constant volume flow rate. The filter aid is applied as body feed, which means that it is continuously mixed with the beer bulk (in regulated amounts) throughout the filtration process. The constant flow rate is necessary since with pressure surges or flow irregularities the bridges formed on the sieve may be broken through and the beer would run turbid. This must be prevented in all circumstance between inflow and outflow. It is desirable that this pressure increase occurs slowly and continuously up to an excess pressure of 2-5 bar. The pressure difference should on average increase at most 0.2 to 0.3 bar per hour. Normally, the continuously dosed mixture consists of 2/3 medium guhr and 1/3 fine guhr. Kieselguhr usage during continuous dosing is between 60 and 120 g/HL.

During filtration, entry of oxygen is very damaging and this should be minimized by removing air from pipes, beer, and kieselguhr before filtration. Only degassed water must be used. Usually, flushing with CO_2 before running the device is advantageous.

Kieselguhr filtration alone is not sufficient to give final clarity to beer. Therefore the beer is further filtered in "polishing" filters (e.g., membrane filters) to give the former a polished appearance.

Flow of liquid in plate and frame filter

Plate and frame filter consists of a unit in which alternating, usually square, plates and frames are suspended. Filter sheets are hung over the plates on both sides and these form seals between the frames and plates. The filter sheets are made of cellulose and ion exchange resins. The sheets are stabilized by mass hardening so that they are washable and can therefore be used for a long time. After filtration the kieselguhr is spayed off and the filter sheet can then be used again. See Fig. 12.33 for a schematic of the plate and frame filtration.

The kieselguhr is circulated (as a slurry) from the precoat tank to produce ~ 1.5 mm thick coating. Recirculation is continued until the liquid becomes clear. Without a drop in pressure, a second coat is applied. The actual filtration entails continuous metering of the earth into the beer (as body feed, explained earlier) before filtration. A normal length of time can be 8-12 hrs.

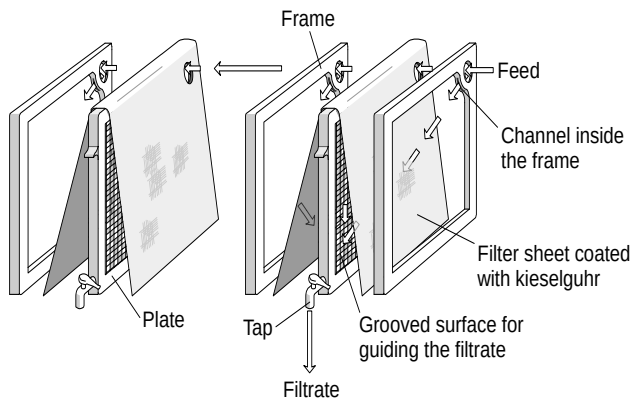


Fig. 12.33 Component parts of plate and frame filter

12.4.10 CARBONATION

After the beer has reached the final degree of clarity, it is stored in a pressurized buffer tank called *Bright Beer Tank* (BBT). CO_2 is injected through an orifice in the pipe during the transfer. The whole is rested at 0°C for 24 hrs for CO_2 dissolution. The final concentration of CO_2 is around 0.5%. CO_2 fulfils various functions, viz.:

- Produces characteristic flavor (bite) of a carbonated beverage

- Produces fizz and *head*
- Prevents in-bottle gushing and foaming
- Creates anaerobic condition, which in turn prevents oxidation and microbial growth

The pressure inside the bottle is around 20 psig. The O_2 level in the beer should be less than 0.1 ppm.

12.4.11 BOTTLING

Packaging can be done in kegs, cans, barrels, and bottles. In bottle- and can filling, the fillers are always under counter- or hyper-pressure. There are two basic methods of filling, viz., (i) Equal pressure filling (isobarometric method), and (ii) Differential pressure method.

In the equal pressure method, the beverage inlet is opened and because there is the same pressure in the bottle and the filler, the bottle is filled as a result of the difference in height. In the differential pressure method, the higher pressure of the filler forces the liquid in.

The metering of beer into the bottle is based on height or level. Beer filling machines are always built as rotating machines with up to 200 filling valves. The bottles are delivered on a conveyor belt, separated to a predetermined spacing by a separating device, and positioned on a lifting platform under the filling elements by a *star wheel* loading device.

The filling is accomplished in phases. The bottle is first positioned for receiving the liquid, raised, and CO_2 injected to displace the air. Air can also be displaced with water (deaerated) jet at high pressure. The bottles are then filled with fillers that operate at three variable speeds: first at a *slow speed* (to avoid frothing), then *rapid speed* (bulk filling), and finally *slow speed* (to achieve fine filling) again.

The bottles are crown-corked to withstand the internal pressure. Since beer is sensitive to light, colored bottles (amber or green) are used. Upon exposure to sunlight, beer tends to become *sunstruck* or *skunky*. Skunk is a polecat known to emanate very bad smell. The UV radiation of the sunlight and iso-α-acids of the hops undergo photochemical reaction to produce *prenyl mercaptan*, the compound responsible for the off odor. The compound is also called *isopentenyl mercaptan* or *3-methyl-2-butene-1-thiol*. The scheme of reaction is shown in Fig. 12.34.

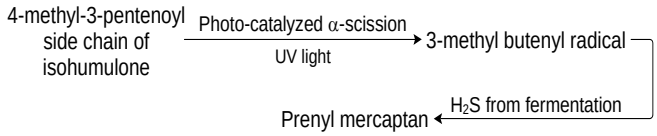


Fig. 12.34 Formation of skunky odor

Selective reduction of the isomerized α-acid with sodium borohydride is one alternative to prevent this defect. The α-acid, however, is less bitter. Reduced iso α-acid does not take part in photochemical reaction and hence colorless bottles can also be used. This, however, could not be successful commercially. The beer packed in colorless bottles was reminiscent of horse urine in color!

12.4.12 PASTEURIZATION

Pasteurization of beer can be done by three main methods:

1. Heat treatment
2. Sterile filtration
3. Chemical treatment

Heat treatment includes (i) Hot Filling (no longer used), (ii) Flash Pasteurization, and (iii) Tunnel Pasteurization (slowly being replaced by flash pasteurization)

12.4.12.1 Flash pasteurization

In flash pasteurization the beer is heated by a plate heat exchanger to at least 68-75°C and held at this temperature for about 50 s. Then it is cooled down again. A regeneration section (similar to milk pasteurization) is used for economizing energy. It is important that the CO_2 saturation pressure is lower than the applied pressure at all times and that pressure at the beer inflow side is greater than that at the outflow side. High pressure pumps for pressures up to 12 bar are necessary for this. The CO_2 saturation pressure follows Henry's law. Keeping temperature constant, the solubility of CO_2 (%) at different pressures is obtained by simply multiplying the solubility of CO_2 at atmospheric pressure (1 bar) with the desired absolute pressure (bar). For example, at a constant temperature, if the solubility of CO_2 is 0.321%, the solubility at 5 bars will be (0.531×5)%.

In beer pasteurization, a term called *pasteurization unit* (denoted by PU or PE) is used. Pasteurization unit is defined as the time in minutes necessary at a particular temperature (for a defined effect). The temperature of 60°C is set as the standard and it is calculated that:

$$\text{Number of PU (or PE)} = \text{time (min)} \times 1.393^{(\text{temperature in the heater} - 60°C)}$$

Thus, when heated for 1 min, the PU at different temperatures is as follows:

For 60°C, PU = $1.393^{(60-60)} = 1.393^0 = 1$

For 61°C, PU = $1.393^{(61-60)} = 1.393^1 = 1.393$

For 62°C, PU = $1.393^{(62-60)} = 1.393^2 = 1.94$

For 63°C, PU = $1.393^{(63-60)} = 1.393^3 = 2.70$

For 64°C, PU = $1.393^{(64-60)} = 1.393^4 = 3.76$

For 65°C, PU = $1.393^{(65-60)} = 1.393^5 = 5.24$

For 66°C, PU = $1.393^{(66-60)} = 1.393^6 = 7.30$

and so on

For beer pasteurization, 5-6 PU are reasonably adequate but 14-30 PU are used to allow margin of safety. If 15 PU is chosen as an example of what is necessary, the time needed at different temperatures is calculated as follows:

At 66°C for 15 PE, the time needed (in min) = 15 PU/7.03 PU = 2.06 min
At 65°C for 15 PE, the time needed (in min) = 15 PU/5.24 PU = 2.86 min
At 64°C for 15 PE, the time needed (in min) = 15 PU/3.76 PU = 3.98 min
and so on

12.4.12.2 Tunnel pasteurization

Tunnel pasteurizers are meant for bottled and canned beers. At present, tunnel pasteurization is being replaced by flash pasteurization or sterile filtration.

In the pasteurizer, the filled bottles are heated in stages, subjected to pasteurization temperature for a fixed time, and then cooled again. The residence time at the pasteurization temperature must be selected such that the core part (which is about 1.5 cm above the bottom of the middle of the base of the bottle) is also heated for an adequate time-temperature regime. Because of the poorly conducting glass material of the bottle, uniform heating is not easy. The space in the bottle during pasteurization must not be less than 5% of the volume of the bottle otherwise the pressure in the bottle can cause breakage. In modern pasteurizers, the temperature is monitored by a recorder. Heating and cooling of the bottles and cans is performed using various water circulation paths in order to utilize recovered heat. Passage through the tunnel takes about an hour.

The main components of the tunnel pasteurizer consist of (i) the transporting drive mechanism and (ii) the spraying and water circulation system. The transportation is done by conveyer chains or "walking beam" conveyor system.

12.4.12.3 Sterile filtration (filter stabilization)

There is an increasing trend to pasteurize beer by sterile filling method. This method of stabilization helps retain the beer flavor to a significant extent. Consequently, beer that has been sterile-filtered is considered organoleptically superior.

Membrane filters and modules are available for such a filtration. The filtration is carried out after kieselguhr filtration followed by a series of 3 additional stages of filtration. The first stage employs module- or cartridge filter with pore size of about 5 μm. The second stage filter (polishing filter) employs cartridge filter with pore size of 1 μm. The third stage filter (sterilizing filter) employs cartridge- or module filter with pore size of 0.45 μm. The installed view of a typical membrane filter (spiral wound) used for sterile filtration is given in Fig. 12.37. The exploded view of the module (functional unit) is given in Fig. 12.35. Spiral wound module consists of thin film composite (TFC) system. A flexible, porous sheet is placed between two flat membranes. This "sandwich" thus produced is sealed on three of its four edges. The folded side is sealed to a cylindrical collector tube on both sides of a distributor with holes drilled in it. Several sandwiches are thus fastened and separated from one

another by a spacer of flexible plastic. The fluid to be treated circulates in the spacer and the porous sheet ensures the drainage of permeate towards the axial collector. The wound components are snugly fit into a housing (Fig. 12.36). The dimension of a spiral wound module is: 10-20 cm (diameter) and 30-180 cm (length).

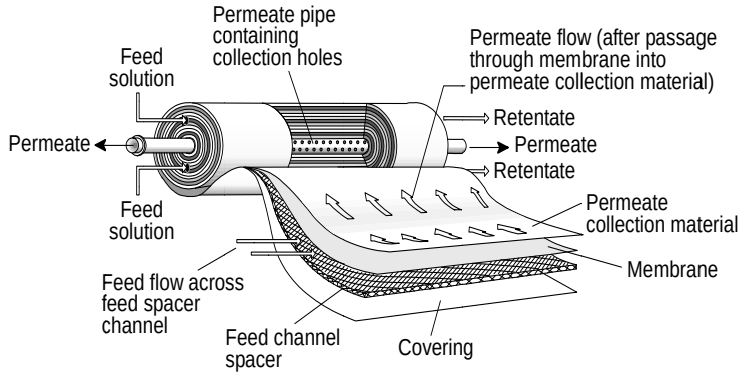


Fig. 12.35 Spiral wound filter packing

Fig. 12.36 A typical housing for spiral wound filter

Fig. 12.37 Installation of spiral wound filter

Initially, sterile filtration was intended for *draft beer* / *draught beer*. The term *draft* is nowadays used in a very loose sense and so the distinction between draft- and other beers is becoming increasingly blurred. Draft beer is usually packed in bulk (in kegs and barrels). It has comparatively short shelf-life.

It entails addition of one the agents listed in Table 12.4. The use of SO_2 in the final stage is not uncommon (70 ppm in UK). Sodium benzoate and sorbic acid are also permitted.

Table 12.4 Chemicals used in pasteurizing beer

Chemical agent	Level (ppm)	Remarks
n-heptyl-p-hydroxybenzoate	Up to 12	Added immediately after filtration
Diethyl pyrocarbonate (DEPC)	70-100	Persistent fruity aroma
Octyl gallate	10-50	

12.5 HIGH GRAVITY BREWING

There are several methods used for the production of special beer types. One particular brewing method of significance is the High Gravity Brewing. High gravity fermentation involves worts of up to 18 °P and even higher. Following fermentation and maturation, the beer is diluted with cool, carbonated water to a prescribed original gravity or to a prescribed alcohol concentration. There are a number of advantages associated with high gravity brewing. It results in beers that are more consistent (% alcohol, original gravity, etc.) and more physically stable since the compounds responsible for haze are more easily precipitated at higher concentrations. Handling more concentrated wort results in increased utilization of equipment and lower energy costs. The disadvantages are longer fermentation times, different flavor characteristics, and poorer hop utilization than normal gravity fermentations.

The reason for introducing the process may be an insufficient brewhouse capacity which can be overcome in this way. However, the chief reason is that the water which is used later in a cold state for dilution does not have to be heated up and boiled with the wort. This gives substantial economic advantage.

12.6 QUALITY CONTROL

The quality control of beer includes both organoleptic and physicochemical analyses. Some of the important parameters to be tested are alcohol content, dissolved O_2, CO_2, haze, bitterness, gravity, diacetyl level, pH, taste, color, head retention, etc.

Despite extreme care, batch-to-batch variations do occur. Alcohol, bitterness and color are generally adjusted. Deareated water can be used for reducing the alcohol content. Caramel may be used for adjusting color. Iso α-acid extract may be added for maintaining the desired level of bitterness.

Finished beer must appear fresh, bright, and without faults to the customer and hence the quality is a matter of great concern. The beer must also be free from microorganisms to ensure wholesomeness and biological stability. The ethanol content must obey fiscal rules but is also of major importance for the flavor of the

beer. This is further influenced by a wide range of compounds that may be present in even very small amounts. Visually the finished beer must form a nice foam on pouring and it must have an attractive color.

Despite use of the choicest raw materials and careful brewing performance the beer is a fragile liquid, especially when not stored cold. The fine, balanced aroma of fresh beer is eventually replaced by a less attractive smell and likewise the taste deteriorates. The basis for this decay is a matter of intense research.

Sensory analysis is the most powerful test of beer quality. Beer flavor is of course the single most important parameter in sensory analysis. Beer flavor is a very complex subject. Over 800 compounds have been identified that contribute to the characteristic flavor of beer. The main flavor characteristics are the bitter taste derived primarily from the hops, an alcoholic note from ethanol, and a carbonation mouthfeel from CO_2. Secondary flavor notes include fruity-estery flavors, alcoholic notes from higher alcohols, and various sulfur components. However, most of the flavor compounds are present in very small quantities (below their individual taste threshold) and act synergistically to provide the balanced and refreshing taste of good beer. Occasionally undesirable flavor components may appear, giving the beer various off-flavors.

The flavor components and their precursors originate from the raw materials, namely malt, adjuncts, water or hops, or are produced by the yeast during fermentation of the wort. Consequently, the selection of raw materials and/or yeast strain has great impact on the flavor. However, the flavor is also influenced by technological factors which affect the composition of the wort, and the conditions during fermentation, maturation, filtration, and bottling.

Flavor assessment is therefore very important in quality control of beer. One of the most important tools is the sensory analysis by a panel of well-trained tasters. To enable a precise description of a beer sample a common terminology has been elaborated. Each flavor impression is quantified on a scale of 0-10 (Fig. 12.38).

In recent years the methods for chemical analysis have improved dramatically. It is now possible to monitor the concentration of many flavor-active components. Quantification of higher alcohols and esters by headspace analysis using a gas chromatograph is now a routine analysis in many breweries. At the Carlsberg Research Laboratory, analyses for a wide range of yeast secondary fermentation products have been established. These analyses are used to study the effect of different raw materials, yeast strains, or brewing processes on the profile of flavor components in the beer.

Beer will deteriorate rapidly unless stored under cold and dark conditions. The stale "cardboard" flavor which may appear upon storage is mainly due to very small amounts of *trans*-2-nonenal, a lipid degradation product formed during malting and wort production. At the Carlsberg Research Laboratory the brewing process - from malting to beer pasteurization - is examined to pinpoint the critical steps of lipid oxidation.

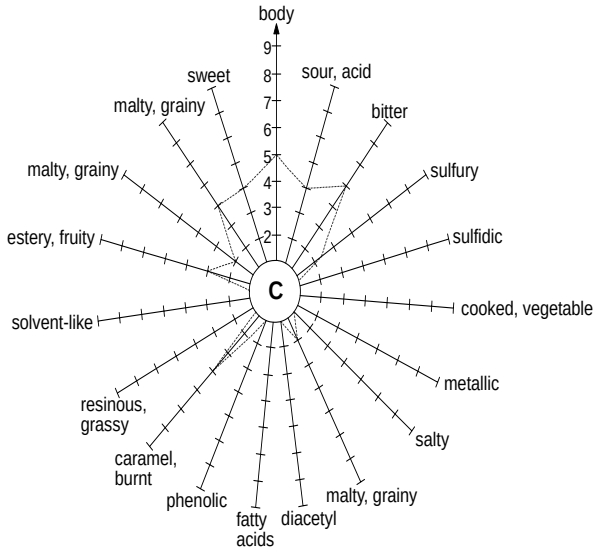


Fig. 12.38 A typical flavor wheel for the sensory analysis of beer

12.7 SOME TERMINOLOGIES

12.7.1 HEAD

Formation of an attractive head of foam is a very crucial aspect of beer quality. A good beer foam can be described as follows:

- Stable head consisting of small, tight, white bubbles
- Adhesion of foam to the side of the glass (so-called *lacing*) whilst the beer is being drunk

Appearance of foam is due to the rising CO_2 but the stability is a combined result of a host of factors. Beer foam is primarily stabilized by adsorbed proteins, polypeptides and β-glucan derived from malt. Because of macromolecular complexity and heterogeneity of the macromolecular components of beer, the adsorbed layer is not dominated by single species or simple mixture of species. High molecular weight glycoproteins and highly hydrophobic polypeptides are usually effective in stabilizing foam, although iso-α-acids from hops and ethanol in beer are also involved in some complex manner. Low ethanol content acts as foam enhancing component by sharply lowering the surface tension, and consequent formation of small bubbles. High alcohol concentration is detrimental to foam stability: it precipitates the proteins.

Recent researches have shown that foam active proteins have molecular weight higher than 8000. *Lipid Transfer Protein 1* (LTP 1), a 10 kDa protein of barley origin, has been identified as the major protein component in beer foam. The transformation of LTP 1 into the more foam-promoting form takes place during wort boiling and involves unfolding of the three-dimensional structure.

12.7.2 HAZE AND CHILLPROOFING

Even after filtration to clarity, beer may develop turbidity or cloudiness upon storage. This turbidity, called *haze*, may be either biological or non-biological. Infection of beer with bacteria and subsequent growth leads to biological haze. This, however, is not of importance in pasteurized beers.

Non-biological haze is the most important index of physical stability of beer. Colloidal materials such as polypeptides and polysaccharides, which form aggregate with each other and with polyphenols, contribute to haze formation. The simple phenolics in beer polymerize to yield *active polymers* (tannins) which complex with beer proteins to form a reversible *chill haze* that precipitates from the solution at 0°C but redissolves at 20°C. Upon further polymerization and complexing, a non-reversible permanent haze is formed. It has been believed that protein serves as a backbone of haze, and the most significant metal ion involved is calcium.

The polyphenols of importance in beer haze are proanthocyanidins from barley grain and hops (see Fig. 12.39 for barley proanthocyanidins). The proanthocyanidins in hops are generally termed tannins due to their greater complexity. Proanthocyanidins show affinity towards certain proteins (hordein in particular) which are characterized by high proline content. During mashing as well as wort boiling, complexing between proteins and tannins occur to give precipitates.

Haze formation in beer is undesirable. It is one of the major factors that determine the shelf-life of bottled or canned beer.

A common method to prevent haze is *chillproofing*. Chillproofing merely implies removal by precipitation or adsorption of residual proteins or protein hydrolysate products from beer, thereby eliminating the backbone of the haze. Some of the methods of chillproofing are:

1. Use of cold cellar temperatures for prolonged period and good, tight filtration. This is an expensive and time-consuming process
2. The use of proteolytic enzymes, such as papain, during aging. Papain hydrolyzes the protein backbone
3. Removal of proteins with adsorbents such as tannic acid, bentonite, silica gel, etc

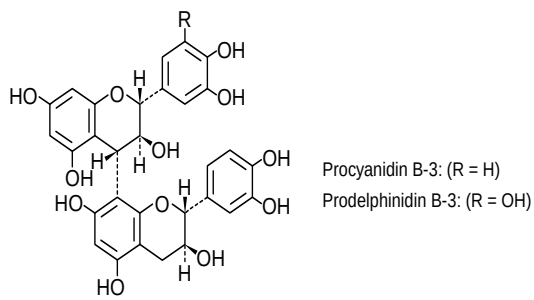


Fig. 12.39 Two dimeric forms of proanthocyanidins in barley/malt

Chillproofing can also be achieved by removing tannins (rather than proteins) using polyvinylpolypyrrolidon (PVPP, Fig. 12.40) which closely resembles polyproline.

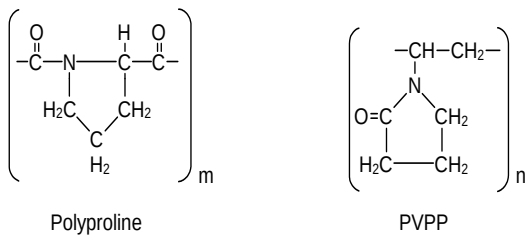


Fig. 12.40 Schematic formulation of PP and PVPP

Polyproline and PVPP both possess a carbonyl group neighboring a proton-free nitrogen atom, a very potent site for hydrogen bonding.

Having thus removed all the haze components, the beer becomes stable even when cooled. That is, the beer is no longer susceptible to chilling, and is hence childproofed.

Another attractive alternative for producing haze-free beer is the use of malt that is free from proanthocyanidin (discussed earlier).

12.7.3 FLOCCULATION

Flocculation is simply a reversible aggregation of dispersed yeast cells into *flocs* (loose clumps). Ideally, brewing yeast does not flocculate at the beginning of the fermentation, but only after all the nutrients have been used up. However, depending on the conditions, the yeast may initiate flocculation either too early or too late, leading to either improper fermentation or the need of centrifugation, respectively. In order to improve the control of flocculation during beer production the genetic mechanisms of flocculation are being studied.

Yeast flocculation is a complicated process that is currently only partly understood. It requires the presence of at least two types of molecules on the yeast cell surface. One type is mannans (carbohydrate chains), which are produced by the gene products of the MNN genes and are present on the cell surface at all times. The other type is flocculins (sugar binding proteins), which are the gene products of the FLO genes, that are activated only after depletion of nutrients. The flocculins bind to mannans through calcium bridges on the surface of neighboring cells leading to the cross binding of cells and ultimately the formation of flocs. Due to the reduced surface-to-volume ratio of the aggregated cells, the flocs sediment much faster relative to the free cells. At a microscopic level, yeasts capable of flocculation have hairy outer surfaces due to the presence of these binding molecules. Flocculation characteristics of yeast cells are of great technological importance. Since yeast clumps tend to be carried to the surface with entrapped CO_2, recovery of the former becomes very easy. Flocculation can sometimes be problematic: it leads to *hung* or *stuck* fermentation, thereby leading to incomplete attenuation of the beer. Hung

fermentations are invariably caused by two factors, *viz*., (i) premature flocculation, and (ii) inability of yeast to metabolize maltotriose.

12.8 SOME FAMOUS BEERS OF THE WORLD

The qualitative ranking of beer cannot be completely objective. The list that appears in Table 12.5 has been taken from Beer International (1997). The largest producer of beer is England. The next largest producers are Germany, China, and Japan. See Fig. 12.41 for world beer consumption.

Table 12.5 Some famous beers of the world

Beer	% Alcohol	Type	Producers
Tetley Mild	3.2	Ale	England
Taylor Landlord	4.3	Ale	England
Marsten Pedigree	4.5	Lager	England
Samuel Adams Boston Lager	4.8	Lager	USA
Budweiser Budvar	5.0	Lager	Czech Republic
Schneider Weiss	5.3	Wheat beer	Germany
Guinness Foreign Extra Stout	7.5	Ale	Ireland

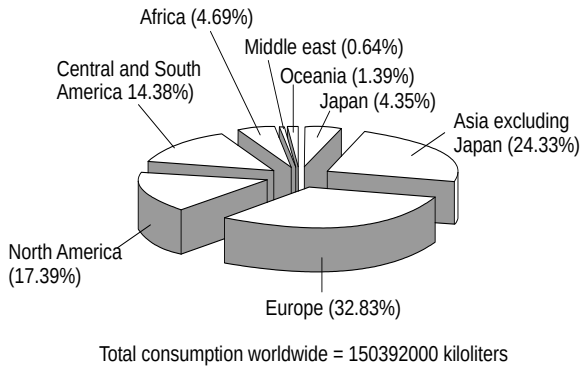


Fig. 12.41 Leading beer consuming countries (2004)

12.9 BEER SPOILAGES

Beer is a poor environment for microorganisms. As such, five intrinsic factors control the microbial growth. These factors are: (i) low pH, (ii) low redox potential, (iii) low nutrient status, (iv) presence of ethanol, and (v) presence of hop components and metabolites.

Any organism that grows in beer should overcome all these hurdles. Extrinsic factors such as addition of preservatives, storage at refrigeration temperatures, pasteurization treatment, etc., further reduce the possibility of beer spoilage. Nevertheless, beers get spoiled, and when this occurs the main causes may be due to few notorious microorganisms, some of which are given in Table 12.6.

Table 12.6 Some beer defects and the associated microorganisms

Spoilage organisms	Defect in beer	Other details
Acetic acid bacteria	Rope formation, acetification, turbidity, discoloration, strange flavor	
Wort bacteria: They include certain members of Enterobacteriaceae, e.g., *Citrobacter*, *Enterobacter*, *Escherichia, Hafnia, Klebseilla.*	Off flavor including phenolics and vegetable taints	These microorganisms normally survive the initial stages of fermentation
Obesumbacterium proteus	Increased level of higher alcohols	This is a fat, rod-shaped bacterium that grows in wort during alcoholic fermentation
Lactic acid bacteria	Increased acidity, foreign flavor	Their nutritional requirement is similar to that of the yeasts and hence compete for growth
Wild yeasts	Foreign flavor	They are versatile and hardy and thus survive fermentation
Zymomonas mobilis	Objectionable stench and heavy turbidity	It produces acetaldehyde and H_2S. It uses the Entner Deuodoroff pathway of metabolism

12.10 PURIFICATION OF CARBON DIOXIDE

Commercial CO_2 is obtained either by the combustion of carbonaceous materials or as a by-product from fermenting processes as well as from a number of chemical processes releasing large amounts of CO_2. Extraction of CO_2 from fermenting processes is carried out in Recovery Based Units (RBU), which come in different designs. Fig. 12.43 shows CO_2 recovery unit designed by GEA-Tuchenhagen Brewery Systems, Germany.

Breweries produce large amounts of CO_2 as a by-product of fermentation. This CO_2 is extracted, purified and used for the carbonation of beer. Details regarding the operation and maintenance of the RBU may vary from manufacturer to manufacturer but the principle involved in the extraction and purification of CO_2 is essentially the same. Through appropriate scrubbing, filtration and separation technology the CO_2 recovery plants meet the strictest CO_2 quality requirements. The key advantages of RBU are:

- Beverage quality CO_2 from own source

- Low operating cost
- Environmental benefits
- Low installation cost
- User-friendly, fully automatic operation

The CO_2 gas developed in the distillery and brewery contains a lot of impurities (such as alcohols, aldehydes, H_2S, NO_x, etc.). The gas is therefore subjected to a series of operations to recover and purify CO_2. A design produced by Witteman Company, India is described in the following paragraphs.

The CO_2 generated from the closed fermenter is led through a vessel called "foam trap" (Fig. 12.42). Here the gas is washed with water to get rid of gross impurities like foam, cells, and denatured proteins. Thereafter the gas is passed through a water scrubber (in some designs, an additional $KMnO_4$ scrubber is also used) to remove water-soluble impurities and aerosols. The gas is now compressed in a double-stage compressor to about 16 bars (gauge), cooled, and passed through a pair of dual tower (only one tower is shown in Fig. 12.42 due to space constraints) that contains activated carbon and a desiccant.

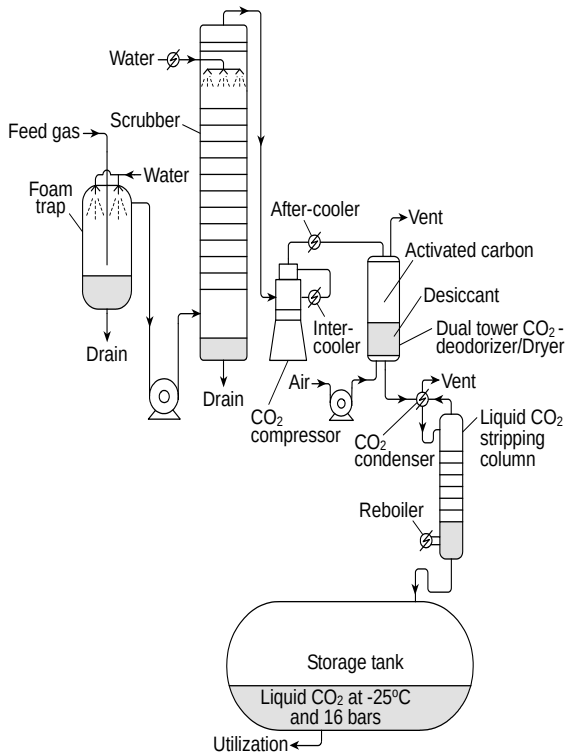


Fig. 12.42 Schematic of carbon dioxide recovery in brewery

Only one tower is operated at a time so that the other can be simultaneously regenerated. Regeneration in this context refers to reactivation of activated carbon by back-flushing the tower with hot air or steam. Activated carbon is used to remove

odor compounds such as aldehydes, H_2S, NO_x, etc. Desiccant is used to dry CO_2 to less than 10 ppm moisture content. In some designs, a "balloon" is installed just before the compressor. Balloon is a tank that serves as a reservoir of CO_2 for an uninterrupted supply to the compressor. Although the compressed gas can be condensed and stored for use, an additional stripping column must be used if a very high purity CO_2 is required. The stripping process involves vaporization and condensation cycles. The incondensable gases like O_2 and N_2 are vented away while CO_2 is collected at the bottom. The purified gas is subsequently stored as liquid CO_2 in thick-walled cylinders at 15-20 bars (gauge) and about -25°C. The volume of CO_2 is reduced by about one-sixteenth. A suitable evaporator unit is used when dry CO_2 is required for the carbonation of beverages.

The purity of CO_2 intended for soft drinks and brewery is very demanding. A typical specification for food-grade CO_2 is given in Table 12.7.

Table 12.7 Standard for food-grade CO_2

Parameter	Specification
Purity	99.9%, v/v min.
Moisture	20 ppm, v/v max.
Oxygen	30 ppm, v/v max.
Carbon Monoxide	10 ppm, v/v max.
Ammonia	2.5 ppm, v/v max.
Nitric Oxide / nitrogen dioxide	2.5 ppm, v/v max. each
Nonvolatile residue	10 ppm, w/w max.
Acetaldehyde	0.2 ppm, v/v max.
Total sulfur content (as S) excluding SO_2	0.1 ppm, v/v max.
Sulfur Dioxide	1 ppm, v/v max.
Appearance in water	No color or turbidity
Odor and taste in water	No foreign taste or odor

Fig. 12.43 Carbon dioxide recovery plant (brewery CO_2)

CHAPTER 13

MICROBIAL PRODUCTION OF ETHANOL

13.1 INTRODUCTION

Ethanol can be produced chemically (> 90%) as well as microbiologically. However, chemically produced alcohol is not used for beverage purpose. The reason for this can be many. For example, there is always some health risk in the use of chemically synthesized alcohol. Another important reason is the lack of congeners. The quality of spirits obtained by a microbiological process can never be obtained by chemical means. Below is given an example of chemical synthesis of ethanol.

$$\text{Ethylene} + H_2O \xrightarrow{H_3PO_4,\ 300^\circ C,\ 60\ atm} CH_3CH_2OH$$

The merits of microbiological production of ethanol can be listed as follows:

- No need of tremendous temperature, pressure, and a multitude of complex reactions
- Uses cheap and readily obtainable raw materials
- Ecologically friendly
- It is the only option when ethanol is to be used for beverage purpose

13.1.1 MICROORGANISMS

Bacteria, yeasts, and molds are all capable of producing ethanol. For example, *Zymomonas mobilis* (bacteria), *Mucor* species (mold), *Saccharomyces cerevisiae* (yeast), *Schizosaccharomyces pombe* (yeast), etc., can be used for the same. However, for industrial fermentations, only yeasts are used. Of them, *S. cerevisiae* and to some extent, *Schizosaccharomyces* are the only commercially used yeasts. *S. cerevisiae* can produce up to 18% alcohol by volume (*abv*). *Schizosaccharomyces* is a fission yeast normally used in continuous process.

13.1.1.1 Desirable properties of yeasts

Not every type of yeast is useful or is used in alcoholic fermentation. The strains used in industrial fermentation are of course highly improved strains. These organisms must possess certain desirable properties, the important ones of which are listed as follows:

- Ethanol tolerance
- Flocculation
- Resistance to killer activity
- Osmotolerance

Killer activity is due to the ability of the yeast to produce a toxin called *zymocin*. Yeasts with ability to elaborate zymocin are not only resistant to other similar killer strains but are also killers to sensitive strains.

13.1.1.2 Preservation and maintenance

Primary stock culture is preserved by low risk methods like lyophilization and desiccation. Working stock cultures are normally prepared in slants or broths. The conventional medium for the isolation and growth (also for maintenance) is MYPG agar adjusted to pH 4.5. After growing for 2-3 days at 30°C, the culture is stored at 4°C. It will remain stable for about 6 months if drying up and/or contamination is checked.

13.2 INDUSTRIAL PRODUCTION

13.2.1 RAW MATERIALS

As such, several raw materials can be used for the fermentation. The main categories of basic raw materials are (i) *saccharine materials*, (ii) *cellulosic materials*, and (iii) *starchy materials*.

Cellulosic and starchy materials require extensive treatment before actual use because the organisms do not possess the suitable enzymes for hydrolyzing these complex polysaccharides. Cellulosic materials are usually hydrolyzed by chemical means, for example, with acids and alkalis. The cost of preparation often comes to over 50% of the total production cost. Unless cheap methods of hydrolysis are available the feasibility of ethanol production using cellulosic materials is remote. One alternative could be development of source of cellulase, either from bacteria, mold, or the yeast itself. However, this possibility has not yet been realized in so far the commercial production of ethanol from cellulosic materials is concerned. The few cellulose processes that exist today (for *fuel alcohol*) have been discussed later.

Starchy materials are nevertheless used in the production of a wide range of alcoholic beverages and spirits. The process is more cost effective than the cellulose process. The hydrolysis can be carried out conveniently using suitable diastatic enzyme sources such as malt and molds. Starchy materials are used for alcoholic beverage production (e.g., beer, whiskey, cereal wines) rather than the ethanol, which has diverse end use.

For large-scale ethanol production, saccharine materials (sugar items) are still the materials of choice. Of them, molasses is the most preferred material. Molasses comes in many types, e.g., *blackstrap* molasses, *high-test* molasses, and *refinery* molasses. High-test cane molasses is simply the concentrate of cane juice (sugar not extracted) and is therefore relatively costly. Refinery molasses comes from the intermediate stage of sugar manufacture. Since it contains significant amounts of crystallizable sugar, refineries do not sell it. Blackstrap molasses is the final by-product of sugar refinery. Nutrients and minerals are available in it in highly concentrated form. It contains as much as 50% of fermentable sugars.

13.2.2 MEDIUM PREPARATION

Molasses as it comes is of about 80° brix (specific gravity: 1.40). Water is added to it to make a solution of 15-16° brix (specific gravity: 1.06). The medium is seldom pasteurized or sterilized. Since nitrogen source can be limiting, $(NH_4)_2SO_4$ can be added to the medium at the rate of 1 kg/50 HL (1HL = 100 liter). The pH is adjusted to 4.5 with concentrated (98%) H_2SO_4.

13.2.3 INOCULUM BUILD-UP

The inoculum is prepared separately in a stepwise fashion (Fig. 13.1). Pure yeast culture from the working stock is first propagated in a shaker flask in MYPG broth for about 2 days at 28°C. It is next transferred to a vessel called *yeast machine*. The vessel contains sterile medium (molasses medium), usually above 10 times the volume of shake flask. There the yeast is grown aerobically for about 2 days at 28°C. The contents are next transferred to a propagator called *bub vat*. It also contains sterile medium, over 10 fold the volume of yeast machine. The vessels are closed ones. Air is supplied at the rate of 1/8 vol/vol/min and propagation carried out as aseptically as possible for 2 days at 28°C. The transfers are made in several stages until the desired amount of inoculum is obtained. The number of yeast cells for pitching should be around 30-50 million cells/ml of the medium in the final fermenter. Care should be taken in the inoculum build up not to shift the yeast towards alcoholic fermentation. Since alcoholic fermentation occurs only at higher sugar concentrations the opposite may be done to shift the yeast towards respiratory growth. The reverse should be the case in the final fermentation, though (i.e., glucose effect must be maintained).

13.2.4 PREPARATION OF THE FERMENTER

Conventionally, fermentation is carried out in batch mode. The vessels, which are generally cylindroconical in configuration, have the capacity of 550 to 1000 HL (Fig. 13.1). The fermenter can be sterilized using live steam or disinfectants such as NaOCl (sodium hypochlorite).

13.2.5 PITCHING

The prepared medium is transferred to the fermenter first. Active inoculum is then added at the rate of 3-4% by volume. In certain cases, the pitching rate can be as high as 20% by volume. Often, yeast is recycled after harvesting of the previous batch, in which case the inoculum takes the form of yeast cream. The yeast should be of good physiological quality, though. Whatever the method, the pitching rate is optimally maintained at about (3-5) $\times 10^7$ cells per ml of the main fermentation medium.

13.2.6 FERMENTATION

The fermentation starts a short while after pitching. The temperature of fermentation is very critical. It must be maintained at 28-35°C and not more. This is achieved by using internal cooling coils. In very small fermenters, water jacket or

even air-cooling (surface cooling) can be used. Heat generation is of the order of 11.7 kcal/kg substrate consumed. A change of 0.75-1°C/h is not unusual. High temperature is undesirable for following reasons:

- Foaming occurs uncontrollably
- Pathogens may multiply
- Alcohol loss may occur (up to 1.5%)
- Yeast viability may decrease
- Premature flocculation may occur, leading to hung fermentation
- Higher alcohols may be produced

The fermentation is usually complete within 40-48 hrs after pitching. Depending on the initial sugar concentration, the final broth (wash or beer) contains 7.5-8% *abv*. The yield is about 92% of the theoretical conversion, because the yeast cells utilize some amounts of sugar for their own cell build-up.

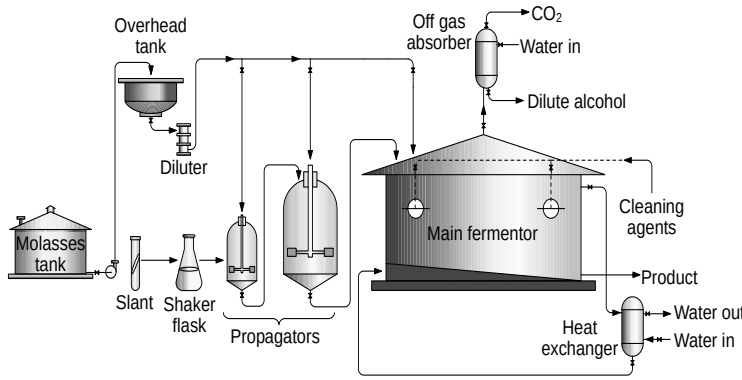


Fig. 13.1 Batch fermentation of ethanol

13.2.7 RECOVERY

The final broth can be treated in many ways. In the following paragraphs, brief descriptions of three common methods are given.

1. Melle-Bionot Process

In this process the yeast is centrifuged (Fig. 11.9) and recovered. While the supernatant is taken away for distillation, the yeast cream is washed, treated with food-grade acid (to kill the contaminants) and returned to the main fermenter. This process is preferred because it does not require repeated inoculum build-up. The yeast is thrown away after a given number of cycles or after it becomes contaminated to unacceptable level.

2. Half broth recycled

Only half of the fermented broth (*wash*) is withdrawn. The other half (along with the yeast mass) serves as an inoculum for the next batch.

3. Yeast not recycled: See later

Whatever the method, the wash is first dropped into a vessel called *beer tank* so that the main fermenter becomes empty for reuse. The wash in the beer tank can now be processed (settling, centrifugation, distillation, etc.).

13.3 CONTINUOUS FERMENTATION

Where there is an inexhaustible source of raw material, continuous process is very efficient. Although there are many variations of continuous fermentations, the *cascade process* is the most successful one. It consists of series of tanks with inlet, overflow, and stirrer facilities. The prepared medium is continuously fed and the corresponding amount simultaneously withdrawn. The flow, medium concentration, and the pitching rate are adjusted such that the fermentation is complete by the time broth leaves the last stage. The ethanol in the final beer is as high as 14%. See Fig. 13. 2 for the scheme of continuous fermentation.

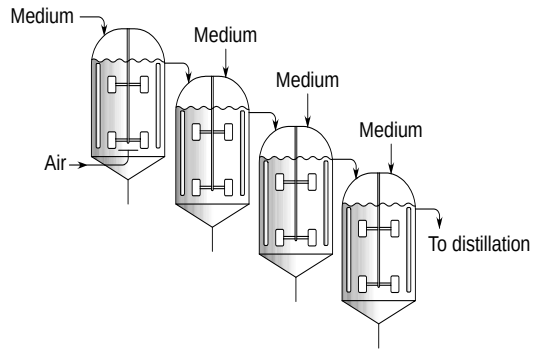


Fig. 13.2 Scheme for cascade process of ethanol fermentation

Like any other fermentations, continuous fermentation also has advantages and disadvantages. Increased productivity, uniformity of operation, ease of automation, etc., are the well-known advantages while the danger of contamination due to prolonged periods of fermentation is the main limitation. The real economy in continuous fermentation results from the elimination of *down time*, the time required for emptying, cleaning, filling, etc., of the fermenter.

13.4 BIOCHEMISTRY OF ETHANOL FERMENTATION

Starting from glucose, yeast uses a set of 12 enzymatic steps. The stoichiometry of the reaction is:

$$C_6H_{12}O_6 \xrightarrow{\text{zymase}} 2CH_3CH_2OH + 2CO_2 + \text{Energy}$$

The organism uses *EMP* pathway, generating 2 ATP per mole of glucose converted to ethanol, plus CO_2. Ethanol, which is the end product, is a primary metabolite. In an industrial fermentation, the basic strategy is to maintain *Crabtree effect* during the fermentation. A truncated form of the metabolic pathway for ethanol synthesis is given in Fig. 13. 3.

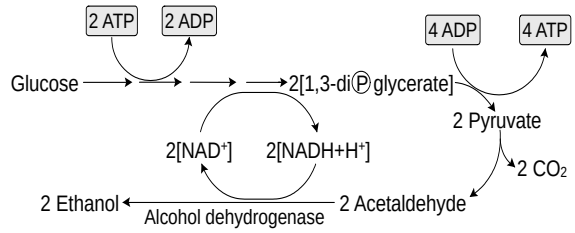


Fig. 13.3 Simplified pathway of ethanol biosynthesis

The theoretical yield of ethanol from glucose is 51.1% (mass/mass). However, because the yeast utilizes some sugar for its own growth (cell build-up), the practical yield is 44-49%, which is 86-95.9% of theoretical value. The productivity of a typical fermentation is 1.9 g/liter/h.

13.5 BIOCHEMISTRY OF HIGHER ALCOHOL PRODUCTION

Higher alcohols in ethanol are responsible for the characteristic aroma. When they are in high concentrations, they can cause *headiness* and *dryness*. Technically, higher alcohols are also called *fusel oils*. They have boiling points of 125-140°C. Unless severely infected, the concentration of higher alcohols is les than 0.5% of ethanol.

The most important higher alcohols found in ethanol are *propanol*, *butanol* and *pentanol* (*amyl alcohol*). The production is limited to exponential growth phase and dependent on yeast strain, pH, and temperature. Fusel oils such as isobutanol, isoamyl alcohol, amyl alcohol, and phenyl ethanol are produced by sequential reactions, *viz., transamination*, *decarboxylation*, and *reduction* of respective substrate amino acids. However, propanol is produced from α-keto butyrate. The general sequence is illustrated in Fig. 13. 4.

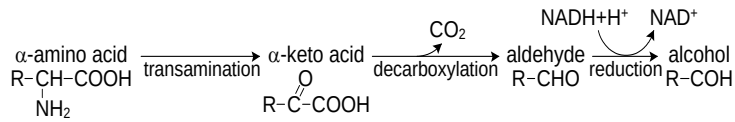


Fig. 13.4 Simplified pathway for higher alcohol biosynthesis

Examples:

Valine → → → isobutanol

Leucine → → → isoamyl alcohol

Isoleucine → → → amyl alcohol

α – ketobutyrate → → propanol

13.6 METHANOL

In alcoholic fermentations, methanol is produced due to demethylation of *pectin* by pectin esterase. Pectin esterase originates from the substrate or molds. There are

certain strains within yeasts also, which can elaborate pectin esterase. The activity of the enzyme rises as pH increases from 1 to 6. When ingested, methanol is oxidized to formaldehyde, which is toxic to living cells. Formaldehyde alters the biological activity of proteins. As little as 30 ml can cause blindness, and even death. An antidote of this poison is ethanol. Ethanol exerts a competitive inhibition and the methanol is excreted away slowly through the urine.

13.7 DISTILLATION AND RECTIFICATION

There are many types of distillation and rectification systems available. Some of the common systems used for producing 95% alcohol (rectified spirit) are:

- One-column system
- Two-column system
- Three-column system, e.g., (a) Barbet system and (b) Othmer system
- Vapor recompression
- Vacuum rectification
- Multiple effect distillation
- Six-column reagent alcohol system

For beverage purpose, 1 to 3 column systems are adequately satisfactory.

13.7.1 TWO-COLUMN SYSTEM

This system consists of two integral parts (a) *Analyzer*, and (b) *Rectifier*. The analyzer is used to exhaust beer, i.e., remove alcohol from the beer. The rectifier is used to purify the alcohol and bring it to a high strength (95-96% *abv*).

13.7.1.1 The analyzer

The analyzer is a cylindrical tank with about 18 plates stacked within. The beer enters the analyzer at the top and follows a zigzag course down the column. Steam enters at the bottom and travels countercurrently, depriving the wash of its alcohol. The exhausted wash is run out. The vapors issuing from the top of the analyzer enter the rectifier somewhere in the middle of the column.

13.7.1.2 The rectifier

The rectifier is composed of specially designed fractionation column with a number of chambers (plate or tray ~ 40, see Fig. 13. 5). In practice, to overcome the shortcoming that may result from under-designing, the number of trays is usually 10% more than the theoretical requirement.

The tray comes in various designs (bubble cap tray, sieve tray, valve tray, etc.) and they are placed at intervals of 60-75 cm up the height of the column. The bubble cap tray is shown schematically in Fig. 13.5. Each tray has a conduit called *downcomer*. Liquid falls through the downcomer by gravity from one tray to the one below it. The flow across each plate is shown in Fig. 13.5.

A weir on the tray ensures that there is always some liquid (holdup) on the tray and is designed such that the holdup is at a suitable height, e.g. such that the bubble caps are covered by liquid. Being lighter, vapor flows up the column and is forced to pass through the liquid, via the openings on each tray. The area allowed for the passage of vapor on each tray is called the *active tray area.*

The feed is introduced somewhere in the middle part of the column. The section above the feed is called *enrichment-* or *rectification section.* The section below the feed line is called *stripping section* (see Fig. 13.6). The steam issuing from the reboiler (Fig. 13.6 and 13.7; several designs are available) takes the volatiles upwards along with it. The vapors are condensed externally and returned to the same rectifier. As the condensed vapors flow down they again meet the rising mixture of steam and alcohol. The repeated condensation and evaporation make the vapor richer and richer in alcohol. There is provision for separating the vaporized alcohol into 3 fractions, *viz.*, *Fraction I, Fraction II,* and *Fraction III.*

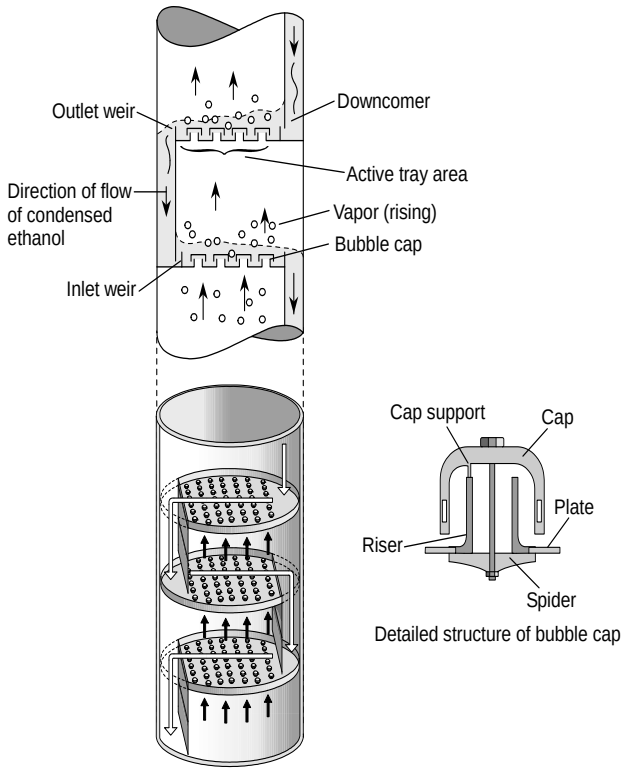


Fig. 13.5 Arrangement of plates in the column

Fraction I

This fraction is also called *heads.* It consists of low-boiling fraction. It is drawn from the top or separately from an aldehyde-stripping column. The fraction consists mainly of aldehyde, formic esters, and a small amount of uncondensed alcohol.

This is the main fraction and contains 96% alcohol (rectified spirit). See Fig. 13. 6 for an idea about the fractions.

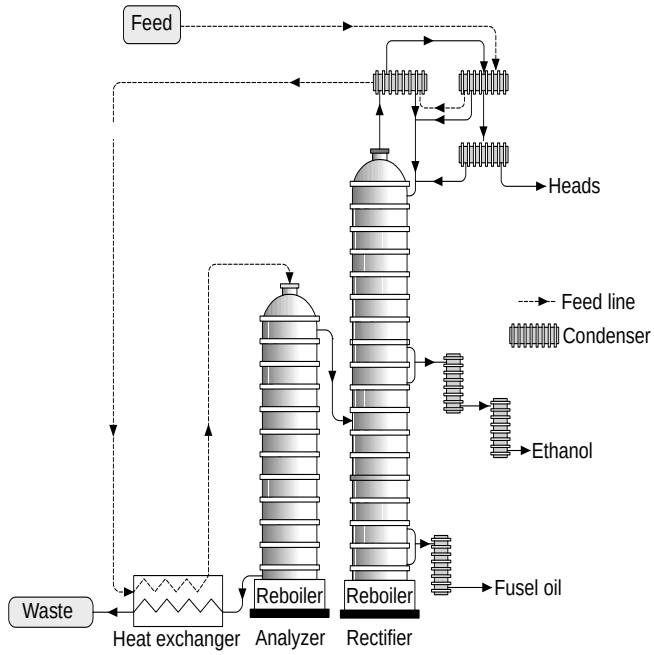


Fig. 13.6 Schematic diagram of ethanol distillation (two-column system)

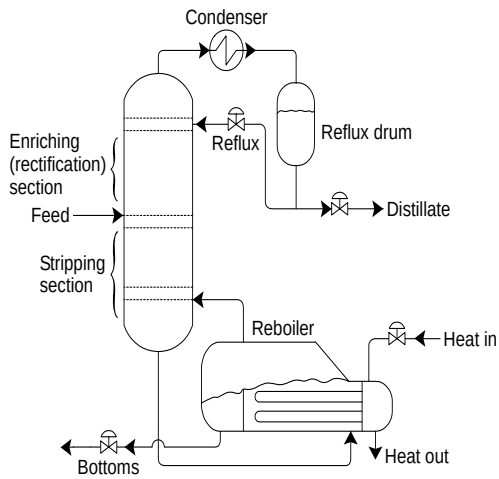


Fig. 13.7 Schematics of rectification and stripping sections

This is a high-boiling fraction (125-140°C) and consists mainly of *fusel oils* (see page 220 also). A small stream is continuously bled from the bottom of the column and condensed outside. The fusel oils become insoluble in ethanol when cooled. The separated ethanol is recycled to the rectification column while the fusel oil is taken out from the receiver (every two to three days).

13.8 USES OF ETHANOL

1. *As a solvent:* ranks second to water
2. *Chemical intermediate*: for the preparation of synthetic rubber, acetaldehyde, acetic acid, ethyl acetate
3. *Fuel*: mixed with gasoline to produce gasohol
4. *Laboratory use*: as reagent, disinfectant, in spirit lamps
5. *Beverage*: blending and fortification

All the acohol which is drunk is absorbed in the stomach (20%) and the small intestine (80%). About 10% of the ingested alcohol is eliminated in the exhaled air and urine. The rest is rapidly diluted in the blood stream and body fluids. Alcohol is metabolized into fat, H_2O, and CO_2 but this occurs at a very slow rate. An adult liver can break down only about 10 g alcohol per hour.

The immediate effect of alcohol is its toxic action on the central nervous system. The risk of alcohol drinking starts at a level of 0.3 g per liter in the blood stream. The increasing amounts of alcohol in the blood stream and their effects are as follows:

2 g/liter leads to drunkenness (intoxication)

3 g/liter results in complete weakness

4 g/liter induces coma

5 g/liter causes certain death

13.9 INDUSTRIAL ALCOHOL

Industrial alcohol (also called *commercial alcohol)* is ethanol produced and sold for non-beverage applications. It is *denatured* to prevent its use as a beverage. Denaturing involves mixing ethanol with small amounts of poisonous or unpleasant substances to make the ethanol undrinkable. The removal of all these substances would involve a series of treatments more expensive than the excise tax on alcoholic beverages. Often, very uncharacteristic colors are added to industrial alcohol to differentiate it from spirits intended for alcoholic beverages.

Industrial alcohol is available in three forms, *viz*., (i) *Completely Denatured Alcohol* (CDA), (ii) *Specially Denatured Alcohol* (SDA), and (iii) *Pure Ethanol.* CDA contains denaturants such as pyridine, wood naphtha (mainly xylene), etc. SDA contains chloroform, acetic acid, ethyl acetate, formaldehyde, etc., as the denaturant. Pure ethanol does not contain any denaturant but still should not be used for alcoholic beverages.

13.10 PROOF AND PROOF SPIRIT

According to the US Official definition, *Proof shall mean the ethyl alcohol content of a liquid at 60°F (15.6°C) stated as twice the percentage ethyl alcohol by volume. Proof spirit* shall mean *that alcoholic liquor which contains 50% ethyl alcohol by volume at 60°F as unity*. In England and Canada, proof spirits contain 49.25% alcohol by weight at 60°F. This is equal to 57.061% by volume. It is to be noted that the remaining percentage in both the definitions (US or UK) is that of water. For alcohol contents below that of *proof spirit*, the concentration may be expressed in terms of *underproof* (UP), and for above 50%, *overproof* (OP). Stated differently, a spirit of 125° proof is 25° above proof spirit. It can therefore be written 25° overproof. Similarly, a spirit with 75° proof is 25° lower than proof spirit and hence can be written 25° underproof.

13.11 DEHYDRATED (ABSOLUTE) ALCOHOL

Dilute ethanol is readily concentrated by distillation because the volatility of ethanol in dilute solution is much higher than that of water. At higher concentrations, ethanol and water form an azeotrope at 89 mole % ethanol (95.7% by weight). The volatilities of water and ethanol mixtures at this particular state are the same. Continued boiling produces vapor of the same composition and no further enrichment is possible. This mixture, which behaves like a pure chemical, thus boils at a constant temperature. It distils over completely without change in composition. Such a mixture is called *constant boiling* mixture or *azeotropic* mixture. It is because of this nature, alcohol cannot be produced in anhydrous form by simple distillation. The production of dehydrated (anhydrous) alcohol requires special techniques such as *azeotropic distillation*, *extractive distillation*, *pressure-swing distillation*, etc. The former two methods require the introduction of a third component into the system. Although treatment of the distillation methods is out of the scope of this book, brief descriptions on the former two methods are given in the following sub-sections.

13.11.1 AZEOTROPIC DISTILLATION

Azeotropic systems generate very intriguing vapor-liquid-equilibrium (VLE) curves. The two VLE plots shown in Fig. 13.8 show two different azeotropic systems, one with a minimum boiling point and one with a maximum boiling point.

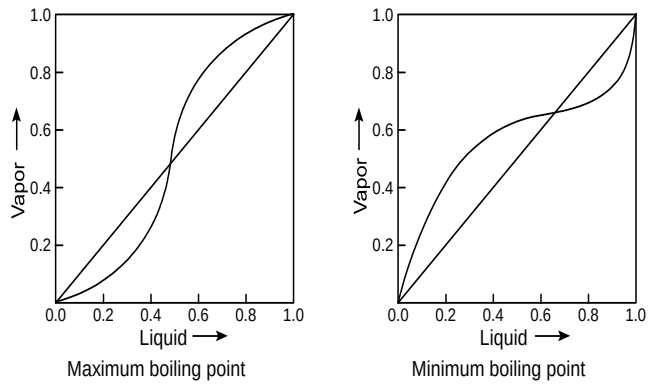


Fig. 13.8 Vapor-Liquid-equilibrium curve of homogenous azeotropic system

In both plots, the equilibrium curves cross the diagonal lines, and these are azeotropic points where the azeotropes occur. In other words, azeotropic systems give rise to VLE plots where the equilibrium curves cross the diagonals. Note the shapes of the respective equilibrium lines in relation to the diagonal lines that bisect the VLE plots.

Both plots shown in Fig. 13.8 are obtained from *homogenous azeotropic systems*. An azeotrope that contains one liquid phase in contact with vapor is called a homogenous azeotrope. A homogenous azeotrope cannot be separated by conventional distillation. However, vacuum distillation may be used as the lower pressures can shift the azeotropic point. Alternatively, an additional substance may be added to shift the azeotropic point to a more 'favorable' position.

When this additional component appears in appreciable amounts at the top of the column, the operation is called *azeotropic distillation*.

When the additional component appears mostly at the bottom of the column, the operation is called *extractive distillation* (described shortly).

The third component added here is called *entrainer* or *material separating agent* (MSA). This component (usually benzene) forms a ternary azeotrope of benzene-ethanol-water in the ratio 24:54:22, which boils at 64.85°C, a boiling point lower than that for alcohol-water binary mixture (78.13°C).

Anhydrous alcohol is produced from rectified spirit. The ternary azeotrope that readily distils over is collected and condensed. Upon cooling, water readily separates out from benzene. Alcohol is miscible in water as well as benzene. The dilute ethanolic water can be rectified before recycling while ethanol-benzene mixture is recycled. Since the water needed for forming the ternary azeotrope must come from the 5% water present in the rectified spirit, the rectified spirit that progressively becomes dehydrated remains at the reboiler from where it is later recovered. See Fig. 13. 9 for the schematic diagram of azeotropic distillation.

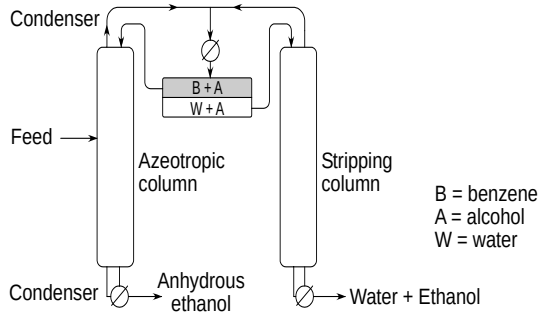


Fig. 13.9 Outline of azeotropic distillation

13.11.2 EXTRACTIVE DISTILLATION

The third component added here increases the relative volatility between the two original components. Ethylene glycol $(CH_2OH)_2$, has been extensively used for this purpose. It associates chemically with water and then causes the volatility of ethanol to increase.

Sometimes, salts (such as potassium acetate) are used to form complex with water and break the azeotrope. At salt concentration of 5 mole%, the azeotrope can be completely broken.

13.12 ETHANOL FROM CELLULOSE

Cellulosic ethanol or *cellanol* is a general term for ethanol fuel produced from lignocellulose, a structural material that comprises much of the mass of plants. Cellulosic ethanol is chemically identical to ethanol from other sources (such as corn, starch, or sugar) but has the advantage that the lignocellulose raw material is available in a great diversity of biomass including waste from urban, agricultural, and forestry sources. Lignocellulose requires a greater amount of processing to make the sugar monomers available to the microorganisms that are typically used to produce ethanol by fermentation.

There are at least two methods of production of cellulosic ethanol, viz., (i) Cellulolytic method, and (ii) Gasification method.

The cellulolytic method entails hydrolysis of cellulose followed by fermentation of the generated free sugars. The gasification method is a chemical process which produces synthesis gas that can be converted to ethanol by fermentation or thermochemical catalysis (e.g., the Fischer-Tropsch process).

Because of the relevance, the first method will be described here. The preparation of ethanol from cellulose-, starch-, and sugar-containing raw materials involves the following general steps:

1. *Pretreatment:* the physical or chemical conversion of the raw material to a hydrolyzable substrate
2. *Hydrolysis:* the enzymatic reaction that converts the starch or cellulose to sugars
3. *Yeast fermentation:* the conversion of sugars to ethanol and carbon dioxide
4. *Purification:* the separation of ethanol from the by-products and wastes

The treatment as well as fermentation can be carried either in a batch- or a continuous process. The continuous process has not reached full degree of perfection. In the paragraphs to follow, both the methods will be briefly described.

13.12.1 PRETREATMENT OF LIGNOCELLULOSE

For acid hydrolysis, wood chips are adequate, but for enzymatic hydrolysis, some chemical or physical pretreatment will usually be required to achieve a reasonable

rate and extent of hydrolysis. The objectives of pretreatment are to reduce crystallinity (of cellulose) and to increase available surface by maximum destruction of fiber structure and interaction between the cellulose molecules.

13.12.1.1 Chemical pretreatment

Considerable attention has been given to agents that will cause swelling of the cellulose and disrupt the crystalline structure. There are two ways in which this occurs:

- Intercrystalline swelling, because of uptake of water between the crystal units, which causes a reversible volume change of up to about 30%
- Intracrystalline swelling, which involves penetration of the crystalline structure by water, leading to unlimited swelling or complete solution of the cellulose.

Current chemical processes include alkali treatment and treatment with sulfur dioxide.

Alkali Treatment

The treatment of cellulose-containing residues with low concentrations of alkali makes them considerably more susceptible to enzymatic- and microbiological conversion. An example of alkali treatment using straw as the cellulose source is described below.

The straw is treated with 20% alkali and, after standing, is neutralized with acetic acid or simply by mixing with silage (which contains lactic acid). This technique has also been applied to wood residues. Straws and hardwood residues, with lignin contents generally less than 26%, respond to the treatment, while softwood residues, with lignin contents higher than 26%, do not.

An alkali treatment method that is claimed to be very effective for straw and bagasse is *dilute alkali treatment* (the Beckmann process). This method involves treatment of the raw material with 1% sodium hydroxide at 45°C for 3 hrs.

Sulfur Dioxide Treatment

Disruption of lignin-cellulose bonds by treating moist wood with gaseous sulfur dioxide under pressure at 120°C for 2-3 hrs appears to offer potential for large-scale processing, though the economics are as yet uncertain. This method increases digestibility to around 60%.

13.12.1.2 Physical pretreatment

Steam explosion, ball- or attrition milling, and two-roll compression milling are effective for many substrates and provide a product of high bulk density, permitting use of 20-30% slurries in the saccharification reactor. This is important if concentrated sugar solutions are to be produced.

The best pretreatment currently available is the *high pressure and high temperature steam*. Stake Technology and Iotech (both in Canada) have pretreatment processes that produce animal feed from straws or hardwoods by steam treatment.

13.12.2 SACCHARIFICATION OF CELLULOSE

Saccharification is the process by which the pretreated cellulosic substrate is converted into a sugar solution. These simple sugars in turn can be used as a substrate by the yeast for alcohol fermentation. The saccharification process can be carried out chemically (by dilute acid hydrolysis) or by enzymic hydrolysis.

13.12.2.1 Acid Hydrolysis

Dilute acid hydrolysis is very effective in breaking the glycosidic linkages between component hexoses, but it also breaks down the sugar hexose units. As a result, the product's acidity must be neutralized, and the amount of sugar is less than quantitative because of sugar degradation. The yield of sugar depends on the relative rates of two reactions that occur when cellulosic materials are treated with dilute acid:

$$\text{Cellulosic material} \xrightarrow{k_1} \text{Sugar} \xrightarrow{k_2} \text{Breakdown products}$$

In simple batch processes, the rates of reactions k_1 and k_2 are approximately equal, so that maximum yields are limited and the hydrolyzate contains as much breakdown products as contaminants. For starch, which is amorphous, the rate of hydrolysis is much faster than degradation, and sugar yields approach the theoretical level. Lignin has apparently little effect on the rate, as most woods hydrolyze faster than cotton or ramie (Asian shrub used for fiber). Crystallinity of the cellulose is thus the governing factor in dilute acid hydrolysis.

13.12.2.2 Enzymatic Hydrolysis

Pretreated substrate, neutralized to approximately pH 4.8, is mixed with enzymes at the required level of activity (see Fig. 13.10 for the assay of cellulose activity). In the commercial fermentation, the highest substrate concentrations that can be stirred (10% or more) are used, and the mixture is incubated at 45-50°C and pH 4.5-4.8. The enzymes are destroyed by even brief exposure to high temperatures (60°C or greater), or by a pH below 3.0 or above 8.0.

The costs of both pretreatment and saccharification are functions of scale: for systems in which mechanical or chemical pretreatment is followed by fermentation, large-volume operation would be attractive in industrialized countries as a means of reducing unit cost. It may, however, be possible to have comparatively cheap methods at the other end of the scale. One such simple process developed by Toyama *et al.* (Fig. 13.11) is described below.

Mixed chopped, pretreated substrate, such as alkali-treated straw, bagasse, or sawdust (adjusted to pH 4.0) is mixed with either crumbled *Trichoderma* koji (solid culture), preferably made from the same substrate as the saccharification substrate, or with commercial cellulase in a *Shocho* jar - a large ceramic jar with a narrow neck to keep out air and prevent contamination (Shocho is a sweet-potato liquor). The

substrate can be in the form of a very thick slurry, since once it is mixed well it will not be stirred. Citric acid (0.5%) is added as a preservative and the jar stored at 45°C. In 5-6 days this should yield 15-20% sugar syrup.

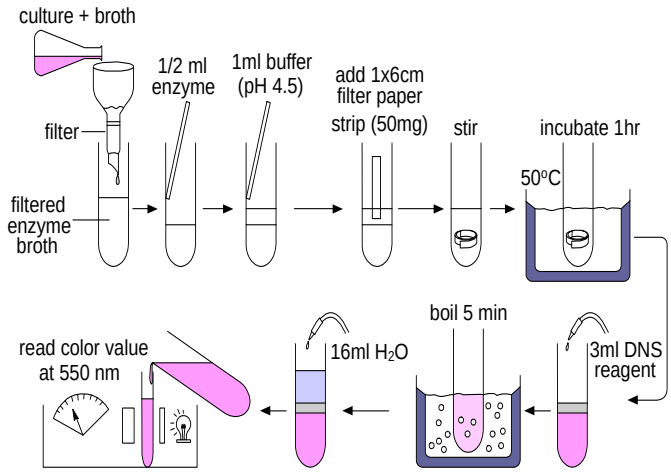


Fig. 13.10 The filter paper assay for determining cellulase activity of enzyme

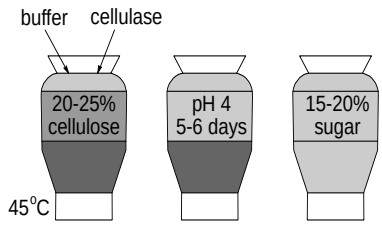


Fig. 13.11 Simplified saccharification process of cellulosic substrates with cellulase

13.12.3 FUTURE OF CELLULOSE FERMENTATION TECHNOLOGY

In its current state, cellulose fermentation technology - except perhaps for limited, small-scale applications such as the Toyama koji method of saccharification - has little likelihood of contributing to the production of alcohol fuels in the immediate future. The processes are either too complex or expensive, or require too much acid and alkali or energy for pretreatment, to be able to compete with other potential sources. However, given the increasing need for liquid fuels other than petroleum, the development of cheap and reliable saccharification technologies is necessary and will undoubtedly be achieved. The research and development will be better done in the industrialized countries, and the developing world should monitor progress and take advantage of improvements.

13.12.4 NEW TECHNOLOGY

Developing technologies that may decrease the cost of ethanol production can be considered in terms of pretreatment, fermentation, alcohol recovery, by-product recovery, and waste treatment.

Much of the research on ethanol processes is aimed at improving pretreatment for lignocellulose feedstocks to enhance the efficiency and reduce the cost of their hydrolysis to sugars. Some of the processes currently being examined are discussed next.

13.12.4.1 Purdue Process

This is a unique process for hydrolyzing crop residues and wood. Hemicellulose is first removed with dilute acid and then the cellulose and lignin are dissolved in concentrated sulfuric acid. The cellulose and lignin are then precipitated from the acid by addition of methanol. Since the precipitated cellulose is in an amorphous form, it is readily hydrolyzed by the appropriate enzymes.

13.12.4.2 General Electic Process

In this process, wood chips are first heated in alkaline aqueous butanol to separate the hemicellulose, cellulose, and lignin. The hemicellulose dissolves in the aqueous phase, the lignin dissolves in the butanol, and the cellulose remains undissolved.

The degraded hemicellulose can be fermented to additional butanol or converted to the sweetener xylitol. The lignin-butanol fraction can be cooled to separate the lignin or used as a fuel. The cellulose can be washed and hydrolyzed to glucose for fermentation to ethanol.

13.12.4.3 Natick Process

The Natick process consists of five steps:

1. Selection of an abundant and inexpensive cellulosic substrate such as municipal waste or aspen chips
2. Pretreatment of this substrate to enhance its enzyme susceptibility, preferably by ball-milling or two-roll compression milling
3. Production of active cellulase. As a result of screening thousands of organisms over the past 40 years, the Natick group has selected *Trichoderma reesei* as the best source of active cellulase
4. Utilization of the cellulase and β-glucosidase to saccharify cellulose. The treatment yields 5-15% glucose syrups in continuous hydrolysis or 10-30% glucose syrups in batch hydrolysis
5. Fermentation of the resulting glucose syrups to ethanol with *Candida* or *Saccharomyces* yeasts

All steps of the Natick process have been carried out at 200-400 liters, pilot-plant scale and a complete description and economic analysis is available from the Natick laboratory

13.12.4.4 Iotech Process

In this process, wood chips are exposed to high-pressure steam for several seconds, followed by explosive decompression.

At the Georgia Institute of Technology, samples of untreated and steam-exploded poplar chips were extracted with water and solvents. Results indicated a fivefold increase in ethanol extractables in the steam-exploded chips over the untreated samples - from about 5% to about 25%. Since lignin is the major component in the extractables, this steam treatment may facilitate degradation of the remaining cellulose.

13.12.5 CONTINUOUS PRODUCTION OF CELLULOSIC ETHANOL

The description given below is the continuous cellulosic fermentation method developed by Bio-Process Innovation (BPI), Inc. (USA). It uses a continuous cascade fermentation system for producing ethanol from waste sugars. BPI is currently working towards applying the technology to waste paper, cornstalks, straw, or sawdust.

As shown in Fig. 13.12, biomass feed is introduced continuously into the first of 3-5 stirred reactors placed in series, with the outflow of one reactor flowing into the next reactor. The liquid stream that moves from reactor to reactor is contacted with a stripping gas to remove the ethanol.

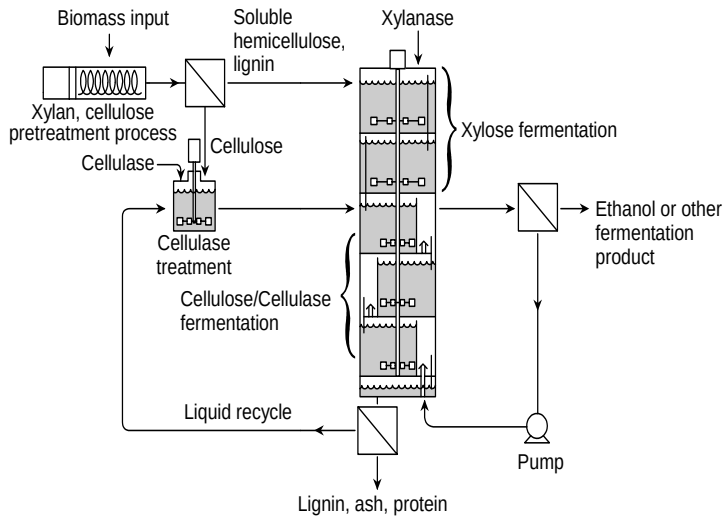


Fig. 11.12 Continuous production of cellulosic ethanol

A low-energy solvent absorption/extractive distillation system extracts ethanol from the stripping gas and recovers the gas for reuse. Separating the ethanol product as it is formed increases the rate of ethanol production. BPI, Inc. has also developed a highly flocculent yeast that further speeds the fermentation by maintaining high cell densities while operating continuously. The final effluent from the fifth reactor consists of waste lignin and ash, which is dewatered so that the water can be recycled.

A five-stage, 40,000-gallon unit has been operating at Permeate Refining Company in Hopkinton, Iowa, since June 1996 on waste starches and sugars. A small pilot unit operating on cellulosics is currently being tested at BPI, Inc. in West Lafayette, Indiana. A small plant in Spring Green, Wisconsin (Spring Green Ethanol), is now using BPI's technology for converting permeate mother liquor to ethanol. Several companies are currently evaluating BPI technology for whey- and molasses-based ethanol plants to be sited and built in the near future.

CHAPTER 14

WINE TECHNOLOGY

14.1 INTRODUCTION

Wine is one of the world's oldest alcoholic beverages, dating back to 6000 BC. It is believed to have originated in Egypt and Mesopotamia (Iraq and Eastern Syria).

14.1.1 DEFINITION

Wine without qualification means the end product of complete or partial alcoholic fermentation of fresh grape juice. Wines produced by the fermentation of juices of fruits, berries, honey, etc., are required to indicate source (the raw material) on the label. For example, orange wine, pear wine, apple wine (cider/cyder), etc.

14.1.2 WORLD PRODUCTION

Around 80% by volume of wine is produced by European countries. There are 7.742 million hectares of vineyards in the world producing 58.681 million MT of grapes each year. About 27 billion liters of wine are produced from these grapes. In 1996, the biggest wine producers worldwide were: France (21.9%), Italy (21.6%), and Spain (12%). The United States produced about 6.8% only. See Fig. 14.10 and Table 14.4 for an idea on world wine statistics.

14.1.3 CLASSIFICATION OF WINE

Wines can be classified on various bases *viz*., (i) *color*, (ii) *relative sweetness*, (iii) *effervescence*, (iv) *alcohol content*, and (v) the system used by *Wine Advisory Board*, USA. However, three basic groups of wines are most easily distinguishable for the consumer. They are (i) *table wines*, (ii) *sparkling wines* and (iii) *fortified wines*. A summary of the classification scheme is given in Table 14.1.

1. Color

Based on color, wines can be classified into (a) red wines, (b) white wines, and (c) pink wines (rosé). Red and white wines are the more important ones. Rosé can be prepared by blending red- and white wines or by using lesser color extracts from grapes.

2. Relative sweetness

Wines may be classified as either *sweet* or *dry* depending on the presence or absence (respectively) of residual sugar in wine. Dry wines contain sugar below 0.12% while

sweet wines may contain sugar as high as 7%. They may be red or white. Off-dry or semi-dry wines are also available.

Table 14.1 Classification of wines

Basis of classification	Class/Type	Description	Example
Color	Red wine	Contains the red coloring matter of skin, pulp and seeds	Burgundy
	White wine	Does not contain the red coloring matter of skin, pulp and seeds	Rhine wine
	Pink wine	Low concentration of red coloring matter is maintained	Rosé
Relative sweetness	Sweet wine	Contains up to 7% sugar	Sherry (sweet)
	Dry wine	Contains less than 0.12% sugar	Sherry (dry)
Alcohol content	Natural	Contains 8.5-16% alcohol by volume (% abv)	Table wines
	Fortified	Contains 17-21 % abv	Sherry
Effervescence	Still	Does not contain CO_2	Chianti
	Sparkling	Contains CO_2 (natural or added)	Champagne
Wine Advisory Board, USA	Dessert wine	Contains sugar; taken after meal	Sherry (sweet)
	Appetizer wine	Dry; fortified; taken before meal	Sherry (dry)
	Sparkling wine	Contains CO_2	Champagne
	Red Table wine	Natural; red in color	Chianti
	White Table wine	Natural; pale yellow to straw color	Rhine wine

There is considerable overlapping among wine types in the classification shown above. For example, a Red Table wine can at the same time be sweet, sparkling, fortified or natural. Similarly, a fortified wine can be sweet, sparkling, red, or white.

3. Alcohol content

Wines may be classified either as *natural* or *fortified*. Fortified wines are those that have received additional distilled spirit (wine spirit or brandy) to bring the alcohol content in the range 17-21% by volume. Due to high alcohol content, fortified wines may be stable even without pasteurization. Natural wines do not contain added spirits. Normally, they have alcohol content up to 16% by volume. Natural wines with alcohol contents in the range 8-10% are called *light wines*.

4. Effervescence

Wines may be classified as *still* or *sparkling*. Still wines do not contain CO_2 while sparkling wines contain CO_2, either natural (resulting from fermentation) or artificial (externally added).

5. System used by Wine Advisory Board

The board has divided wines into five main groups, *viz*,, (i) *White Table Wine*, (ii) *Red Table Wine*, (iii) *Dessert Wine*, (iv) *Aperitif Wine* (Appetizer Wine), and (v) *Sparkling Wine*. See Table 14.1 for the properties of these wines.

14.1.3.1 Red Table Wine

It contains the red coloring matter of the grapes. It is mostly light and dry or semi-dry (up to 14% *abv*). Red wines usually contain more flavor and aroma components. They are served at room temperature to release aroma characters. Red wines being more robust may be served in a glass with a generous, wide bowl and a narrower mouth. The bowl enables the wine to be easily swirled in the bowl without spilling to encourage evaporation of some of the volatile compounds. The smaller mouth of the glass concentrates the ensuing bouquet. Some of the more important examples of red wine are *Claret, Burgundy* and *Chianti* (pronounced as: *kee áantee*). See Fig.s 14.7-14.9 for an idea about the glasses.

14.1.3.2 White Table Wine

It is colorless, still, light, dry to sweet. A white table wine is not in the true sense white: the color actually ranges from *straw* through *brown*. White wines are usually served chilled because at warmer temperatures they quickly lose their volatile characters and become flat and tasteless. Examples: *Rhine wine, Sauterne*.

14.1.3.3 Dessert Wine

It is a still wine fortified in the range 17-21% *abv*. It is, as the name implies, taken after meal and is sweet in taste. Examples: *Tokay, Muscatel, White port, Angelica, Sherry* (sweet).

14.1.3.4 Appetizer Wine

This wine is taken before meal as an aperitif. It is *still* and *fortified*. Examples: *Sherry* (dry), *Vermouth* (pronounced as: *vər moóth*), *Port*.

14.1.3.5 Sparkling Wine

This wine contains CO_2, either natural or artificially added, and can be natural or fortified. It is usually sweet. Sparkling wines are served in tall, narrow glasses that clearly display the beautiful bubbles as they rise to the surface. Examples: *Champagne* (natural), *Madeira* (fortified).

14.1.4 SOME NOTED WINES

Wines from France are considered to be among the highest-quality wines in the world. The most renowned wine-producing regions are Burgundy and Bordeaux.

- *Burgundy:* any of various types of red or white wines from Burgundy area of Eastern France. *Burgundy* implies dark purplish color.
- *Chianti:* (particular type of) red or white wine from central Italy.
- *Sherry:* A type of yellow or brown, uniquely processed fortified wine, originally from southern Spain. It may be sweet or dry. In the sweet type, sugar may be present at concentrations up to 7%.
- *Claret:* (any of various types of) dry red wine, especially from Bordeaux area of France.
- *Port:* Strong, sweet, red wine made in Portugal
- *Champagne*: (any of various types of) sparkling straw-colored wine from eastern France
- *Madeira:* White dessert wine from the island of Madeira. It can be still or sparkling
- *Vermouth:* Strong, white wine flavored with herbs, drunk as an aperitif wine (often in strong cocktail).

14.2 WINE YEASTS

Wines can be prepared using either natural yeast flora of the grapes (spontaneous fermentation) or pure cultures (culture yeasts). Many manufacturers still depend on spontaneous fermentation. Spontaneous fermentation has following advantages and limitations:

Advantages:

The wine will be of unique quality in terms of bouquet because the end product is the result of interaction of diverse yeast types. Each yeast type will contribute unique flavor to the wine.

Disadvantages:

- Since the yeast profile is diverse, spontaneous fermentation may sometimes lead to failure
- Most strains (and also genera) do not produce large amounts of alcohol while a few strains produce undesirable organic compounds such as organic acids, H_2S, higher alcohols, etc., that may affect the flavor.

Grapes harbor both desirable and undesirable yeasts, e.g., *Hansenula*, *Kloeckera*, *Pichia* (film formers), *Saccharomyces*, *Candida*, *Metschnikowia*, to name a few. The most important in winery, however, are *Saccharomyces* spp especially, *S. cerevisiae* and *S. uvarum*. In general, they are not present in dominant numbers initially. However, as the fermentation progresses, they quickly outnumber other groups thereby creating environment for vinification.

14.2.1 PURE CULTURES

Pure cultures can be obtained either from commercial suppliers or by isolation in the laboratory from grapes. In the latter case, isolation can be done by streaking on *grape juice agar*. At present, a number of wine yeasts (pure culture) are available commercially: champagne, burgundy, sauterne, pommard, to name a few. Each confers a distinctive flavor to a particular wine. The all-purpose wine yeast is, as the name implies, suitable for many wines. Bakers and brewers yeast are suitable only for home wine-making: they yield low amounts of alcohol. Of the two yeasts, *viz*., *S. cerevisiae* and *S. uvarum*, the former contains strains that yield much larger amounts of alcohol (18-20% *abv*) than the latter (7-8% *abv*). In industry, often a combination of natural flora and pure culture is used. While the natural flora contributes to flavor, pure culture controls the direction of fermentation.

14.2.1.1 Desirable properties of wine yeasts

For producing a good quality wine, the choice of yeast is as important as the choice of grapes. The desirable characteristics of an ideal wine yeast are:

- High alcohol production
- Appropriate settling characteristics
- Osmotolerance
- Minimal by-product formation
- SO_2 tolerance

14.3 GRAPES

For wine production, *Vitis vinifera*, *Vitis labrusca*, and to a smaller extent, *Vitis rotundifolia* are used. *Vitis vinifera* is the European wine grape and is by far the most important. There are over 5000 varieties of *Vitis vinifera* today. Natural factors make wine from a particular region unique. Known in the wine industry as *terroir*, these factors include local climate (temperature, rainfall, sunlight), location of grapevines (altitude and slope), and soil (structure, composition, and water drainage).

The single most important factor that contributes to wine's character is the grapes that are used. Grapes influence the wine's flavor, alcohol content, acidity, and even its color.

Grape juice is highly variable in composition. Its major constituents are: water, carbohydrates (glucose, fructose, pentose, pectins), nitrogenous compounds (protein and protein-split products), vitamins, enzymes, and aroma compounds. The color may range from white, green, pink, red, to purple (see Fig. 14.1 also). Some of the more important physicochemical characteristics of matured grapes are given in Table 14.2. Grape juice with very high sugar concentrations are used in the production of strong or sweet wines.

Table 14.2 Physicochemical parameters of grapes

Variable	Range	Comments
Sugar	120-250g/L	Pentose: 1g/L; glucose:fructose =1:1
Acid	5-15g/L	As tartaric acid
Pectin	0.02-0.6%	
pH	3-4	

Fig. 14.1 Purple grapes

14.4 PRODUCTION OF RED TABLE WINE

The outline of red table wine production is given in Fig. 14.2. The main steps in red table wine making are as described in the following paragraphs.

14.4.1 GRAPE SELECTION

The grapes used for the production of red table wines are selected varieties of *Vitis vinifera*. Since the color of red table wine comes from the grapes, the berries should be of purple or red color (see Fig. 14.1).

14.4.2 CRUSHING AND SULFITING

In modern wine production, the grapes are harvested from the vineyards and taken to winery where they are passed though destemmer-crusher machine. Three types of crushers are generally used: (i) *Roller type*, (ii) *Disintegrator*, and (iii) *Garolla type*. The last one is more generally used. The crushed grapes fall in the *sump* beneath the crusher. The whole is then taken to fermentation tanks using special plunger pumps.

Sulfiting (addition of sulfur dioxide) is done during crushing, and this is primarily to (i) inhibit the growth of undesirable microorganisms (acetic acid bacteria, some lactic acid bacteria, and some grape yeasts) and (ii) bind acetaldehyde (acetaldehyde has undesirable organoleptic properties).

The toxic effect of SO_2 is due to the dissolved molecular SO_2. The effect of SO_2 on yeasts varies from species to species, and even within strains. In general, SO_2 is

seldom used at a rate above 150 ppm. Moldy grapes may need 200 ppm, though. Higher concentrations of SO_2 markedly delay fermentation (sometimes as long as 2 months). The most commonly used source of SO_2 is potassium metabisulfite (KMS).

14.4.3 MUST TREATMENT

The grape juice meant for wine fermentation is called *must*. For consistent wine quality, the quality of must should also be consistent. If the must does not meet the requirement, grape juice concentrate, sugar, acid, etc., must be added for the adjustment. This manipulation to standardize the must is called *amelioration*. Addition of sugar is supposed to produce *substandard* wine and is prohibited in some countries. *Gallization* is a term used to imply addition of water and sugar prior to fermentation in order to (i) increase alcohol content, (ii) increase total volume, and (iii) decrease acidity. *Chaptalization* is another term used to imply addition of sugar only.

14.4.4 WARMING

If the weather is cold, the must is warmed to 27°C. This facilitates color extraction and yeast action.

14.4.5 STARTER PREPARATION AND ADDITION

Typically, the pure culture kept in a slant is filled with sterile grape juice and incubated at 27°C for 12 hrs with occasional shaking. This is then transferred to a large flask containing sterile grape juice and again incubated for 3-4 days with occasional shaking. The whole is then transferred to a propagator (about 25 liter capacity). After aerobic growth for 4-5 days, this in turn is transferred to a final propagator that contains 25 HL of sterile grape juice. Aeration is accomplished by supplying sterile air (filtered air). Three to four hours after inoculation, 100-125 ppm (parts per million) SO_2 is added for *acclimatization*. The propagators are all closed vessels. The vigorously growing yeast is now used to inoculate the main fermenter at a rate of 2-3% by volume.

14.4.6 FERMENTATION AND COLOR EXTRACTION

The fermenters are mostly of stainless steel or wood. The type of container used and the temperature of fermentation influence the characters of the wine. During fermentation, the temperature is kept at about 27°C. Low temperatures slow down yeast activity and color extraction, and promote lactic acid bacteria. High temperatures lead to alcohol and aroma loss due to evaporation. High temperatures may also lead to *stuck* or *hung* fermentation in which the yeasts flocculate prematurely: the yeasts are weakened and will leave 1-6% sugar unfermented. Temperature control is achieved through cooling coils. The fermentation is monitored by periodic testing of temperature and taking Balling degree (1° balling = 1% sucrose solution in terms of gravity). The test is done 2-3 times a day.

The skins, pulp, and seeds rise to the surface during fermentation to form a *cap* of one to several feet in thickness. The cap is thoroughly punched and mixed with the juice using a pole, several times a day. Alternatively, the juice may be drawn from the

bottom by a pump and sprayed over the entire surface of the cap, again several times a day. The color is extracted due to the solvent action of alcohol (generated during fermentation) and the temperature of fermentation. Most pectic substances are destroyed or precipitated by alcohol. Tannins are also extracted. The duration of fermentation can be judged by the amount of color and tannins extracted. It generally takes 3-4 days.

Color can also be extracted by HTST heating of crushed grapes (to 85°C). The color is primarily *anthocyanin*. Since the yeast flora is killed during heating, pure culture must be used for fermentation.

Another interesting process for color extraction-cum-fermentation is *maceration carbonique*. In it, the injured berries are kept for 8-10 days in a vessel under layer of CO_2. During this time, intracellular fermentation occurs in grapes thereby forming 1.5-2.5% ethanol by volume. In the meantime color will be extracted. The berries are later on pressed and fermentation carried out for additional 48 hrs. Following advantages have been claimed for this method:

- The aroma of wine is more intense
- The concentration of polyphenols is low
- Bacterial fermentation of malic acid is favored

14.4.7 DRAWING OFF

Drawing off is done to remove *pomace* from the primary-fermented must. It consists in allowing the *free run* wine to flow into tub through a large bronze spigot (trap/valve/plug) and pumping the wine to secondary fermentation tanks. The pomace is strained off in stainless steel screens during pumping.

14.4.8 PRESSING

Pressing is done to remove residual wine from the pomace and this is done in a vertical or horizontal basket or *Williams press*, the pressure being applied by a hydraulic ram. This wine is normally kept separately to produce *press wine* (low-grade wine: contains tannins, sediments, etc.). Mixing *press wine* with *free-run wine* lowers the quality of the latter.

14.4.9 AFTER-FERMENTATION

Primary fermentation normally leaves some amounts of residual sugar. This is fermented in storage tanks by the surviving yeasts. Aeration is done to invigorate the yeast. Temperature control is critical again because low temperatures also lead to stuck fermentation. To avoid this, temperature control and bottom aeration (to intermix the contents) is done. Chemical analysis is done until sugar content falls below 0.2% (in case of dry wine).

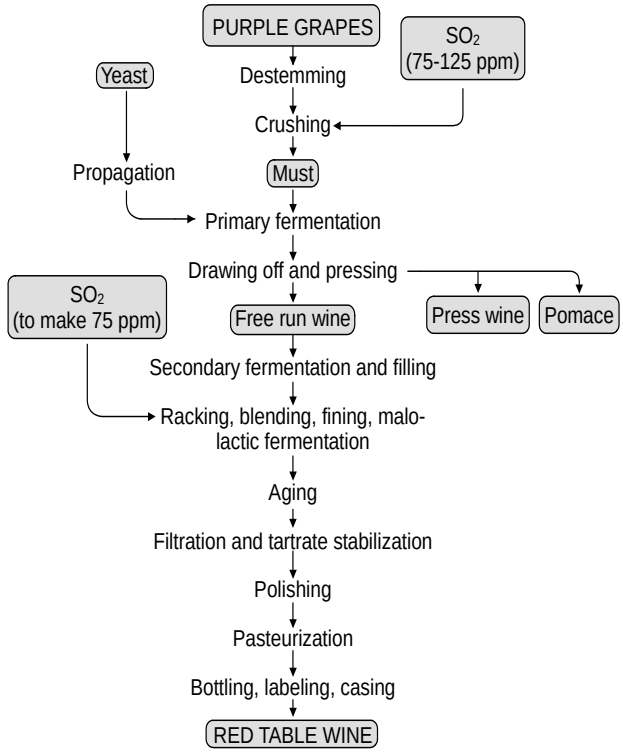


Fig. 14.2 Industrial production of Red Table Wine

14.4.10 FILLING AND MALO-LACTIC FERMENTATION

Wine from secondary fermentation is carefully drawn out and filled in tanks of table wine, and sealed. Final phase of fermentation takes place here also. In very obstinate cases, it may be necessary to add yeast foods: ammonium phosphate or urea. The stage is taken care to protect wine against vinegar bacteria by means of a *fermentation bung*. This may be prepared by boring a small hole in a cellar bung and inserting through it a U-tube. The bung is inserted in the bunghole in the tank and one arm of the tube is immersed in a jar or bottle containing dilute KMS solution. This helps build a slight positive pressure of CO_2 in the tank, which prevents the growth of vinegar bacteria or film-yeasts. After bubbling in the fermenter bung practically ceases, indicating that the fermentation is nearly complete, the bung is replaced by a plain, soled, cellar bung. The process is complete within about 6 weeks at most after crushing of berries. The wine is now assumed to be dry.

14.4.10.1 Malo-lactic fermentation

Malo-lactic fermentation refers to secondary fermentation in which lactic acid bacteria are allowed to metabolize malic acid to (lactic acid + CO_2). This fermentation is particularly useful if the titrable acidity of the wine is to be reduced. Wines with low levels of acidity should be protected from malo-lactic fermentation: wine quality decreases if the acid level falls too low. Malo-lactic fermentation can be

easily prevented by early *racking*, cool storage, and maintaining 100 ppm or more of SO_2. On the other hand, if such a fermentation is desired it can be facilitated by leaving the wine on the *lees* (yeast sediments) for prolonged periods at higher temperatures. This storage causes lysis of yeast cells and releases amino acids and other nutrients needed for the growth of the 'contaminant' lactic acid bacteria. The biochemistry of fermentation is given in Fig. 14.3.

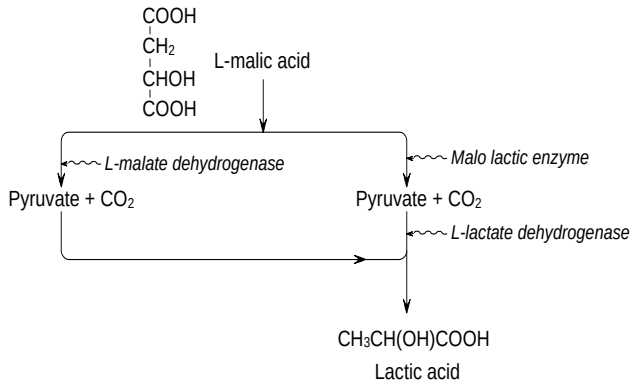


Fig. 14.3 The malo-lactic pathway

Malo-lactic fermentation has an important bearing in the quality of wine. It is a natural way of reducing acidity in wine. Besides, the fermentation also results in wines with greater softness and mellowness. The bacteria implicated for malo-lactic fermentation are *Leuconostoc oenos, Lactobacillus*, and *Pediococcus*, the first one being the most important.

14.4.11 BLENDING, RACKING, AND FINING

After the yeasts have settled down well in storage tanks the clear wine portion is carefully siphoned away from the lees. After carrying out routine chemical and organoleptic tests, blending is carried out. Blending is one of the most important cellar operations the main purposes of which are to: (i) *develop specific types*, and (ii) *maintain character and quality of wine types*. Usually, the wine of a given *vintage* is blended and earlier this is done, the better. Wines labeled *vintage* should not be blended.

Following blending, the wine is amended with a total of 100 ppm SO_2. Sediments are allowed to settle and the clear wine siphoned to yet another vessel. This process is called *racking*. Racking serves many purposes, viz., (i) *removes considerable amount of* CO_2, (ii) *raises redox potential*, and (ii) *clarifies wine*.

One danger of leaving the new wine in contact with its yeast sediment is that it may lead to yeast autolysis and, at low redox potential, formation of H_2S. Normally, wine should be racked within a month of the end of fermentation. Racking process normally entails a sacrifice of 2-3% wine in lees.

Clarification by conventional racking is a long process. To hasten this, certain agents, commonly called *fining agents*, are added during racking. Fining is a traditional method

of bringing about clarification. Fining agents may be used during aging as well. They not only clarify the wine (by physical adsorption) but also help remove excess tannins. Some of the more important fining agents are gelatin, tannins, isinglass, and bentonite. Typically, bentonite can be used at a rate of 1.5 g/liter. However, it is essential that the fining agents be tested for dosage optimization before use.

14.4.12 AGING

This is one of the most interesting and one of the most important, yet one of the most complex processes of wine making. Newly fermented wine is cloudy, harsh in taste, yeasty in flavor and odor, and without the pleasing bouquet that develops later in its history. Aging (also spelt *ageing*) can be done in oak barrels as well as in bottles, the latter being called *binning*. Depending on the type of wine, aging can be done for 6 months to several years. If fining agents are used in the aging, racking is done at least twice a year. Aging, however, can be hastened by aeration, refrigeration, and pasteurization. Since aging is simply a maturation or mellowing process it can occur throughout the storage period. Aging can be done after clarification, post-pasteurization, or even after final bottling.

The principal changes in flavor and bouquet during aging in the wood are generally believed to be due to slow oxidation process: wood extractives also have a material role on flavor. Several changes, e.g., formation of esters, etc., also occur.

14.4.13 FILTRATION

Filtration is carried out in plate and frame filter using filter aid such as *Hyflo super cel*, diatomaceous earth, etc. This helps remove suspended solids, clouding agents, etc.

14.4.14 TARTRATE STABILIZATION

New wines are supersaturated with respect to potassium tartrate. Storing in cold temperatures allows crystallization and sedimentation of this compound, which can finally be removed. The crystals are called *wine diamonds* and are not hazardous. In commercial practice, tartrates are removed by passing wine through ion-exchange resin.

14.4.15 PASTEURIZATION

Wines with alcohol contents less than 17% are susceptible to spoilage. The shelf-life can be increased by pasteurization. Pasteurization of wine can be done by following techniques:

1. *HTST*: the wine is heated at 80°C for 1 sec, cooled, and filled
2. *Fill Cold*: the wine is first filled and heated in water bath or by hot water spray to an internal temperature of 60°C
3. *Hot Bottling:* the wine is heated to 55-70°C and filled hot.

In some wines, the quality is reduced by pasteurization while that of others may be improved. The response is related to grape variety.

The alcohol content of wine has a significant effect on the heat resistance of yeasts and has been found to be more important than other variables like pH and residual sugar. High level of alcohol content reduces *D* and *Z* values.

An alternative to pasteurization is *filtration sterilization*. In this method, membrane filters of pore sizes 0.65-1.2 μm are used, the former being more general in use. This filter removes bacteria also. However, the wine should be highly polished by *prefiltration* through various types of *depth* filters prior to using this method.

14.4.16 BOTTLING, LABELING, AND CASING

These are the final operations that the wine receives at the cellar. The objectives of bottling are to protect wine against spoilage organisms, oxygen, light, and to provide bottle-aged wines (Fig. 14.4).

Fig. 14.4 Wine Cellar

A wine cellar in the Bordeaux region of France contains thousands of bottles of wine. Fine wines are best stored under dark, cool, and moderately dry conditions. The bottles are stored horizontally to prevent the corks from drying out.

14.4.17 SOME MAJOR COMPONENTS OF WINE

Being produced from complex material, wine contains innumerable components. The most important (qualitatively) of them are given in Table 14.3.

Table14.1 Some major components of wine

Component	Concentration
Ethanol	14%
Methanol	Traces to 0.6 g/L
Higher alcohols	0.15-1.0%
Acetaldehyde	200-500 ppm
Esters	200-400 ppm
Volatile acidity	0.03 g/100 ml as acetic acid; maximum limit = 0.14 g/100 ml
Pectins and gums	0.3-0.5 g/100 ml
Water and sugar	Variable
Glycerol	Trace

The dominant higher alcohols in wine are 1-propanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 1-pentanol, and 1-hexanol.

14.5 PRODUCTION OF WHITE TABLE WINE

White table wine is a grape wine which does not contain the red coloring matter of the skins, juice, or pulp of grapes. The wine color ranges from straw to brown.

White table wines differ fundamentally from red wines in production, composition, and sensory quality. Since white wines are not produced by fermentation on skins, the tannins and extract contents are lower. White table wines are more delicate in flavor and are usually sweet. On the other hand, red table wines are more flavorful and are usually off-dry to dry.

14.5.1 GENERAL PROCESS OF WHITE TABLE WINE PRODUCTION

Except for some steps, the technique of white table wine production is similar to that of red table wine. The process starts with the selection of white grapes. During crushing, sulfiting is done at the rate of 75-150 ppm SO_2. The crushed grapes are allowed to stand in the vat at cold temperature overnight. A small amount of tannin gets extracted and sliminess is lost. The loss of sliminess, which is due to the action of pectic enzymes, is advantageous because it increases the flow of free-run juice. Without sulfiting, particularly during warm weather and long standing, oxidation as well as other enzyme-catalyzed reactions may occur. These can affect the color and organoleptic properties of wines unfavorably. Further, sulfiting is particularly important for vinification of grapes with low concentration of acids. Sulfiting helps prevent bacterial fermentation of malic acid to lactic acid.

The settled juice is separated from the sediments and skins by many different methods, including drawing off, centrifugation, and filtration. The sediments may be pooled and fermented separately for low-grade wines. Settled-and-drawn off must produces earlier-clarifying wines but some believe that the wines are less flavorful. Settling is particularly desirable with musts intended for sweet table wines. Must

from grapes infected by *Botrytis cinerea* (a mold) should always be settled. Natural or artificial infection with *Botrytis* is sometimes desirable. This mold develops on grapes exposed to a rainy and foggy climate. Its action on grapes causes a rapid evaporation of moisture and consequent increase in sugar content. The infected berries are said to be *botrytized.* The mold produces glycerol (up to 2%), which gives a unique botrytized flavor in wine.

14.5.2 AMELIORATION

Acid, if needed, may be added. Addition of acid before fermentation promotes cleaner fermentation and apparently gives smoother wines than if added later during aging: but losses of acid in the lees are greater if the acid is added before fermentation. Tartaric acid is preferred. Citric acid should not be used if acidification is to be done before fermentation. Some wine makers add a small amount of tannins also. It aids in stabilization but the wine will usually be slightly darker. Sugar concentration can be adjusted as described for the red wine.

14.5.3 STARTER ADDITION

Starters can be added with or without heat treatment of the must. Usually, HTST (85°C) is carried out where heating is used. Starter is added at the rate of 2-3% vol/vol. The yeast should preferably be of granular (i.e., agglomerating) type, such as champagne- or burgundy strains. The propagation method is similar to that described for red table wine.

14.5.4 FERMENTATION

White wine fermentation is done at 10-15°C, which is lower than that for red wine. At temperatures exceeding 20°C, the fermentation not only becomes stuck but the bouquet, aroma, and flavor are also damaged. Fermentation is carried out in lined steel, concrete- or wooden tanks, ovals, or puncheons.

After fermentation, the wine is removed from the lees as soon as possible before yeasts begin to autolyze. The rest is the same as for red wine. The wine is bottled for a storage period, which may extend up to 2 years for high-quality wines.

14.6 FORTIFIED AND SWEET WINES

Production of fortified wines and sweet wines does not need special procedures. Fortification can be carried out by addition of distilled spirits either to a fully fermented or partially fermented (or fermenting) wine. The final alcohol concentration is made 17-21% by volume. The high alcohol content makes the wine more stable and may keep well even without pasteurization. Fortification of stuck wines is another way of producing fortified sweet wines. Sweet wines can also be produced by adding appropriate amounts of sucrose solution (commonly called *dosage*) to dry wines.

14.6.1 SWEET TABLE WINES

Example: sauterne

Sauterne (pronounced: *sō túrn*) is prepared from sweet grapes and fermentation arrested at some point by racking and adding a heavy dose of SO_2 (250 ppm or more). Another method of producing sweet sauterne is to add, after aging, the required amount of grape juice preserved in unfermented condition with about 1000 ppm SO_2. This preserved juice is called *mute* (a French term).

14.6.2 SHERRY

The origin of sherry is Spain. Sherry is a type of wine containing fortifying grape spirits or added alcohol, having the taste, aroma, and characteristics generally attributed to this product, and an alcohol content of not less than 17% by volume. Sherry is the most important Californian wine type. There are three methods of sherry production:

1. *The solera process:* bouquet is due to flor yeasts
2. *Submerged fermentation:* bouquet is due to flor yeasts
3. *The baking process:* bouquet is due to baking

Sherry can be either sweet or dry. Dry sherry is used as an appetizer while sweet sherry is used as a dessert.

14.6.2.1 Solera process

Selected grapes are first sun-dried to concentrate the sugar. They are then crushed and *plastered* by the addition of $CaSO_4$ in the form of finely powdered gypsum in order to increase the acidity (and lower pH). $CaSO_4$ reacts with potassium tartrate with the formation of free tartaric acid. This reduces susceptibility of the must to the growth of lactic acid bacteria.

Fermentation is carried out as with other wines. The base wine, which has alcohol content between 14.5-15.5% by volume, is partially filled in horizontal barrels. The contact of the wine with air allows the development of flor yeast, *Saccharomyces bayanus* by today's designation. The yeast metabolizes wine components, principally alcohol, and produces characteristic flavor compounds, notably acetaldehyde and some other compounds such as acetals, esters, and higher alcohols.

Sherry flavor develops in an interesting manner. Barrels are arranged in ranks, usually 3-6. Flor formation occurs about two weeks after the filling step. Some amount of matured wine is drawn from the last rank without disturbing the flor. This rank is now filled with wine from the preceding rank. The sequence goes on and the first rank receives young wine. In a five-rank system, by the time wine leaves the last rank it will have an average age of 12 years. Refilling is done at fixed intervals. The bottom row is called *solera* (which means floor). The other layers are called *criadera* (which means nursery). The wine that is drawn from the bottom row is now blended,

fortified, clarified, and filtered. See Fig. 14.5 for an idea about the arrangement in ranks.

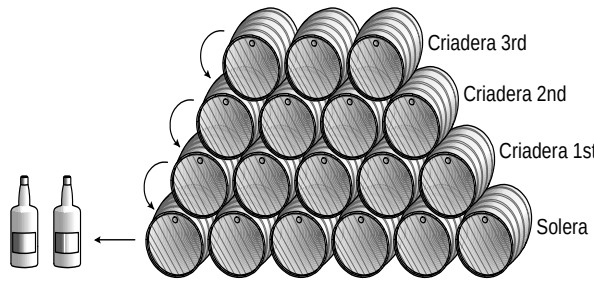


Fig.14.5 Example of arrangements of barrels in solera process

14.6.2.2 Submerged fermentation

This method is used for the production of cheap sherry. The quality does not approach that of solera process. In this method, the sherry process is accelerated by developing flor yeast in submerged culture. Air requirement is met by aerating the base wine.

14.6.2.3 Baking process

The sherry bouquet is developed by *baking* the base wine at 50-60°C for 10-20 weeks. The base wine, after fortification, should be left in tanks for 24 hrs. This allows the separation of yeast cells and colloids that flocculate during fortification. The process brings about slight browning and certain degree of oxidation. The quality is improved in oak barrels. Fortification is done before baking and sugar can be added at the rate of 1-10%. Baking is followed by cooling and stabilizing (with bentonite), racking, filtering, polishing, and aging. Light colored sherry is produced by charcoal treatment. The whole may finally be blended before bottling. The wine is stored for 6 months to 3 years.

14.7 SPARKLING WINES

Sparkling wines are those which readily foam because of high concentration of CO_2, either natural or artificially introduced. The CO_2 pressure is 4-5 atm at 20°C. However, in the US, wines containing a pressure of slightly more than 2 atm may be called sparkling wines.

Sparkling wines may be prepared by various methods:

1. *Champagne process:* bottle fermentation, yeast removal by disgorging
2. *Transfer process:* bottle fermentation, transfer to a tank, and removal of yeast by disgorging
3. *Bulk fermentation:* fermentation in large tanks
4. *Carbonation:* carbonation of base wine obtained by normal fermentation.

14.7.1 THE CHAMPAGNE PROCESS

In the classical bottle fermentation, a dry wine (*cuvé*) is taken for secondary fermentation. The cuvé has 10-12% *abv*. Blending is done to give an acidity between 0.7 and 0.8% as lactic acid. Before fermentation, the cuvé receives some sugar (18 g/bottle), typically at the rate of 25 g/liter and champagne yeast (0.3 g/bottle). The secondary fermentation takes place in thick-walled, tightly corked bottles at 9-12°C. The fermentation requires several months. After that, the wine remains on yeast for several months or years (2-3 years). All the bottles are kept half-way inverted (~ 45º) so that the yeasts collect in the neck of the bottles. The settling down of yeast is aided by gradual shaking and by increasing inclination of the bottles so that they reach a vertical position after several days. This shaking-and-inclination process is called *riddling* or *remuage* (Fig. 14.6). Riddling is done daily until all the sediments come to the cork. The bottles are then cooled to -1.1 to -5.5°C.

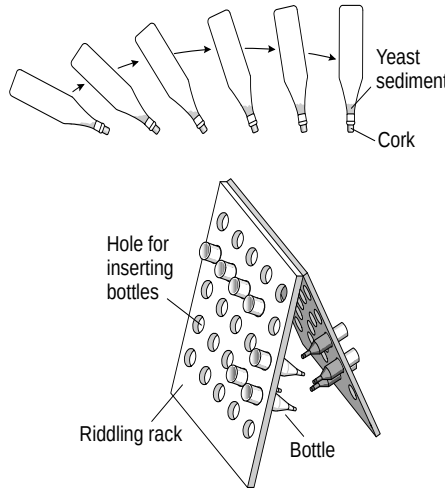


Fig.14.6 Example of riddling in champagne making

The neck of the bottle (containing the yeast) is then frozen by placing it in brine or other freezing solution. When the cork is removed the solid plug containing the yeast is ejected. This is called *disgorging*. When skillfully done, only 1-3% of wine is lost. The lost amount is replaced by adding sucrose solution (*dosage*). The sucrose concentration depends on the end product desired. The bottle is again tightly closed with a wooden cork.

14.7.2 THE TRANSFER PROCESS

In this process the fermentation is carried out in a manner similar to that for classical champagne process. Riddling may not be done. The yeast is removed by transferring the wine from the bottles to a tank under nitrogen pressure (closed system). Dosage is added and filtration carried out in a closed system under CO_2 or nitrogen counter pressure. Bottling is done as in champagne process.

14.7.3 THE BULK FERMENTATION PROCESS

This method is suitable for the mass production of sparkling wines. The wine has somewhat poorer quality. Secondary fermentation is carried out in pressurized vessel. A certain amount of unfermented residual sugar is retained in the wine so that there is no need for addition of dosage. After filtration, the wine can be filled into bottles. In this process the CO_2 generated during the secondary fermentation is also retained.

14.7.4 THE CARBONATION PROCESS

This method entails impregnation of the base wine with CO_2. The quality is largely determined by the quality of the base wine. In contrast to secondary fermentation, artificial carbonation allows only weak binding of CO_2 in the wine. Consequently, the gas escapes more quickly when the wine is opened.

Fig. 14.7 Champagne Flute

Fig. 14.8 White Wine Glass

Fig. 14.9 Snifter

Sparkling wines are often served in tall, flute-shaped glasses called champagne flutes. White wine glasses are typically smaller than red wine glasses. Distilled liquors, particularly brandy or cognac, are traditionally served in balloon-shaped snifter glasses.

Table 14.4 Major wine-producing countries of the world, 1996

	Wine production, million L	Wine exports, million L	Wine consumption, million L	Total grape, '000 MT	Area of vines, '000 ha
France	5965	1229	3479	7701	917
Italy	5877	1511.5	3562	9459	922
Spain	3267	672.9	1475	4846	1224
USA	1864	163.8	2046	4935	311
Argentina	1268	125.4	1355	2040	211
S. Africa	1000	99.6	406	1440	106
Portugal	953	200	580	1270	259
Germany	830	300.8	1866	1297	105

14.8 WINE SPOILAGES AND DEFECTS

As with other alcoholic fermentations, wine fermentation is also a robust process. Because of the alcohol content, low pH, and low nutrient status, most spoilage organisms fail to thrive in wine. The extrinsic factors such as pasteurization and storage under cold condition further reduce the chances of spoilage in wine. Some of the more important spoilages that may occur in wine are given in Table 14.5.

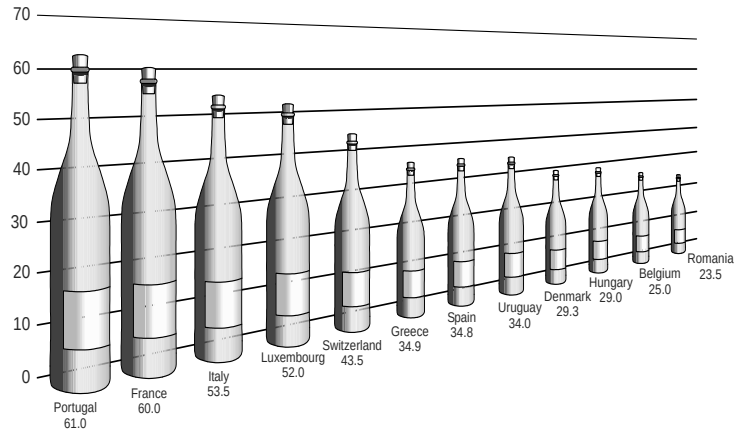


Fig. 14.10 World Wine Consumption in 1997 (in liters per capita)

Table 14.5 Wine defects

Defects	Organism involved	Remarks
Acetification	*Acetobacter* sp	Formation of vinegar
Mousy	*Gluconobacter suboxydans*	Formation of gluconic acid from glucose
Tourne	Lactic acid bacteria	Production of lactic acid
Amertume	Fructose and glycerol fermenters	Fermentation of glycerol and fructose. Fermentation of fructose to mannitol gives bitter taste and this particular fermentation is called mannitic fermentation.
Malo-lactic fermentation	Lactic acid bacteria	Conversion of malic acid to lactic acid, thereby reducing the titrable acidity

CHAPTER 15

BRANDY AND WHISKEY

15.1 BRANDY

Brandy is a distillation product of grape wine. Brandy can also be prepared from other wine types. Some of the more important brandies are given in Table 15.1. The name brandy comes from the Dutch word *brandewijn*, meaning "burnt wine."

Table 15.1 Some important brandies

Brandy type	Country	Fruit used
Cognac	France	Grapes
Armagnac	France	Grapes
Kirsch (pronounced: *kee.ə.sh*)	France/ Switzerland/ Germany	Black morello cherry
Slivovitz	Balkans	Plum
Perry	Britain and Sweden	Pear
Applejack/ cider brandy	America	Apple juice
Framboise	France	Raspberry
Fraise	France	Strawberry
Mirabelle	France	Yellow plum

15.1.1 GENERAL METHOD OF BRANDY PRODUCTION

Generally, brandy is produced from white grapes. Brandy from red grapes is somewhat inferior to that from white grapes and contains larger concentrations of higher alcohols

It is desirable to avoid treatment of the must with SO_2. Among other things, SO_2 corrodes the copper distillation column. The fermentation temperature is kept below 24°C. Distillation can be done in batch or continuous mode. The distillate is aged in oak barrels for several years. Some prefer to add caramel to adjust the color. The color of brandy comes from the wood in which it is aged.

15.1.1.1 Cognac

Cognac (pronounced: *con.yak*) comes from the Cognac region in France, and is double distilled using pot stills. Popular brands include Hine, Martell, Rémy Martin, Hennessy, Ragnaud-Sabourin, Delamain, and Courvoisier.

Selected grapes are pressed immediately and only slightly for the production of high quality cognac. Emphasis is given on clean wine which has been stored for as short time as possible, and which must not be oxidized. Traditionally, distillation is carried out in *direct-fired copper pot stills*, 150-500 liter capacity. See Fig. 15.1 for an idea about the distillation pot.

The new wine (with lees, but not more than 8% of added lees) is placed in the still and brought to boil. Distillation is carried out along with lees to provide bouquet to the brandy. Distillation continues until the vapor contains negligible amount of alcohol. This takes 8 hrs or more and the main distillate (wash) contains 24-32% *abv*. A tail fraction may be separated (it contains fusel oils). A second distillation is carried out after *pooling* the distillates from three other similar batches of distillates. This distillation lasts for 14 hrs. About 1-2% heads (containing aldehydes) are separated. The main distillate averages 60-70% *abv*. The new brandy is placed in *limousin* oak casks. The oak is well dried before being made into casks (see Fig 5.8 and 5.9). The casks are washed several times with water and once with brandy. Before too much tannin is extracted the brandy is transferred to used casks or tanks. It acquires best quality after 15-20 years in the wood. The matured cognac is diluted with water before bottling so that the alcohol content is above 40% by volume.

Fig. 15.1 Copper stills for batch distillation of whiskey and brandy

15.1.1.2 Armagnac

Armagnac is made from grapes of the Armagnac region in Southwest of France (Gers, Landes, Lot-et-Garonne). Some of the popular brands of armagnac are Darroze, Baron de Sigognac, Larressingle, Delord, Laubade, Gélas, and Janneau.

In contrast to cognac, armagnac is obtained in single distillation from wine which does not contain lees. The brandy has an alcohol content of 52-53% by volume. It is produced generally by continuous distillation.

Brandy possesses a wide range of flavor components. It has complex aroma and flavor, with at least 546 identified compounds. The main components of brandy are given in Table 15.2.

Table 15.2 Components of brandy

Components	Concentration
Ethanol	>40%
Methanol	Traces to 0.188%
Fusel oil	0.3%
Aldehydes	10-107 ppm
Esters	400-700 ppm

15.2 WHISKEY

Whiskey is also spelt as *whisky* in Scotland, England and Canada. According to US definition, whiskey is a category of distilled alcoholic beverage obtained from a fermented mash of grain.

15.2.1 KINDS OF WHISKEY

Whiskeys are broadly divided in to two categories, (i) *straight,* and (ii) *blended.* The term *straight* is somewhat misleading.

Straight whiskey can be a *mixture* of whiskeys, so long as the same distiller produces it during the same period, or it may be made from any mixture of grains, provided at least 51% of the total is accounted for by the grain with which the finished product is later identified. Thus, corn must make up at least 51% of the *mash bill* to be called straight bourbon. The same is the case with rye, another important mash ingredient. Straight scotch is a pure malt whiskey. This is sparingly produced since 1853.

Some of the principal whiskey types are:

- *Scotch whiskey:* malt is the basic raw material
- *Irish whiskey:* uses malt plus 5 other grains
- *American whiskey:* mainly rye whiskey, corn whiskey, and bourbon whiskey
- *Canadian whiskey:* uses a blend of cereals
- *Japanese whiskey:* uses blended grains, including some amount of rice.

Corn whiskey is distilled at less than 160° proof. It is made from mash containing 80% or more of corn. It is primarily aged in used, charred oak barrels. Bourbon whiskey also contains corn as the main ingredient but the quantity is lesser than that for corn whiskey.

15.2.2 GENERAL PRODUCTION METHOD

Corn and malt, and very often rye, are the principal ingredients of the mash bill. Malt is an indispensable ingredient of whiskey. It serves as an enzyme source, substrate for yeast, and provides characteristic flavor. Wheat and rice are also used in some whiskeys.

See Fig. 15.2 for the outline of whiskey production. Distillation of whiskey can be done either in pot stills or continuous stills, the former being classical.

After the malt and other ingredients have been obtained, the main production steps are: (i) Mashing, (ii) Fermentation, (iii) Distillation, (iv) Filling and maturation, and (v) Bottling

15.2.2.1 Mashing

The malt is ground to grist in a mill and is then fed into mash tuns (Fig. 15.2) as in brewing. Mash filtration produces wort (that contains assimilable nutrients for yeasts) and spent grain called *draff*. The wort is taken for fermentation while the draff is used as cattle feed.

Fig. 15.2 The inside of mash tun

15.2.2.2 Fermentation

The finished wort is cooled and fermented in vessels called *washbacks* (which can be of wood or steel, capacity ranging from 6000 to 45000 liters; see Fig. 15.4). The pH, temperature, and sugar concentration, all are important. The fermentable sugar content is maintained between 12 and 20% to give alcohol content of 5-8% in the *wash* (fermented mash). The pH is maintained by adding food grade acids like lactic acid. Alternatively, a bacterial souring can also be employed. Fig. 15.7 gives a flow diagram of whiskey production using bacterial fermentation for maintaining the acidity. Where bacterial fermentation is used, pasteurization is essential to kill these bacteria before the next stage of yeast fermentation. Whatever be the process, the washbacks are never filled to the top since the wort froths significantly during fermentation. The fermentation usually lasts for 2-5 days at 27°C.

The fermentation is done using improved strains of *Saccharomyces cerevisiae*. Yeast management is an important part of the process and this is similar to other alcoholic fermentations. The yeast is propagated first in the laboratory. Thereafter it is built up in a series of propagators called *dona* tub and *yeast tank* (Fig. 15.3). The propagation proceeds aseptically under aerobic condition. The final fermenter (Fig. 15.4) receives

the culture at the rate of 2-3% of the fermenter volume. Yeast tanks contain su pitch several fermenters.

Dona tub

Yeast tank

Fig. 15.3 Yeast propagation tanks

15.2.2.3 Distillation

Distillation can be carried out by continuous- (in towers similar to those used in ethanol distillation) or batch method (copper stills). Continuous distillation is carried out mostly for bourbon production in the US.

The copper pot stills in which the wash is distilled have become the ultimate symbol of whiskey distilleries (Fig. 15.5 and 15.6). The stills are made from copper since it is a material that is easy to work with, it does not rust, and it is an efficient heat conductor.

Fig. 15.4 Main fermenter

In general malt whiskey is distilled twice. The stills used for the first distillation are called *wash stills*. The resulting *low wines* spirit has an alcohol content of 20-26%. The low wines spirit is distilled second time in *spirit stills*. The *stillman* (person in charge of distillation) should be very careful during the second distillation (the *spirit run*). The more volatile *heads* called *foreshots* are collected separately so that they can be redistilled together with the next batch of low wines.

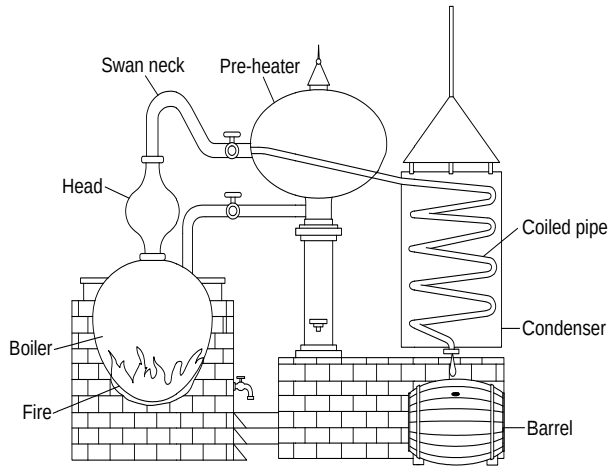


Fig. 15.5 Schematic of open-fired kettle distillation

Copper pot still

Alcohol safe (Spirit safe)

Fig. 15.6 Equipment required in whiskey distillation

The desired spirit (the part of the spirit to be used for making whiskey) is called *the middle part* or *the heart of the run* and starts to come through as the alcohol content reaches about 75%. The stillman has to regularly measure the alcohol content with an alcohol meter during the distillation. For this, the distillate is led through a section called *spirit safe* (where the alcohol is continuously measured; Fig. 15.6) and then to the collection tanks.

After the alcohol content in the distillate decreases to about 64%, the stillman performs what is called *cutting on spirit*. He now diverts the spirit into a separate

container. This fraction of distillate is called *fients* or *tail* and contains appreciable amounts of higher alcohols.

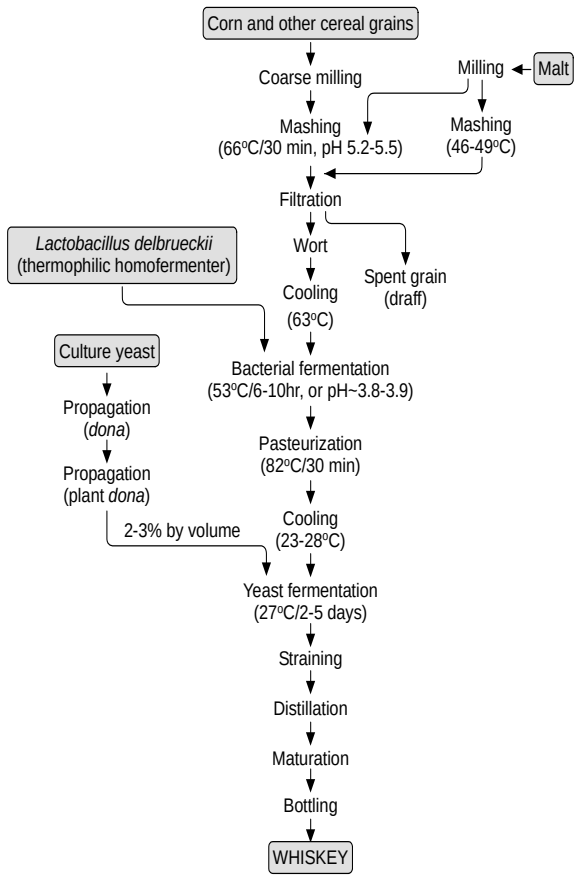


Fig. 15.7 Outline of whiskey production

15.2.2.4 Filling and maturation

All casks used to store whiskey are made from oak. Most distilleries use oak casks (Fig. 15.8) that have contained sherry or bourbon. Whiskey receives its natural amber color from interaction with the wood, although it has become increasingly common to artificially add color by using the E150 additive.

The spirit is not legally considered to be whiskey until it has been stored in wood for at least three years. Some of the whiskey evaporates through the wood during storage (Fig. 15.9). About 1-2% of the whiskey evaporates each year in a natural process which is called the *angel's share*. Since the alcohol content must be at least 40% in order for whiskey to be called whiskey, this means that there is a theoretical limit to how many years a whiskey can be stored before it has to be bottled. For example, if a whiskey looses 1.5 % of its alcohol content each year it may only be stored for 32 years before the alcohol content drops below 40%. Because of this it is unusual for whiskey to be stored much longer than 30 years. Yet another reason for

the limited maturation period is that whiskey constantly picks up tannin from the wood, and too much tannin ruins the whiskey. The greater part of all single malt whiskey is stored between 8 and 12 years.

Fig. 15.8 Filling whiskey in barrels

Fig. 15.9 Whiskey being aged in casks

15.2.2.5 Bottling

Before the whiskey is bottled it is usually filled into large tanks to be cut with de-mineralized water to 40, 43 or 46% alcohol content. After the whiskey is cut it is common to chill-filter it. This is done to remove slight impurities from the whiskey which otherwise would cause a clouding effect at low temperatures. Not all distilleries practise chill-filtering since they believe that it removes some of the character of the stored whiskey. See Fig. 15.10 for bottling line and bottled whiskey.

Fig. 15.10 Whiskey being filled in bottles

15.2.3 SCOTCH WHISKEY

Barley malt is the key component of Scotch whiskey. Special methods are used for malting. Drying of barley is accomplished in burning peat. This imparts characteristic *peatiness* to the whiskey. The five principle steps of scotch whiskey making are: (i) Malting, (ii) Mashing, (iii) Fermentation, (iv) Distillation, and (v) Maturation.

15.2.3.1 Malting

As usual, this entails steeping, germination, kilning, and milling. The sprouted *green malt* is cured in a tower-shaped kiln that has a furnace at the base. Anthracite and peat are burned in the furnace and the upward draught of hot air is assisted by a powered fan. Among other things, kilning on peats provides a very characteristic peaty flavor to the malt. Peat is formed from decomposed vegetable matter and the peat *reek* or smoke given off from combustion is imparted to the malt. In the early drying stages whilst the grain is in a soft moist condition the peat reek permeates into the barley. In the latter drying stages the outer skin of the grain will also be flavored.

The malt is stored for a week or two and then milled in a four-roller mill. An ideal grist should have a very coarse appearance (this assists in mashing and filtration). Formation of flour should be minimized because this impairs mashing and filtration. A typical composition of the grist is: 14% coarse materials and husks, 78% fine grits, and 8% flour.

15.2.3.2 Mashing

Mashing is done in mash tuns as in the case of other whiskey types. The quality of water is important here also. The process involves the application of three waters. The first water is introduced at around 70°C to get a striking temperature of 65°C. After mixing, the mash is allowed to sit for 1 hr for the enzymatic conversion of carbohydrates into simple forms. Thereafter the wort is drained, cooled to 22°C and

pumped to washbacks (fermenting vessels). Second water is applied at 78°C so that the mash attains a temperature of 70°C. Quantitatively, the second water is usually about half that used for the first water. After mixing, the mash is left for 30 min. The wort is processed as earlier. Third water is applied at 90°C in the form of sparge to recover the residual sugars. The extract is again pooled to the washbacks while the draff grain is fed to cattle.

15.2.3.3 Fermentation

Highly improved strains of *Saccharomyces cerevisiae* are used for the fermentation. The pitching rate is about 1% by volume. This means that the yeast must be propagated separately before the main fermentation. The pH and sugar concentration of the wort must be carefully maintained at 3.8 and 16-20% respectively. A temperature of 22°C is optimum for the fermentation. The fermentation may proceed up to 50 hrs. See Fig. 15.11 for the outline of scotch whiskey preparation.

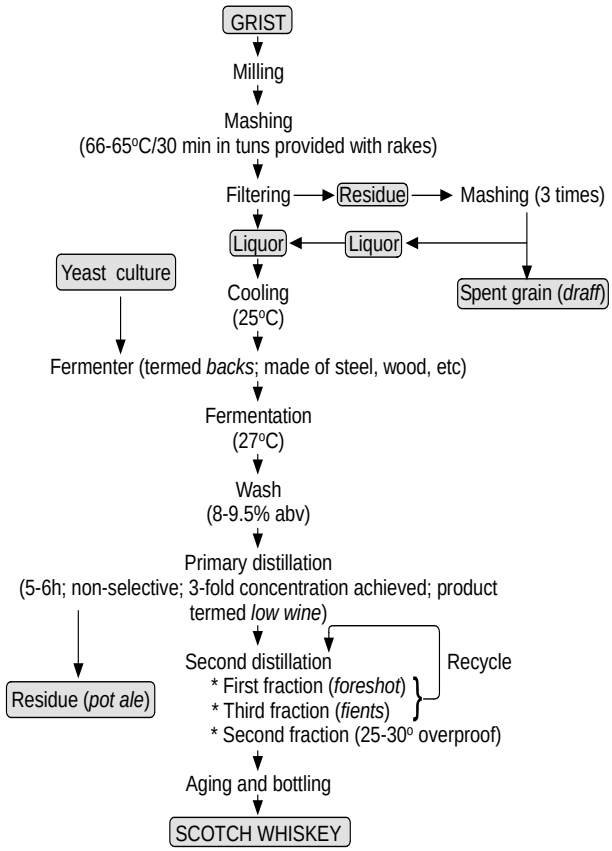


Fig. 15.11 Outline of Scotch whiskey production

15.2.3.4 Distillation

The fermented mash (wash) is pumped along with the yeast sediments and distillation is carried out in copper stills (called *wash stills*) with tall *swan-necks*. The stills are either direct-fired or electrically heated. The solids present in wash are prevented from settling during distillation by a rotating arm called *rummager*. The alcoholic vapors are condensed in a series of pipes in a cold water jacket to give a distillate of about 21%. This distillate is termed *low wines*.

The wine then passes through the second still, the *low wine still* or *spirit still*. The same process is repeated in the second distillation, but the stillman must be careful to separate *foreshots* (early part of the run, which is pungent and impure) and the *fients* (latter part of the run that contains higher alcohols). Only the middle part of the distillate (at about 70-75% alcohol) is collected for whiskey making. The timing for removing different fractions is based on the alcohol content, which is monitored regularly with an alcohol meter. The foreshots and fients are once again mixed and redistilled.

15.2.3.5 Maturation

The distillate thus produced must be matured in oak casks for at least three years to be legally termed Scotch whiskey. For single *Malt Whiskey*, at least 8-10 years of maturation is needed.

CHAPTER 16

VINEGAR PRODUCTION

16.1 INTRODUCTION

The word *vinegar* originated from the French word *vyn egre* whose literary meaning is *sour wine*.

Vinegar is the product obtained by acetic acid fermentation of alcohol-containing solutions. It must contain at least 4 g of acetic acid per 100 ml at 20°C and may not contain more than 0.5 ml (v/v) ethanol. The pH is usually 2-3.5. It is customary to express the strength of vinegar in terms of *grains* where 1% (m/v) is equal to 10 grains.

Technologically, vinegar is the product of a two-stage fermentation. In the first step yeast converts sugar to ethanol anaerobically, while in the second step ethanol is oxidized to acetic acid (ethanoic acid) aerobically by bacteria of the genera *Acetobacter* and *Gluconobacter*.

16.1.1 TYPES OF VINEGAR

Vinegar may be classified on the basis of raw material from which it has been prepared. In fact, anything that contains enough sugar or alcohol and is in no way objectionable as food may be used to make vinegar. As a result, vinegar can be: *cider vinegar*, *whey vinegar, alegar* (from ales*), malt vinegar*, *grain vinegar, spirit vinegar*, and so on.

By far the largest percentage of vinegar is the spirit vinegar. It is more commonly called *distilled vinegar* and less usually, *white vinegar* or *alcohol vinegar*.

16.2 PRODUCTION OF DISTILLED VINEGAR

16.2.1 THE MASH

The raw material for vinegar production is called *vinegar stock*. The vinegar stock for distilled vinegar can be (i) dilute, purified ethanol, or (ii) fusel oil containing pure spirit.

It is customary in almost all countries to denature the ethanol that serves as a raw material for vinegar industries. One of the common denaturant is ethyl acetate. This is split into acetic acid and ethanol during fermentation. Denaturation can also be done with distilled vinegar.

All mashes must contain ethanol, water, and some nutrients for the growth and metabolism of acetic acid bacteria. The water used should be bacteriologically clean and chlorine-free. Most *natural* raw materials (e.g., in the case of rice vinegar, malt vinegar, etc.) do not need the addition of extra nutrients. The composition of the vinegar stock (besides ethanol) for distilled vinegar is given in Table 16.1.

Table 16.1 Composition of vinegar stock (besides ethanol) for distilled vinegar

Material	Amount (g/liter)
Glucose	0.5-1.0
$CaCO_3$	0.1g/L (for demineralized water)
NaCl	0.1 (for demineralized water)
K, Mg, $(NH_4)_2HPO_4$, SO_4^-	0.3 (total)
Commercial nutrient mix*	0.07-0.2

*Commercial nutrient mix refers to supplements such as malt extract, dried yeast, Acetopep®, etc.

16.2.2 THE ORGANISM

Acetification, the oxidation of ethanol to acetic acid, is performed by members of the genera *Acetobacter* and *Gluconobacter*. These are Gram-negative, catalase positive, oxidase negative, strictly aerobic bacteria. *Acetobacter* species are better acid producers and are common in commercial vinegar production. However, their ability to oxidize acetic acid to CO_2 + H_2O (called *overoxidation*), a property that distinguishes them from *Gluconobacter*, can cause problems in some circumstances. Fortunately, overoxidation can be repressed by ethanol and this fact can be used for controlling it. In practice, ethanol content of the stock is not allowed to fall to the level whereby overoxidation is induced.

Most acetic acid bacteria associated with commercial acetification are difficult to culture on conventional solid media. They are very unstable, subject to variation, and die rapidly under cultural conditions other than specific for them.

To preserve and maintain them, they are always maintained continuously in laboratory fermenters. Under correct condition, the organism can retain their property indefinitely. This is possible even in large fermenters. Therefore, despite the propensity of *Acetobacter* towards variation, it is quite possible to perform vinegar fermentation year-in year-out without interruption.

16.2.2.1 Biochemistry of acetification

Acetic acid bacteria, depending on species and cultural conditions, can use several pathways to utilize sugar for energy. The oxidation of ethanol to acetic acid, however, is the relatively simpler pathway by which acetic acid bacteria derive their energy. It occurs in two stages, mediated by *alcohol dehydrogenase* and *aldehyde dehydrogenase*. Both the enzymes are associated with the cytoplasmic membrane and

have *pyroquinoline quinone* (PQQ) as a coenzyme. PQQ acts as a hydrogen acceptor, which then reduces a cytochrome. The consequent electron transport establishes a proton-motive force across the membrane which can be utilized to synthesize ATP. See Fig. 16.1 for the biosynthetic pathway of acetic acid during vinegar production.

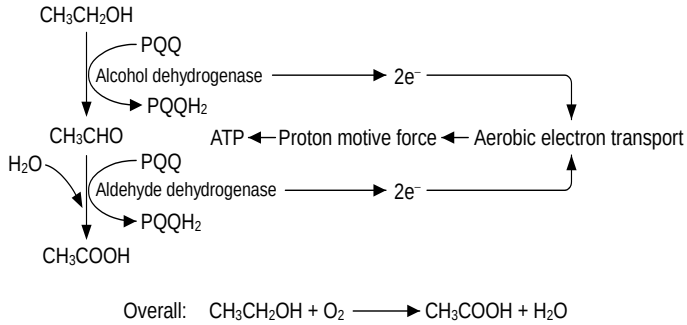


Fig. 16.1 Biosynthesis of acetic acid in *Acetobacter*

From stoichiometry of the equation (Fig. 16.1) it can be calculated that 1 liter of ethanol should yield 1.036 kg of acetic acid and 0.313 kg of water. This leads to the approximate relationship that *1% v/v ethanol* will give *1% w/v of acetic acid*, and this relation is used to predict the eventual acidity of vinegar and to calculate fermentation efficiency. It implies that, in absence of overoxidation, evaporative loss and conversion to biomass, the sum of concentration of ethanol (%v/v) and the concentration of acetic acid (%w/v), known as the *total concentration* or GK (from German term, *Gesammte konzentration*), should remain constant throughout acetification. The GK yield is the GK of final vinegar expressed as % of the GK at the start of the fermentation.

16.2.3 FERMENTATION PROCESS

There are three main methods of vinegar production, *viz*., (i) *The Orleans Process* (open vat process), (ii) *The Quick Vinegar Process*, and (iii) *Submerged Fermentation* (bubble process). These are described in the subsections to follow.

16.2.3.1 The Orleans process

This is also called French method, slow process, etc. This is a surface culture method. The process is no longer used and the equipment can be seen only in museum of factories. The equipment consists of a barrel, about 200 liter capacity, with several openings (see Fig. 16.2). The vinegar produced by this method is supposed to be of the finest quality.

For starting, one-fourth to one-third of the barrel is filled with a good-grade active vinegar, preferably from previous fermentation. This serves as an inoculum. 10-15 liters of wine may also be introduced along with the vinegar. Vinegar stock is then introduced through the top of the barrel via a pipe whose one end rests at the bottom of the barrel. The barrel is about half filled. Air passes freely though the

muslin-covered hole. The whole is left at 21-29°C for 14 days to a month, or until the acetification reaction reaches the predetermined GK.

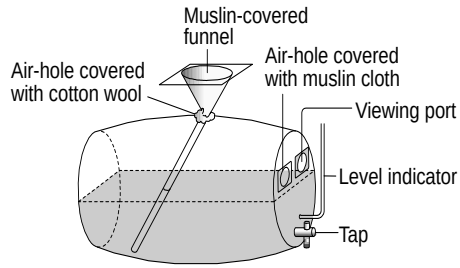


Fig.16.2 Vinegar production by Orleans process

A film of *Acetobacter*, called *mother of vinegar*, forms on the surface. Now, about one-third of the vinegar is slowly drawn out through the tap (at the bottom) and equivalent amount replaced with vinegar stock (from the funnel) without disturbing the film. The cycle can be repeated several times.

16.2.3.2 The quick vinegar process

This method is also called *German process*, *Fring's generator process*, *Trickling process* (solid-state), etc. The process was discovered by Boer and later modified by Schutzenbach. The quick vinegar process derives its name from the faster rate of acetification achieved by increasing the area of active bacterial film and improving O_2 transfer to the acetifying stock. The acetic acid bacteria grow as a surface film on an inert support material packed into a false-bottom vat. The size of the vat varies from 20 to 60 m^3.

The acetifying stock is sprayed onto the packing material. The stock trickles down against a current of air, which is either pumped through the bed or drawn up by the heat of reaction within it. The packing material normally consists of some lignocellulosic materials such as birch twigs, vine twigs, beech wood, etc. The vinegar stock is collected in a sump at the bottom of the vat and recirculated until the desired level of acidity has reached. The faster rate of reaction means that wash heats up during passage through the bed and, depending on the size of the fermenter, some cooling may be required. Fring's generator is an improvement over the older trickling process. Automatic systems are used here to control the temperature at 26-28°C. The recirculated stock is sprayed back through a sparger onto the beech wood shavings. The new stock usually contains 10.5% ethanol, 1% acetic acid, and 284 ppm special nutrient mix, e.g., acetopep®. The new stock gets mixed with vinegar absorbed in the shavings to produce a microbiologically favorable condition (8% acetic acid + 4% ethanol) for the production of acetic acid. The operation is semi-continuous. Addition of stock is carried out in 2-3 steps over a period of several days. The total time is between 4 and 10 days. The tank is largely emptied when the residual ethanol reaches 0.3%. The yield is between 85-90% and 5 liter vinegar/m^3 of shaving can be obtained per day. Fring's generator can also be run in tandem. See Fig. 16.3 for schematic diagram of Fring's generator.

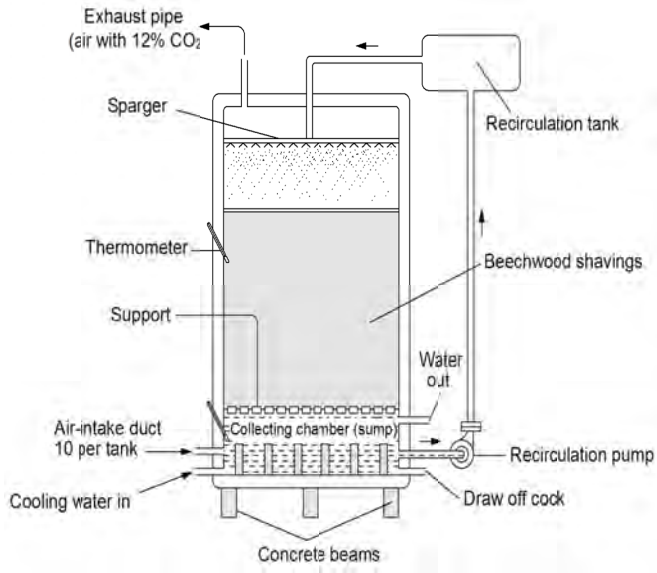


Fig. 16.3 Schematic diagram of Fring's generator

Disadvantages of quick vinegar process over submerged process

- Uniform distribution of stock is not possible. Due to ethanol concentration gradient, overoxidation is possible at certain sections of the bed
- Mashes high in nutrient content and low in ethanol concentration aggravate this problem by forming slime deposits on the carrier material thereby plugging up the column
- Non-homogeneity of the carrier column makes it impossible to distribute air
- Variations in temperature cannot be corrected
- Low yield

Advantages of quick vinegar process over submerged process

Performance is less affected because the acetification rate is slow, and it rarely stops. This has one important consequence: *bacteriophage-attack* (attack by bacteria-eating viruses) is less severe. The possible reasons could be the heterogeneity of the culture present, which allows organisms with different phage-susceptibilities to take over in the event of phage attack.

16.2.3.3 Submerged process (Frings acetator)

This method is the most widely used process for the production of distilled vinegar. Not only is the acetification rapid, the total concentration that can be handled is also significantly higher (≈ 15%).

The *acetator* vessel (Fig. 16.4) can be of wood or stainless steel with sizes that can handle 75-1200 liters ethanol in 24 hrs. The acetic acid bacteria grow suspended in

the medium which is oxygenated by sparging air. The process requires uninterrupted and a very high rate of aeration. Aeration is carried out using a special device called *Frings aerator*. The aerator is a hollow body turbine surrounded by a stator. As the turbine rotates, air is sucked in and distributed through outlets. The equipment does not need compressed air and is therefore called *self-priming* or *self-respiratory*. Baffles are provided for uniform mixing.

The Frings aerator is specifically designed for submerged mode of vinegar fermentation keeping in view the extreme sensitivity of the acetic acid bacteria to lack of air. At a pH of 2.5 and 10-14% acidity, a stoppage of mere one minute is sufficient to completely arrest the acetification: the effect is so extensive that the acetification will not resume when aeration is resumed.

The Frings aerator has fully automatic systems. A mechanical defoamer is provided at the top of the fermenter. Foams occasionally form due to dead cells. The defoamer consists of a horizontal, rotating device that sucks in foam through a port and throws it through a pipe leading to the tank-bottom.

The method is semi-continuous. The temperature is closely controlled at around 29°C. Before the alcohol level falls below 0.2% (and 13.3 g acetic acid/100 ml) about 40% of the fermenter content is pumped out. Without interrupting aeration, the fermenter is refilled with new mash of total concentration 15% (1% acetic acid and 14% ethanol). The vinegar stock is added in such a way that it is rapidly mixed by the aerator. Concentration gradient should be avoided, as this is detrimental to acetic acid bacteria.

The method is advantageous in that it is rapid and high concentration of acetic acid can be produced. A semi-continuous run takes 24-48 hrs.

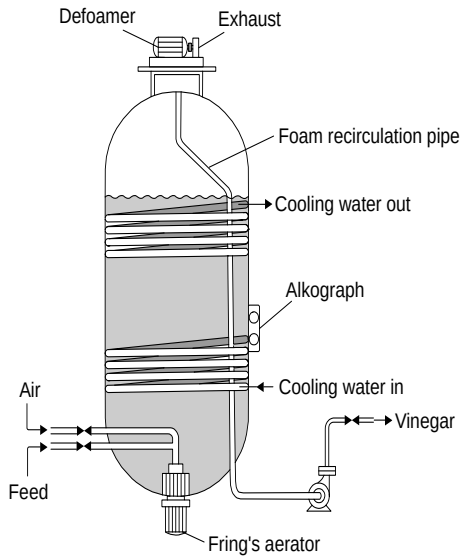


Fig. 16.4 Schematic diagram of Fring's acetator

16.2.4 DOWNSTREAM PROCESSING OF VINEGAR

Vinegar produced from natural raw material (e.g. cereals, fruit juice, etc.) is turbid due to suspended solids. A rest period of several months is therefore recommended for such vinegar. The insoluble materials will precipitate out over this storage period.

In the distilled vinegar, turbidity in the raw form is due to bacteria. The bacteria settle down on standing the vinegar. An aqueous suspension of bentonite may be added to the vinegar for hastening the clarification. This particular step is called *refining*. The upper supernatant is now easy to filter and this is carried out with suspensions of diatomaceous earth. Filtration must remove all suspended materials such as vinegar bacteria or occasionally appearing *vinegar eels*. Simple plate-and-frame filter is satisfactory but membrane ultrafiltration has also been used.

Vinegar is often pasteurized by short heating, e.g., 60-70°C for a few seconds before bottling. In some countries, 50 ppm SO_2 is permitted.

16.2.5 USES OF VINEGAR

- Vinegar is used in the home for the preparation of salads and vegetables.
- In food industry, it is used for the production of pickles, other vegetables, fish, mustard, mayonnaise, and salad dressings.
- Occasionally, it is also used as antiseptic. The acid restricts the spoilage microflora such as yeasts, molds, and lactobacilli. The mold *Moniliella acetoabutens* is very resistant to acetic acid, though.

It is well established that, although addition of strong acids has a more pronounced effect on pH *pro rata*, they are less inhibitory than weak lipophilic acids (e.g., acetic- and lactic acid) at the same pH. This is because microbial inhibition by weak acids is not solely due to the creation of a high extracellular proton concentration, but is also directly related to the concentration of undissociated acid (Fig. 16.5).

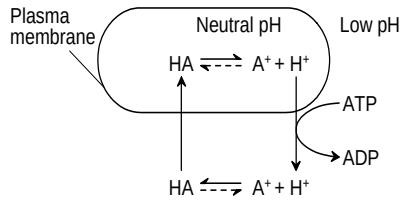


Fig. 16.5 Microbial inhibition by weak organic acids

Unlike protons and other charged particles, undissociated lipophilic acid molecules can pass freely through the membrane; in doing so they pass from an external environment of low pH (where the equilibrium favors the undissociated molecule) to the high pH of the cytoplasm (around 7.5 in neutropliles). At this higher pH, the equilibrium shifts in favor of dissociated molecule, so the acid ionizes producing protons which will tend to acidify the cytoplasm and break down the pH component

of the proton motive force. The cell will try to maintain its internal pH by expulsion of the protons leaking in but this will slow growth as it diverts energy from growth-related functions. If the external pH is sufficiently low and the extracellular concentration of acid high, the burden on the cell becomes so great, the cytoplasmic pH drops to a level where growth is no longer possible and the cell eventually dies.

16.2.6 DIFFERENTIATION OF SYNTHETIC AND NATURAL VINEGAR

Artificial (synthetic) vinegars are made by diluting and coloring concentrated acetic acid. They are also labeled *non-brewed* condiment. Biologically produced vinegar can be distinguished from artificial vinegar by measuring the ^{14}C content with a scintillation counter. The basis is that fossil fuels have lower ^{14}C content.

16.2.7 MICROBIAL SPOILAGE OF VINEGAR

Sometimes, undesirable strains of *Acetobacter*, e.g., *Acetobacter acetii* sub-species *xylinum*, can cause sliminess. Other organisms such as film yeasts, molds, and algae may sometimes cause problem by oxidizing acetic acid and/or producing foreign flavors.

CHAPTER 17

MICROBIAL PRODUCTION OF CHEMOTHERAPEUTIC AGENTS

17.1 INTRODUCTION

Despite being diverse, microorganisms exist together. This association is termed *symbiosis*. Basically, microbial associations are of three types: (i) positive, (ii) neutral, and (iii) negative. The last association can be further subdivided into (a) competition, (b) predation, (c) parasitism, and (d) antagonism. Antagonism is a classical example of antibiosis. Antagonism in microorganisms refers to the phenomenon whereby one microorganism adversely affects the environment of the other by killing, injuring, or inhibiting growth.

Antibiotics are a special category of chemotherapeutic agents that are administered to fight infections (usually microbial) of humans and animals. Although the classical definition of antibiotics associates it with secondary metabolite, the term *antibiotic* today includes many similar but chemically synthesized chemotherapeutic compounds also. Over 8000 antibiotics are known and several hundred discovered yearly. Nearly 3000 antibiotically active substances have been detected in lichens, algae, higher animals, and plants. Each year about 300 new antibiotically active substances are detected of which 30-35% are secondary components from fermentation with known antibiotics. Of the large number of known antibiotics of microbial origin, only 123 antibiotics are currently being produced by fermentation. In addition, some 50 antibiotics are produced as semi-synthetic antibiotics. Three antibiotics, *viz.*, *phosphonomycin*, *pyrrolnitrin* and *chloramphenicol* are produced completely synthetically. It generally takes 7-10 years of time and investment of 40-80 crores of rupees for a new antibiotic to touch market from its first discovery in laboratory. Bacteria as well as fungi elaborate antibiotics. Some of the common examples are given in Table 17.1.

Not all antibiotics qualify as chemotherapeutic agent. Some of the fundamental requirements of an antibiotic to be recognized as a chemotherapeutic agent are:

1. *Wide spectrum:* it must be active against a wide range of pathogens
2. *Prevent the development of resistant forms:* pathogens should not easily gain resistance to the antibiotic in question
3. *Selective nature:* it must act only against the target and not the host organism
4. *Not disturb the normal gut flora when orally administered*

Table 17.1 Examples of organisms capable of producing antibiotics

Organism type	Organism	Antibiotic	Property	Use
Fungi	*Penicillium chrysogenum*	Penicillin	[a]G+	Pneumonia, pharyngitis
	Penicillium griseofulvum	Griseofulvin	Antifungal	Skin and hair lesions
	Cephalosporium acremonium	Cephalosporin	G+and [b]G−	[c]UTI, pneumonia
Bacteria	*Bacillus polymyxa*	Polymyxin	Antitumor	UTI, gastroenteritis
	Streptococcus lactis	Nisin	G+	Non-medical use
Actinomycetes	*Streptomyces griseus*	Streptomycin	G+ and G−	Tuberculosis, UTI
	Streptomyces aureofaciens	Tetracycline	G+ and G−	UTI, cholera
	Streptomyces erythrus	Erythromycin	G+	Cholera
	Streptomyces norsei	Nystatin	Antifungal	Skin lesions
	Micromospora purpurea	Gentamicin		UTI, abscess

[a]G+: active against Gram positive organisms; [b]G−: active against Gram negative organisms; [c]UTI: active against urinary tract infection

17.1.2 CLASSIFICATION OF ANTIBIOTICS

Antibiotics can be classified in several ways. The important schemes for the classification are given in Table 17.2. Table 17.3 gives the addresses of some pharmaceutical companies that produce antibiotics.

Table17.2 Schemes for the classification of antibiotics

Scheme	Examples of class	Examples of antibiotics
Based on extent of effect	Bacteriostatic	Penicillins
	Bactericidal	Tetracycline
	Fungicidal	Griseofulvin
	Fungistatic	Nystatin
Based on chemical nature	β-lactams	Penicillin, Cephalosporin
	Aminoglycoside	Streptomycin
	Tetracycline (Anthracycline)	Tetracycline
	Polypeptide	Gramicidin

continued….

….. continued

Target	Antiviral	Interferon
	Antitumor	Nalidixic acid
	Antibacterial	Streptomycin
	Antifungal	Nystatin
Mode of action (the manner in which the effect is manifested)	Inhibition of cell wall synthesis	Penicillin, Cephalosporin
	Inhibition of protein and nucleic acid synthesis	Tetracycline, Streptomycin, Gentamicin, Erythromycin, Chloramphenicol
	Inhibition of specific enzymes	Sulphonamides
	Damage to cytoplasmic membrane	Nystatin

Table 17.3 Pharmaceutical companies that produce antibiotics

Antibiotic	Company	Country
Penicillin G	Hoechst AG	FRG
	Pfizer Inc	USA
	NOVO Industri A/S	Denmark
	Merck & Co	USA
	Sarabhai Chemicals Ltd	India
	Hindustan Antibiotics Ltd	India
Penicillin V	Hoechst AG	FRG
	Pfizer Inc	USA
	NOVO Industri A/S	Denmark
	Glaxo Laboratories	UK
Semi-synthetic penicillins	Pfizer Inc	USA
	Merck & Co	USA
	CIPAN	Portugal
	Bristol Myers Co	USA
Streptomycin	NOVO Industri A/S	Denmark
	Pfizer Inc	USA
	Glaxo Laboratories Ltd	UK
	Merck & Co	USA
	Sarabhai Chemicals Ltd	India
Tetracyclines	CIPAN	Portugal
	Pfizer Inc	USA
	Hindustan Antibiotics	India
	Hoechst AG	USA

17.2 STREPTOMYCIN

Streptomycin is an *aminoglycoside* antibiotic produced by selected strains of *Streptomyces griseus*. The antibiotic works by inhibiting the synthesis of DNA and proteins. It was first isolated in 1944.

17.2.1 CHEMISTRY

It is basic in nature, with solubility in water at the rate of 20 g/liter. It is stable to pH changes. It can withstand boiling temperature. Being a base, streptomycin is usually produced as salt, normally of HCl and sulfate. One unit of streptomycin is equal to 1 μg of free base.

Streptomycin is composed of 3 subunits: (i) *aminocyclitol* (= streptidine, which is an inositol derivative), (ii) L-*streptose*, and (iii) N-*methyl*-L-*glucosamine*. See Fig. 17.1 for the general structure of streptomycin. Some of the common examples of this antibiotic and the respective R groups are shown in Table 17.4. Streptidine is most accurately characterized as a guanidinocyclitol (rather than aminocyclitol).

Dihydrostreptomycin is a semi-synthetic streptomycin. It is produced industrially by catalytic reduction of streptomycin. It is more suitable than streptomycin.

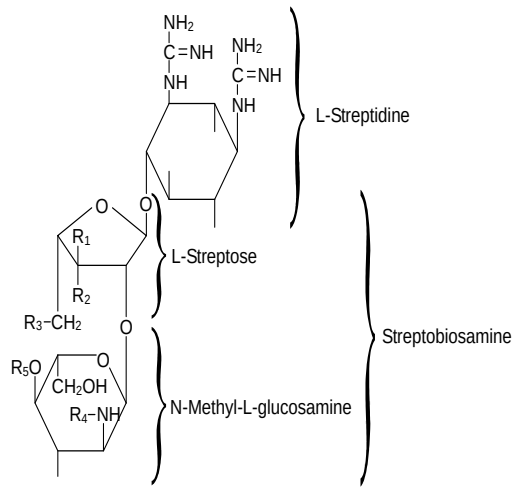


Fig. 17.1 General structure of streptomycin

17.2.2 USES

The antibiotic is used in the treatment of *tuberculosis*, *urinary tract infection*, and *systemic infection* by Gram positive bacteria, and against bacteria that have gained resistance to penicillin. Non-medical uses include preparation of selective media, in cloning experiments, and as laboratory standard for quantitative analysis of streptomycin.

Table 17.4 Some streptomycins and their R groups

Antibiotic	R group				
	R_1	R_2	R_3	R_4	R_5
Streptomycin	CHO	OH	H	CH_3	H
Hydroxystreptomycin	CHO	OH	OH	CH_3	H
Dihydrostreptomycin	CH_2OH	OH	H	CH_3	H
Deoxydihydrostreptomycin	CHO	H	H	CH_3	H
Mannosidostreptomycin	CHO	OH	H	CH_3	M*

M*: Mannose moiety

17.2.3 LIMITATIONS

The antibiotic exerts a *neurotoxic* reaction upon prolonged use. It can lead to hearing loss and loss of balance (that is, it is *ototoxic*). Streptomycin may sometimes damage kidney also. *Dihydrostreptomycin* has lesser side effects than streptomycin.

The drug may lead to development of streptomycin-resistant forms of pathogen. It is therefore advisable to use the drug along with *p-aminosalicylic acid* or *isoniazid*. The resistant forms of microorganisms can develop through mutation giving rise to: (i) strains with chromosomal resistance (immune to streptomycin), and (ii) strains with enzymes capable of inactivating the antibiotic.

Medical use of streptomycin has diminished in the recent decades due to widespread use of other aminoglycoside antibiotics (e.g., gentamicin, tobramycin) and is now generally reserved for medical treatment (via intramuscular injection) in combination with other antibiotics (e.g., penicillin).

17.2.4 MODE OF ACTION

All aminoglycosides affect protein synthesis. The target of this antibiotic is the 30*S* subunit of the 70*S* ribosome of the prokaryotes. It strongly inhibits initiation and elongation of peptide chains. It also causes misreading of *m*RNA thereby leading to insertion of wrong amino acids (and therefore production of faulty polypeptides). Finally, under the influence of streptomycin, some molecules of nucleic acids (e.g., *r*RNA, *t*RNA, and denatured DNA) also act as *m*RNA although they ordinarily do not have this property. Accumulated evidence shows S_{12} (a small ribosomal protein of 30*S* subunit) is the ultimate target. See Fig. 17.2 for the explanation.

The notations P and A in Fig. 17.2 refer to *peptidyl*-and *aminoacyl* site respectively. 30*S* and 50*S* refer to physical characteristic of prokaryotic ribosome. The suffix *S* refers to sedimentation coefficient expressed in *Svedberg* unit. Prokaryotic ribosome (70*S*) can be broken down into 30*S* and 50*S* subunits. The 30*S* subunit contains 21 small proteins designated s_1, s_2,…s_{21}. Each of these proteins has very important functions in protein synthesis and some of them are actually enzymes. The 50*S* subunit contains 33 large proteins, designated L_1, L_2, L_3….L_{33} (see page 57 also).

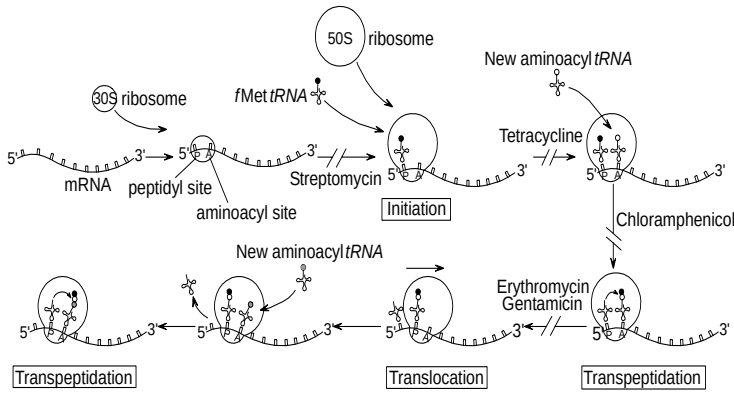


Fig. 17.2 Inhibition of protein synthesis by antibiotics

17.2.5 BIOSYNTHESIS OF STREPTOMYCIN

Study in cell-free system shows involvement of several enzymes for the synthesis of streptomycin. All the three subunits of streptomycin (Fig. 17.1) originate from glucose but entail distinctly different pathways. According to available experimental evidence the assembly of the streptomycin molecule can be envisaged to include the following major steps:

1. Transfer of dihydrostreptose to streptidine-6-phosphate to form dihydrostreptose streptidine-6-phosphate
2. Transfer of N-methyl-L-glucosamine to the pseudodisaccharide with the formation of dihydrostreptomycin-6-phosphate
3. Oxidation of dihydrostreptomycin-6-phosphate to streptomycin-6-phosphate
4. Hydrolysis of streptomycin-6-phosphate to streptomycin

The final intermediate of the pathway, streptomycin phosphate, is biologically inactive but becomes active following removal of the phosphate group. See Fig. 17.3 for the outline of biosynthesis.

Many organisms synthesize *mannosidostreptomycin* (which has much lower antibiotic activity and is therefore undesirable) before the actual formation of streptomycin. This is not an obligatory intermediate, though. In the course of synthesis, mannosidostreptomycin is degraded by the organism's own enzyme *mannosidostreptomycinase* to yield streptomycin. In a typical fermentation, the concentration of mannosidostreptomycin can reach up to 40%.

Biosynthesis of streptomycin is regulated by an inducer called *A-Factor* (γ-butyrolactone). In *Streptomyces griseus*, A-Factor is an autoregulatory factor that at extremely low concentration triggers streptomycin biosynthesis and cell differentiation by binding a repressor-type receptor protein. The antibiotic is synthesized in the idiophase and this occurs only after A-Factor has reached a *critical* concentration.

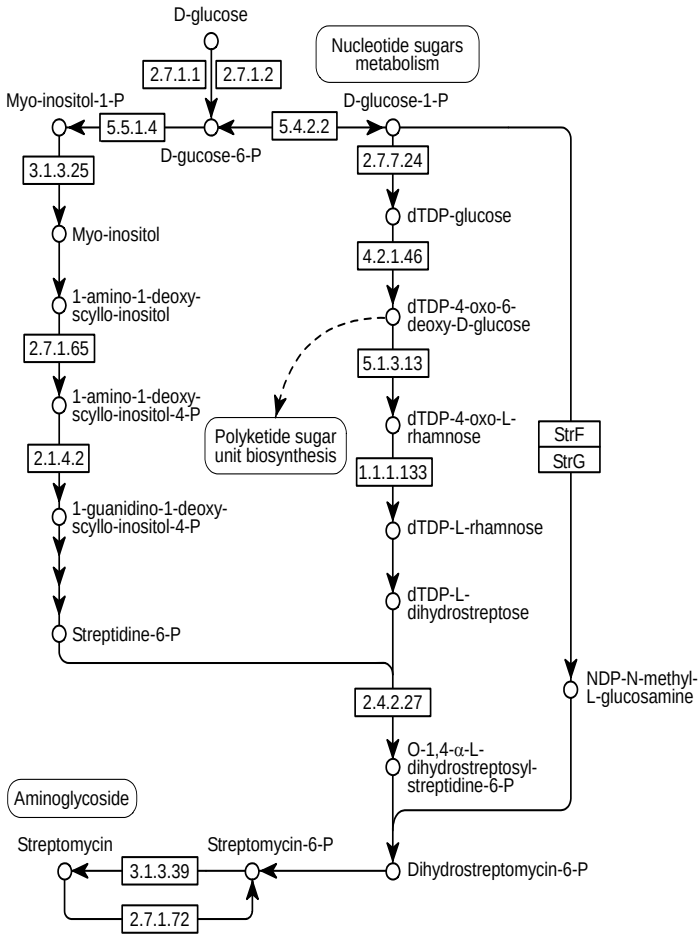


Fig. 17.3 Biosynthesis of streptomycin

2.7.1.1 = hexokinase; 2.7.1.2 = glucokinase; 5.5.1.4 = inositol 3-P-synthase; 3.1.3.25 = inositol-P-phosphatase; 2.7.1.65 = scyllo-inosamine kinase; 2.1.4.2 = inosamine-P-amidotransferase; 5.4.2.2 = phospho-glucomutase; 2.7.7.24 = dTDP-glucose synthase; 4.2.1.46 = dTDP-glucose-oxo-reductase; 5.1.3.13 = dTDP-L-rhamnose synthetase; 1.1.1.133 = dTDP-4-dehydrorhamnose reductase; 2.4.2.27 = dihydrostreptosyl transferase; 3.1.3.39 = streptomycine-6-phosphatase; 2.7.1.72 = streptomycin kinase; StrF and StrG = streptomycin biosynthesis proteins; dTDP = deoxythymidine diphosphate.

17.2.6 GENERAL PRODUCTION METHOD

17.2.6.1 Microbial strain

Improved strains of *Streptomyces griseus* are used for the industrial production of streptomycin. From Walksman's discovery up to the present day, the productivity of *Streptomyces griseus* has increased by over 100 fold. Classical mutation programs are used for the improvement of the strains.

17.2.6.2 Culture medium

Glucose is the carbon source of choice. This has to be so because glucose is the precursor of streptomycin. The preferred nitrogen source is soybean flour meal. Ever since Rake and Donovick's experiment with soybean flour, no better nitrogen source has been found. Minerals are automatically inclusive because of the complex nature of the medium. Sodium chloride is always thought necessary, though. A typical composition of the medium is given in Table 17.5. It has been shown that streptomycin production is inhibited by phosphate concentration as low as 1.5×10^{-2} M. So, phosphate is not normally added in complex media containing glucose.

Table 17.5 Typical composition of production medium for streptomycin

Component	Amount (g/L)
Glucose	60 + (10)[a]
Soybean meal	30
Cornsteep (100%) solids	4
$(NH_4)_2SO_4$	9 + (1.5)[a]
NaCl	2.5
KH_2PO_4	0.025
$CaCO_3$	0.5
Soybean oil	7

Figure in parenthesis with a superscript (-)[a] denotes complementary addition during main fermentation. This means that the above composition can also be used for inoculum build-up.

17.2.6.3 Production

The inoculum is built up in a stepwise manner at 27°C. The process starts with the plate-culturing of lyophilized spore cultures in soy flour agar medium. Incubation is done at 27°C for 2-3 weeks. The spores are then transferred to a shaker flask. After growth for some time the contents are again transferred to propagator for biomass build-up. The medium is sterilized as usual. The fermenter is inoculated at the rate of 5-10% vol/vol. The process is aerobic. Inadequate supply of air (O_2) leads to accumulation of lactate and pyruvate, which is undesirable. The pH is maintained at around 7 and the main fermentation is carried out at 27°C.

The fermentation is triphasic. Trophophase lasts for 24 hrs. The pH increases because of preferential utilization of soybean meal. Growth and concomitant accumulation of A-Factor (also written, Factor A) is also rapid. Idiophase lasts for 2-7 days during which streptomycin is rapidly synthesized. Glucose utilization is very rapid. The third phase marks the cessation of antibiotic synthesis. Cells begin to lyse, and pH rises due to NH_3 liberation. Harvesting is done before the third phase commences. The yield is about 1200 μg/ml.

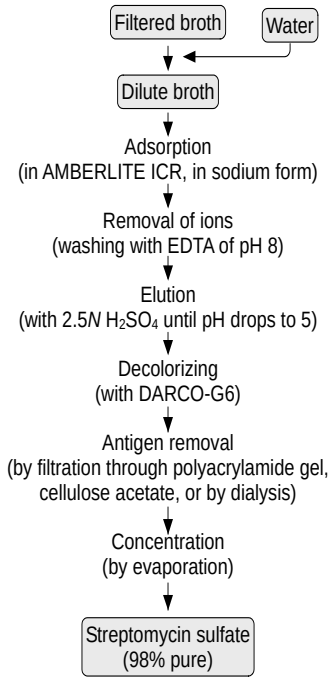


Fig. 17.4 Recovery of streptomycin

17.2.6.4 Recovery

The broth (*beer*) is filtered in rotary vacuum filter to remove mycelia (Fig. 17.10a and 17.10b). Water is added to the liquor in the ratio 1:1 and passed through adsorption column. Through the same column, EDTA solution is passed to remove metal ions. The adsorbed, pure streptomycin is eluted from the column with 2.5 *N* H_2SO_4. See Fig. 17.4 for the outline of recovery process. Further processing entails decolorizing with carbon, antigen removal by filtration, concentration, and drying. The final product is either sulfate- or hydrochloride salt of streptomycin. The purity will be of the order of 98%.

During fermentation, streptomycin can be found both in the culture fluid and bound to the mycelia. The bound antibiotic is released from the cell walls by treatment with acids, alkali, or ionizable salts or sonication.

17.3 PENICILLIN

Penicillins are a class of *β-lactam* antibiotics of related structure with slightly different properties and activities. They are produced by *Penicillium* molds. All penicillins have a common nucleus called *6-aminopenicillanic acid* (6-APA). 6-APA (Fig. 17.5) is a fused β-lactam-*thiozolidine* ring. The side chain attached to this ring gives each penicillin its unique characteristic.

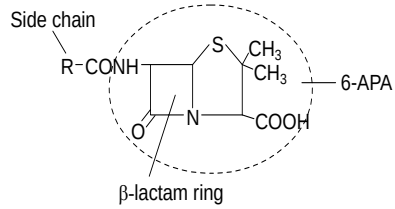


Fig. 17.5 6-aminopenicillanic acid

17.3.1 THE ORGANISM

Although many *Penicillium* molds are capable of producing penicillin, not many of them are of commercial value. The trade fermentation today employs mutated strains of *Penicillium chrysogenum* Wisconsin Q 176. See Fig. 17.6 for the genealogy.

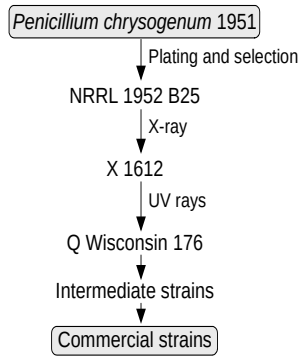


Fig. 17.6 Genealogy of commercial strains of *Penicillium chrysogenum*

17.3.2 CLASSIFICATION OF PENICILLINS

Penicillins can be classified on two main bases: (i) *application point of view*, and (ii) *production point of view*. From application point of view, penicillins can be divided into 4 categories:

1. *Penicillin G type:* These are used to combat streptococcal and staphylococcal diseases
2. *Ampicillin and its relatives:* These are active against Gram-positive- and some Gram-negative bacteria
3. *Penicillinase-resistant types:* These are used to combat bacteria that have developed resistance to penicillin G
4. *Extended-spectrum antibiotics:* These are active against pseudomonads (a group of proteolytic bacteria)

From production point of view, penicillins can be categorized as (i) *natural penicillins*, (ii) *biosynthetic penicillins*, and (iii) *semi-synthetic penicillins*.

1. Natural penicillins

Penicillins obtained by fermentation in the usual medium are called natural penicillins. The side chain, R, comes from precursors present in the fermentation medium. Some of the common natural penicillins are penicillin-G, V, X, F, K, and dihydropenicillin. Penicillin G and V are by far the most important natural penicillins (See Fig. 17.7). Since the fermentation medium is complex in composition, a wide variety of precursors may be available to the organism. This can result in several chemically different penicillins in a single fermentation.

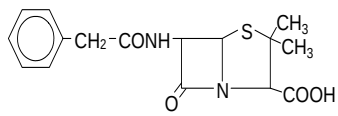
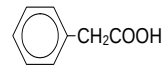
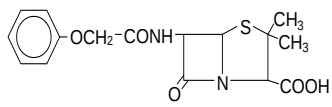
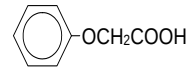
Penicillin	Structure	Precursor
Penicillin G (benzyl penicillin)		 Phenyl acetic acid
Penicillin V (phenoxypenicillin)		 Phenoxy acetic acid

Fig. 17.7 Chemical structure penicillin G and V

2. Biosynthetic penicillins

Biosynthetic penicillins are in fact natural penicillins. The only difference is in the manipulation of fermentation so that the desired type of natural penicillin is obtained in large amounts. In essence, the production of biosynthetic penicillins entails external addition of the precursors of side chain so that the fermentation becomes more selective. For example, addition of *phenoxyacetic acid* during natural fermentation produces penicillin V in large amounts.

3. Semisynthetic penicillin

Production of semisynthetic penicillin entails fermentation as well as enzymatic and/or chemical steps (discussed earlier, page 133). The 6-APA is produced by fermentation and the side chain is later added by chemical reaction.

17.3.3 USES OF PENICILLIN

Penicillin finds wide use in medical as well as other fields. Its therapeutic application in general includes treatment of syphilis, gonorrhea, meningitis, anthrax, pneumonia, and pharyngitis. The side effects are relatively rare but can include immediate or delayed allergic reactions – specifically skin rash, fever, and less frequently, *anaphylactic* shock. Anaphylactic shock refers to allergic reaction in which the release of *histamine* can be widespread, leading to edema. In extreme cases, heart and circulatory failure can occur.

Penicillins also find wide use in cloning experiments, especially for selecting clones on the basis of *reporter* or *marker* genes. In general microbiology laboratory, it is used for *auxanography* and for the preparation of selective media.

17.3.3 PROPERTIES OF PENICILLIN

Penicillins are markedly sensitive to pH, temperature and *penicillinase* enzyme. The sodium and potassium salts are freely soluble in water and alcohol but only slightly soluble in benzene and chloroform. They are colorless in crystalline form.

17.3.5 BIOSYNTHESIS OF PENICILLIN

Penicillin is synthesized as a classical secondary metabolite from three amino acid precursors, viz., L-α-aminoadipic acid, L-cysteine, and L-valine. L-α-aminoadipate is an intermediate of the L-lysine biosynthetic pathway and is also provided by catabolic conversion of L-lysine. However, the importance of the catabolic degradation of lysine for penicillin has not been clarified yet.

Starting from these amino acids, penicillin biosynthesis is catalyzed by 3 enzymes, namely: (i) δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthase, denoted by ACVS, (ii) isopenicillin N synthase, denoted by IPNS, and (iii) Acyl-SCoA: isopenicillin N acyl transferase. The outline of penicillin synthesis (Brakhage, 1997) is shown in Fig. 17.8.

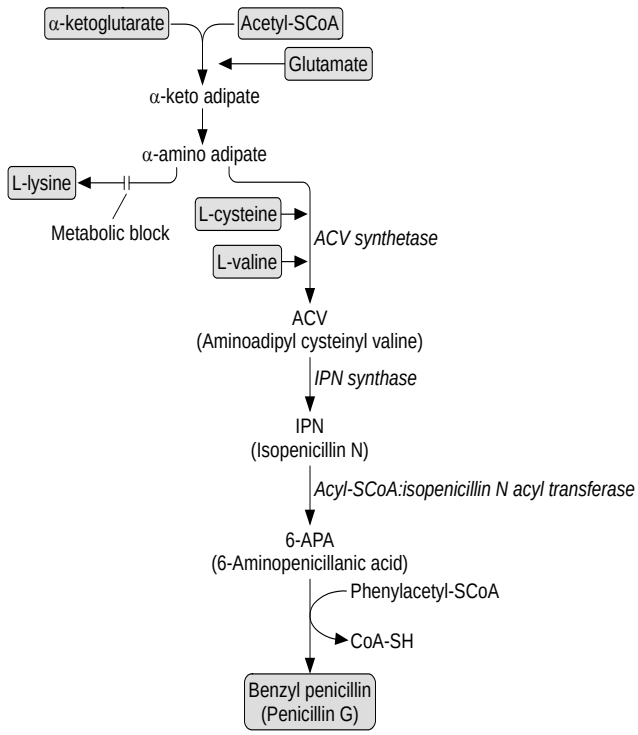


Fig. 17.8 Penicillin biosynthesis by aminoadipate pathway

Thus, in *Penicillium chrysogenum*, L-lysine and penicillin share the same biosynthetic route up to *a-aminoadipic acid*. The mutated strains can be supposed to be *lysine auxotrophs*. Alpha aminoadipic acid route is one of the two routes used by

microorganisms for the synthesis of lysine. The other route, called *diaminopimelic acid pathway*, is the main pathway used by commercial strains of lysine-producing bacteria.

In *P. chrysogenum*, L-lysine is known to be a potent inhibitor of penicillin synthesis. Since L-α-aminoadipic acid is a branch point between the lysine and penicillin biosynthetic pathway, lysine inhibition seems to operate at one or more steps of lysine pathway.

17.3.6 MODE OF ACTION

Gram-positive bacteria, particularly *Staphylococcus aureus,* are sensitive to penicillin. The antibiotic works by inhibiting biosynthesis of peptidoglycan of the bacterial cell wall: it obstructs the cross-linkage between *N-acetyl muramic acid* and *N-acetyl glucosamine*. The antibiotic also interferes with the interpeptide cross-links. The bacterial growth is arrested as a result of weakened cell wall. See Fig. 17.9 for the mode of action.

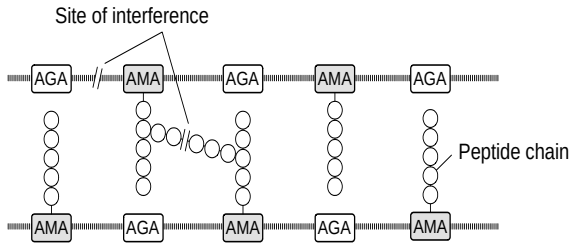


Fig. 17.9 Mode of action of penicillin on cell wall of Gram-positive bacteria

The notations AMA and AGA refer to N-acetyl muramic acid and N-acetyl glucosamine, respectively. In particular, the antibiotic inhibits the enzymes *transpeptidase*, *transglycosylase*, and *D-D-carboxypeptidase*.

17.3.7 PRODUCTION OF NATURAL AND BIOSYNTHETIC PENICILLINS

17.3.7.1 Medium consideration

The medium formulation depends on whether the fermentation is carried out by surface liquid culture method (classical) or the fed-batch culture method (modern).

In the classical method (which is still used in many places), lactose is the universally preferred carbon source. Glucose is also included in the medium but this is primarily for mycelial growth. Abundant growth is very essential for the main fermentation. Glucose is a readily assimilable carbon source and so the organism does not use it for the production of penicillin, which is a secondary metabolite. The production of penicillin occurs in the *idiophase*. The biosynthesis is the result of depletion of one or more nutrients. The mold produces penicillin only when it is forced to live on lactose (which is not readily metabolizable). Stated differently, the organism should experience a condition of *starvation* for the penicillin to be synthesized. A typical fermentation medium for batch fermentation of penicillin is given in Table 17.6.

Cornsteep liquor (CSL) is universally used as nitrogen source. Nitrogen is often supplied as NH_3 because the cornsteep nitrogen may not be adequate. CSL also contains growth factors, minerals, and above all, precursors of side chains. CSL is a concentrated form of steepwater in which corn has been steeped for the manufacture of starch, gluten, and other corn products. CSL contains 50% solids. Although the composition is highly variable, the concentrations of total nitrogen, lactic acid, amino nitrogen, reducing sugars and ash are around 7.4-7.8%, 12-27%, 2.6-3.3%, 1.5-14%, and 18-20%, respectively (on dry basis).

Table 17.6 A typical fermentation medium for penicillin production

Components	Amount (%)
Cornsteep liquor	3.5
Lactose	3.5
Glucose	1
$CaCO_3$	1
KH_2PO_4	0.4
Edible oil	0.25
Penicillin precursor	--

For the production of biosynthetic penicillins, the desired precursors must be added externally. The addition is continuous and in regulated amounts (0.5-1.5 g/liter). Usually, the uptake of precursors is 80-90% because the attachment process does not take place with absolute specificity. Addition of a good amount of precursor is desirable for directing the synthetic reaction towards the desired direction but the toxicity of excess precursor that may result on the microorganisms must also be seriously considered. This calls for regulated feeding of precursors.

17.3.7.2 Fermentation

Following sterilization, the medium is transferred to the main fermenter, which may be as big as 250 m^3 in capacity. The initial pH is kept at 6.5. Inoculum is built up the normal way and 10-20% vol/vol is added to the main fermenter. The pure culture (which normally comes as lyophilized spores) is initially grown in special sporulation agar. This is then transferred to shaker flask, which contains a supplement of 2% sucrose. Incubation is done at 25°C for some days. The culture is then transferred to propagator of considerable size. Aseptic- and aerobic fermentation ensues here also. The main fermenter may be inoculated with either spores or mycelium. The methods of inoculum build up, as also the method of inoculation, may vary here. A spore concentration of about 5×10^5/ml of medium has been used in various studies.

Classical fermentation

In the classical method, the main fermentation is triphasic in nature. During the active growth phase, glucose and cornsteep components are rapidly utilized. The pH remains constant. At the end of this phase, however, glucose and cornsteep

components deplete. Ammonia is liberated due to deamination reaction. Consequently, pH rises to 7-7.5. This pH is optimum for the synthesis of penicillin. The antibiotic is very sensitive to pH changes. The latter can be controlled by adding suitable amounts of H_2SO_4 or $CaCO_3$. The late growth phase is followed by idiophase, the phase in which penicillin synthesis occurs. Growth ceases in this phase because the nutrients have depleted. The organism begins to utilize lactose. This condition forces the mold to synthesize penicillin. Care must be taken not to allow the late idiophase to proceed too far, as this leads to cell lyses and consequent degradation of penicillin.

The duration of fermentation is typically 6 days. Since the organism is aerobic, aeration should be done at the rate of 0.5-1 vol/vol/min. Turbine agitators are used to facilitate aeration because the rheology of the medium becomes very complex as the mycelial growth progresses. Precursors are batched or added constantly in regulated amounts. The fermentation is normally carried out at 26°C.

During fermentation, periodic checks must be carried out for penicillin yield and contamination. The penicillin yield (harvest titer) for a typical fermentation is about 40-50 g/liter.

Fed-batch fermentation

Today, trade fermentations are carried out in fed-batch mode, and lactose is replaced by sucrose, glucose, or other crude sugars. The process involves 2-3 initial seed growth phases followed by a fermentation production phase having a time cycle ranging from 120 to 200 hrs. High dissolved O_2 levels are crucial, especially during the peak growth period that occurs at 40-50 hr time-period cycle.

About 65% of the carbon is metabolized for cellular maintenance, 20-25% for growth, and 10-12% for penicillin production. Sugar and precursors are fed continuously and the sugar is also used to help regulate the pH of the fermentation between 6.4 and 6.8 during active penicillin fermentation phase.

Cornsteep liquor and cottonseed- or soybean meal, ammonia and ammonium sulfate represent the major nitrogen sources. The essential precursors are phenylacetic acid (for penicillin G) and phenoxyacetic acid (for penicillin V) that are either fed or batched.

Mini-harvest protocols are often used in penicillin fermentations. This "batch-fill and withdraw" system involves the removal of 20-40% of the fermenter contents with replacement with fresh sterile medium. This procedure can be repeated several times during the fermentation without yield reduction and, in reality, can enhance the total penicillin yield per fermenter. Thus, although a single batch can be complete in about 50 hrs, the extended culture protocol requires a time cycle of 120-200 hrs.

17.3.7.3 Harvesting

Harvesting is done before the mycelia begin to lyse. The mycelia are filtered in rotary vacuum filter (Fig. 17.10a and 17.10b). Washing is done repeatedly to elute the antibiotic. Filter aids such as *hyflow* may be used during the filtration. The broth is cooled promptly to 0-4°C to minimize enzymatic- and chemical degradation of the antibiotic.

17.3.7.4 Purification

Penicillin can be purified by two methods, *viz*., (a) *carbon process*, and (b) *solvent extraction*. The carbon process is an obsolete process. Solvent extraction is now used for purification/concentration of penicillin. This latter method is thus an industry standard.

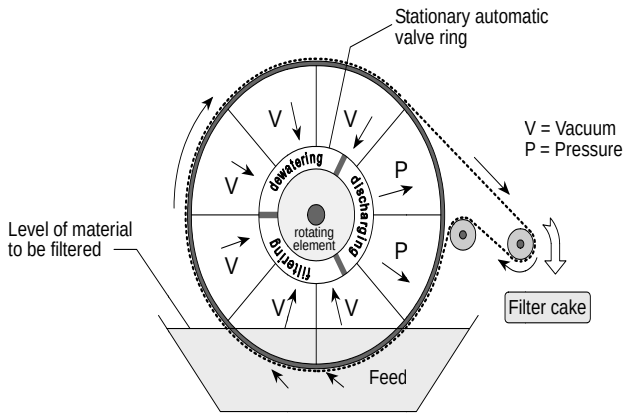


Fig. 17.10a Rotary vacuum filter of mycelial broth

Fig. 17.10b Industrial-scale rotary vacuum filter

Penicillin is very sensitive to temperature and pH. At pH 2 and 20°C, the half-life is only 15 min. It is therefore very important that extraction cycles be very fast. In practice, each extraction cycle takes not more than 60-90 sec.

The temperature during the extraction is maintained at 0-3°C. The most common solvents used for the extraction are amyl- and butyl acetate. Basically the process entails extraction with organic solvent followed by back-extraction in alkaline aqueous phase.

To begin with, the pH of the penicillin broth is adjusted to 2.5-3.5 with H_3PO_4 or H_2SO_4 (dilute, 10% vol/vol). Emulsifiers may be added at the rate of 0.003-0.1% in the organic solvent. At this pH, penicillin is preferentially soluble in the solvent.

The extraction takes place in continuous, countercurrent, multistage, centrifugal extractor (Podbielniak D-36 is normally used). The ratio of broth to solvent is kept at around 5-7:1. A single extraction will produce 5-7 fold concentration and the concomitant 5-10% loss of penicillin.

This extraction in organic solvent is immediately followed by back-extraction in water. Water is added to the penicillin-rich solvent in the ratio water:solvent = 0.1-0.2. The pH of the mixture is adjusted to 5-7.5 by adding NaOH or KOH in the water. At this pH, penicillin shifts its selectivity towards water. The resulting aqueous extract is rich in Na- or K-salt of penicillin. At neutral pHs in water, penicillin is ionized. In acid conditions this ionization is suppressed and the penicillin is more soluble in solvents.

The extract is again dissolved in organic solvent by adjusting the pH to 2.5-3.5. This time the amount of solvent is reduced. This is again followed by aqueous extraction. The cycle is repeated until the required degree of concentration is achieved. The final extract will necessarily be an aqueous extract of Na- or K-salts of penicillin. The spent solvent is recovered for reuse.

The resulting aqueous solution is treated with 0.25-0.5% carbon to remove pigments and other impurities. The solution is subjected to filtration to remove trace impurities, bacteria, and pyrogens. Crystallization is done by salting out using sodium acetate as the salt. The crystals are washed with water and dried in anhydrous isopropanol, butanol, etc. The final drying is done in warm air, vacuum, radiant heat, or large horizontal bed driers.

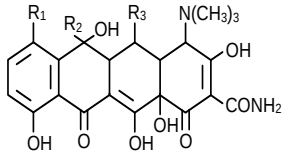
The penicillin thus produced must conform to the FDA standards. The strength and dosage of penicillin is expressed in terms of international units. Each of these units is equal to 0.0006 g of the crystalline fraction of penicillin G.

The yield from modern strains can be as high as 40-50 g/liter. The loss figure (during handling, filtration, extraction, crystallization, drying) is about 22%.

Semi-synthetic penicillins are produced by a combination of microbial and enzymatic or chemical process. 6-APA is the principal intermediary in the manufacture of semi-synthetic penicillins. See immobilized enzyme (Section 10.19.2, page 133) for a full treatment of the topic. Approximately 75% of the total bulk penicillin is used for the production of semi-synthetic penicillins and cephalosporins.

17.4 TETRACYCLINE

Tetracyclines are a group of broad-spectrum antibiotics which are closely related chemically and very similar in biological action. They all have hydronaphthacene skeleton. They can be prepared microbiologically as well as chemically. Some well-known members of this family are given in Table 17.7. The chemical structure is given in Fig. 17.11. Tetracycline has low toxicity and good oral absorption. It is bacteriostatic and requires high dosage. This antibiotic is used in the treatment of shigellosis, salmonellosis, typhoid fever, brucellosis, etc. It is also used in feed to eliminate parasites (and thus help gain weight in animals). The antibiotic also finds use in the preservation of fish (the ice in which the fish is kept is treated with tetracycline).



Antibiotic	R group		
	R_1	R_2	R_3
Tetracycline (TC)	H	CH_3	H
Chlortetracycline (CTC)	Cl	CH_3	H
Oxytetracycline (OTC)	H	CH_3	OH

Fig. 17.11 General structure of tetracycline

Because of the development of strains of microorganisms resistant to the tetracyclines, these antibiotics have lost some of their usefulness. They are no longer the drugs of first choice for treatment of staphylococcal-, streptococcal-, or pneumococcal infections. Nevertheless, they are still useful for the treatment of UTI, trachoma (chronic eye infection), gonorrhea, and Rocky Mountain spotted fever. Tetracyclines can be taken orally or applied topically. The recommended maximum dose is 4 g/day.

Table 17.7 Some members of tetracycline

Antibiotic	Organism
Tetracycline	*Streptomyces aureofaciens*
Chlor(o)tetracycline (= Aureomycin, Biomycin)	*Streptomyces rimosus*
Oxytetracycline (= Terramycin, Geomycin)	*Streptomyces antibioticus*

17.4.1 BIOSYNTHESIS OF TETRACYCLINE

Biosynthesis of tetracyclines, particularly in the latter stages, has been studied with the use of mutant cultures. It has been established that pretetronid (Fig. 17.12) intermediates are involved in the synthesis. These intermediates are converted by *Streptomyces aureofaciens* to tetracyclines.

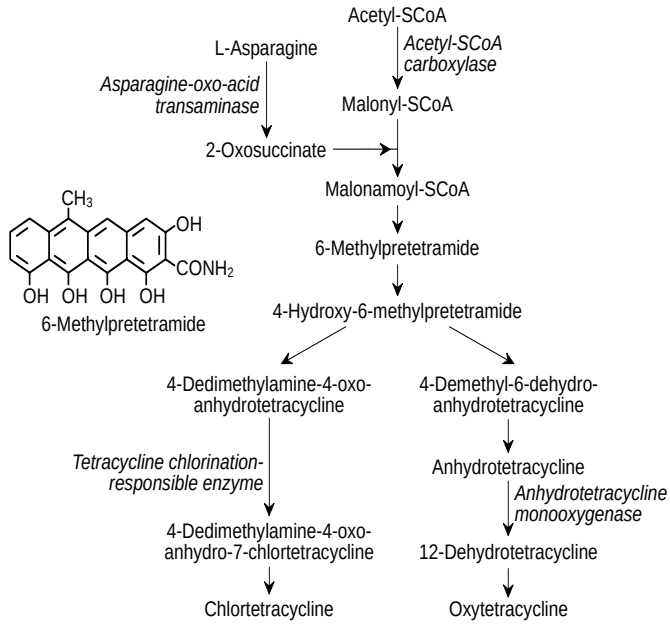


Fig. 17.12 Biosynthesis of tetracycline

17.4.1 PRODUCTION OF TETRACYCLINE

Tetracycline can be produced chemically as well as microbiologically. The microbial production of all tetracyclines is similar. Chlortetracycline production, however, is comparatively simpler than the production of other tetracyclines. In particular, production of tetracycline is very sensitive to chloride content in the medium: it leads to the production of chlortetracycline rather than the tetracycline as shown in the examples and figures elsewhere.

17.4.1.1 Fermentation

For trade fermentations, UV mutants of *Streptomyces aureofaciens* are used. The organism comes in the form of lyophilized spores. The inoculum preparation requires several stages. Starting from the spores dried on sand or lyophil vials, one or more shaker flask stages may be used and then one or two inoculum tank stages. The sporulation medium, inoculum build-up medium, and the production medium have different compositions. A summary of the composition is given in Table 17.8.

Table 17.8 Medium used in tetracycline production

	Purpose		
	Spore production	*Inoculum build-up*	*Production*
Component	Meat extract: 2% Asparagine: 0.05% Glucose: 1% KH_2PO_4: 0.05% Agar: 1.3%	CSL: 2% Sucrose: 3% $CaCO_3$: 0.5%	Sucrose: 1% CSL: 1% $(NH_4)_2PO_4$: 0.2% KH_2PO_4: 0.2% $CaCO_3$: 0.1% $MgSO_4.7H_2O$: 0.25% $ZnSO_4.7H_2O$: 0.005% $CuSO_4.5H_2O$: 0.00033% $MnCl_2.4H_2O$: 0.00033%
	CSL = Cornsteep liquor		

During inoculum build-up, the organism remains in the shake-flask for 24 hrs at 28°C. The final propagator uses medium of the same composition. About 5% inoculum is added and propagation carried out for 19-24 hrs at pH 5.2-6.2. The main fermenter receives 2-10% of inoculum from the final propagator. The fermenter has a nominal capacity of 25,000 to 75,000 liters. Fermentation is carried out in sterilized medium (121°C for 1-2 hr). The main fermentation runs for 60-65 hrs at 28°C. The pH is around 5.8-6. It is a submerged fermentation requiring 0.5-2 vol/vol/min of aeration. Agitation is carried out with mechanical agitators. Lard is used as an antifoam. Glucose is generally not used in the main fermentation as this exerts catabolite repression. The yield is around 15000 units per ml.

17.4.1.2 Harvesting and purification

The mycelium in the beer is removed in a rotary vacuum filter (Fig. 17.10a and 17.10b). The liquor can be purified and concentrated in several ways, including adsorption in activated charcoal, solvent extraction (amyl alcohol), etc. An example of extraction is given in Fig. 17.13.

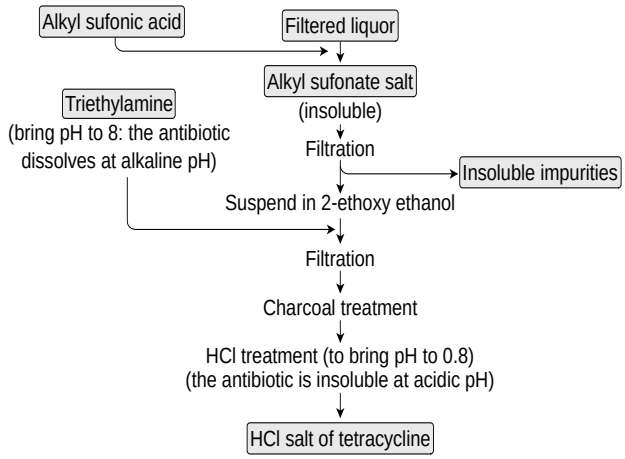


Fig. 17.13 Purification of chlortetracycline

17.4.2 MODE OF ACTION OF TETRACYCLINE

Tetracyclines inhibit a lot of enzyme reactions essential for the vital processes of bacterial cells. The most sensitive biochemical reaction that is inhibited is the synthesis of proteins. Tetracyclines work by binding specifically to the 30S ribosome of the bacteria, preventing attachment of the aminoacyl *t*RNA to the RNA-ribosome complex. It simultaneously inhibits other steps of protein biosynthesis. Tetracycline can also alter the cytoplasmic membrane and this in turn causes leakage of nucleotides and other compounds out of cell. This does not directly kill the bacteria but instead inhibits them.

CHAPTER 18

MICROBIAL PRODUCTION OF FLAVORS AND FRAGRANCE

18.1 INTRODUCTION

Microorganisms are capable of producing a wide range of compounds that have flavor and fragrance value. These compounds can be used in food items as well as non-food items, such as perfumes. Not all of the potential of microorganisms in this regard has been fully tapped. Even for those that have apparently reached commercial success, details are not available. Some of the important flavor and fragrance materials that can be obtained from microorganisms are *diacetyl*, *lactones*, *butyric acid*, etc.

18.2 DIACETYL

Diacetyl (= 2,3-butanedione) is a naturally occurring chemical characterized by a powerful and diffusive odor resembling butter when dilute. It is extensively used in imitation butter and other dairy flavors and in other numerous flavors where butter notes are desirable. Diacetyl also finds limited use in perfumes, primarily in reconstituting essential oils. The chemical structure of diacetyl is shown in Fig. 18.1.

18.2.1 PRODUCTION

Diacetyl can be formed via two different methods. The first one involves chemical synthesis. For example, diacetyl can be synthesized from methyl ethyl ketone by conversion to an isonitroso compound which is then decomposed by hydrolysis with hydrochloric acid to diacetyl.

A second method for producing diacetyl is by bacterial fermentation. For example, glucose can be fermented to methylacetylcarbinol which is then oxidized to form diacetyl.

In the microbial process, diacetyl can be produced from two groups of microorganisms, *viz*., bacterial starters, and bakers- or brewers yeast. When brewers- or bakers yeast is used, the organism is grown aerobically on sucrose. Bacterial process is probably more widely practiced. Good candidates in this group are: *Streptococcus lactis*, *Streptococcus diacetylactis*, and several *Leuconostoc* species. The easiest method for natural diacetyl production is probably from starter culture (lactic cultures from dairy industries). The outline given by Reed and Peppler is shown in Fig. 18.2. Lactose or citrate can be used as the substrate in this process.

Citric acid is a precursor for both diacetyl and acetoin (see Fig. 18.1), and often, the final fermentation product is a mixture of diacetyl and acetoin (= acetyl-methyl-carbinol). In addition to the precursor citrate, the production of diacetyl is enhanced

by a pH below 5.5, low temperature, and aeration. A pH below 5.5 favors citric acid *permease* activity and restricts *diacetyl reductase* activity. Aeration promotes both the formation and accumulation of diacetyl by increasing oxidation-reduction potential of the culture. This results in enzymatic stimulation and spontaneous oxidative decarboxylation of α-acetolactic acid to diacetyl. Addition of humectants (e.g., glycerol and sucrose) to lower the water activity (a_w) to about 0.96 has been found to increase the amount of diacetyl (Toller, 1981).

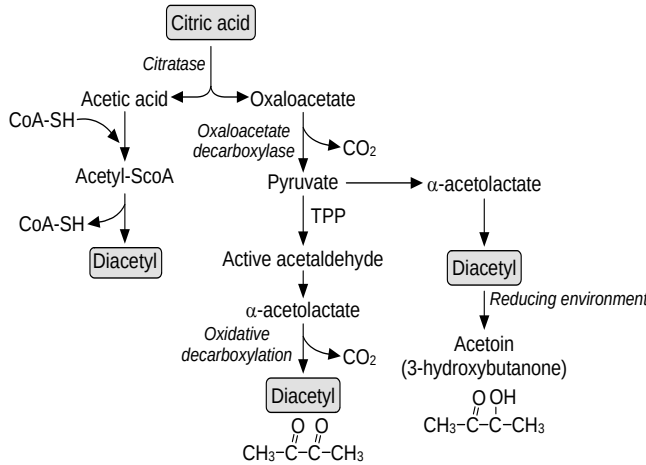


Fig. 18.1 Microbial synthesis of diacetyl

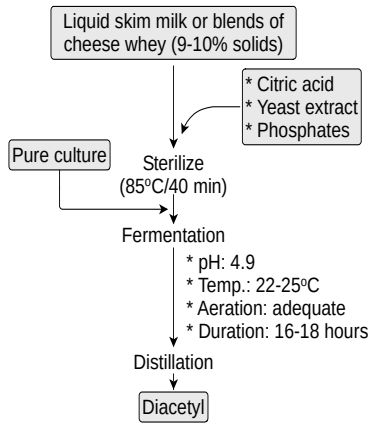


Fig. 18.2 Diacetyl production from starter culture

18.3 LACTONES

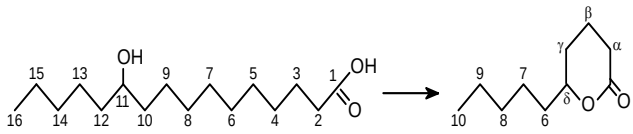
A lactone is a cyclic compound containing an ester group in the ring, and those having 3, 4, 5, 6 and 7 members are referred to as α-, β-, γ-, δ-, and ε-lactones, respectively. Lactones are useful as starting materials or solvents for the synthesis of various compounds, such as pharmaceutical preparations, flavors and fragrance, and

agricultural chemicals, etc. Lactones are ubiquitous in food, contributing to taste and flavor nuances. Numerous odor and taste characteristics have been attributed to lactones. Among these are oily-peachy, creamy, fruity, nut-like, coconut, honey, and so on. γ-octalactone and γ-nonalactone possess coconut aroma and are highly desired by flavorists.

Many strategies are known for synthesis of lactones, including those using chemical methods and fermentation. Because of relevance in industrial microbiology, only the microbial process will be discussed here. The use of microbial process to produce lactones would appear to have advantages over synthetic methods because the former combines into a single step the multiple reactions required by a synthetic method. Moreover, the microbial process would satisfy the desire to obtain flavor and fragrance material from natural sources.

There are several methods (patented or in industrial operation) available for the microbial production of lactones. An example for the production of δ-decalactone is described next.

Boog and coworkers (1993) have described a method for the production of δ-lactone from 11-hydroxy fatty acid or its ester by aerobically culturing *Saccharomyces cerevisiae*. The conversion of 11-hydroxypalmitic acid into δ-decalactone is given below.



11-Hydroxypalmitic acid δ-Decalactone

According to the description, the production of δ-decalactone from 11-hydroxypalmitic acid or its ester is as follows:

The medium consists of 2% (w/w) soy peptone, 0.5% (w/w) yeast extract, and 1% (w/w) 11-hydroxypalmictic acid. The medium is steam-sterilized at 121°C for 20 min. The hydroxy fatty acid used as the substrate may be conveniently added as the only carbon source. To facilitate the dispersion of the substrate in the culture medium, a suitable emulsifier may be added in an amount up to 1% (w/w) of culture medium. The substrate may also include an ester of 11-hydroxypalmitic acid but this will require introduction of lipase (for hydrolysis) during the fermentation.

The fermentation is carried out either in batch or fed-batch mode. In the latter case, 11-hydroxypalmitic acid is the component for fed-batching. Yeast cells are added at the rate of 1000 cells per kg of medium. The pH of the medium is adjusted to 6.5 with 1 *M* NaOH. Aeration and agitation is carried out such that the oxygen level is above 10% of saturation level. Fermentation is carried out at 28°C. Foaming of fermentation broth may be prevented by the addition of conventional antifoaming agents (e.g., silicone oils, diglycerides, etc.). If an ester of 11-hydroxypalmitic acid has been used, a suitable lipase is added during the fermentation. The fermentation

duration is dependent on the metabolic characteristic of the organism. If the organism is itself able to metabolize the δ-lactone, the fermentation must end before this degradation occurs. The general duration of fermentation in such a case is about 10 days, but the amount of δ-lactone in the broth must be regularly checked to ensure that the degradation phase is not initiated. Usually, pasteurization of the broth is done to arrest the degradative reactions. On the other hand, if the organism does not have the ability to metabolize δ-lactone, the fermentation duration is not crucial.

The reaction mixture usually consists of δ-hydroxy alkanoic acid and the corresponding δ-lactone. For lactonization, some additional conversion steps must therefore follow the main fermentation. Lactonization can be done in the fermenter itself by acidifying the broth to pH 3 with glacial acetic acid. The lactone is then extracted with butyl acetate. Finally, after removal of the solvent, the residue is distilled to obtain δ-decalactone. The yield is about 85%. For this particular protocol, the amount of δ-decalactone formed is 1-1.5 g/kg of broth.

The lactone thus produced is suitable for adding to flavorings or foodstuffs (e.g., margarine, vegetable fats and oils).

18.4 BUTYRIC ACID

Butyric acid at low concentrations is used to supply butter-like note to flavors. It finds particular application in natural cheese flavors. The esters of butyric acid may also contribute to the flavors of various products. Pentyl butyrate provides a strong, ethereal, fruity odor reminiscent of apricot, banana and pineapple; isobutyl butyrate supplies an ethereal, fruity, somewhat pungent odor suggestive of pear, pineapple, and banana.

Although natural butyric acid as an ester may be found at concentrations of 2-4% in butter, its isolation is an expensive and difficult process. As a result, the fermentative production of natural butyric acid is a valuable alternative.

Butyric acid is produced primarily by obligate anaerobes (bacteria of genera *Clostridium*, *Butyrivibrio*, and *Eubacterium*) and *Fusarium* (mold). The clostridia, particularly *Clostridium acetylbutyricum* (= *acetobutylicum*), have been studied in detail. The latter's ability to produce organic solvents such as acetone and butanol has led to commercial processes which may be modified and adapted to produce butyric acid. The mechanism for butyric acid synthesis in microorganisms has been summarized by Gottschalk (1979) as in Fig. 18.3.

Besides proper selection of microorganisms, it is necessary to maintain the pH of the medium above 5.0 in order to direct the fermentation away from solvent formation and towards butyric acid formation. Calcium carbonate may be used to control the pH above 5.0. $CaCO_3$ plays another role as well, possibly that of serving as a point of attachment for the microorganisms. Using a simple medium consisting of *cerelose* (commercial dextrose), dried yeast, and $CaCO_3$, yield of 1% butyric acid was obtained.

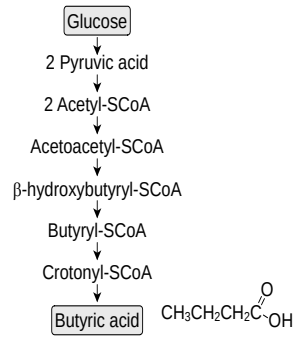


Fig. 18.3 Biosynthesis of butyric acid

Additional work by Sharpell and his co-workers showed that increased yield of butyric acid could be possible with further modifications of the medium and operation conditions. The toxic nature of butyric acid towards *Clostridium* species, however, appears to be a limiting factor.

One of the derivatives of butyric acid that have found use as flavoring compounds is α-keto butyrate (= 2-oxo-butyrate). Its production using a mutant strain of *Neurospora crassa* (a mold) has been described by Zurbriggen and coworkers (2004). The mutant strain is so chosen that it is capable of accumulating α-keto butyrate in the medium and exhibits very low or no acetolactate synthetase activity.

In the preferred embodiment, the fermentation is carried out in two stages, the first one for the biomass build up and the second one for the production of α-keto butyrate.

First of all, abundant spores of *Neurospora crassa* are produced on an agar plate. The spores are suspended in sterile water and used as a starter culture to inoculate a synthetic medium (e.g., Vogel-saccharose medium; composition not given here) for the production of abundant vegetative cells (not spores) and relevant enzymes. The fermentation (the first stage) takes place at a pH of 4.5-6 for 2-5 days at 20-30°C. The biomass is collected by filtration and the second stage of fermentation carried out in a different medium. For the second stage fermentation, the cells are suspended in phosphate buffer (0.1 *M*) of pH 9-11. The buffer is supplemented with threonine (0.2-4%, as a substrate) and valine (0.01-0.5%, as an enzyme trigger) to convert threonine into α-keto butyrate. The fermentation is carried out at a temperature of 23-30°C and it proceeds for 18-48 hrs. The bioconversion of threonine to α-keto butyrate is up to 90%.

The medium is filtered to remove the fungal biomass. The broth is then subjected to suitable ion-exchange resin for purification. When α-keto butyrate is to be used for the synthesis of other flavor compounds, such as emoxyfurone, the filtrate may be used directly (without further purification).

CHAPTER 19

MICROBIAL PRODUCTION OF POLYSACCHARIDES

19.1 INTRODUCTION

Depending on function, microbial polysaccharides are of 3 main types:

1. *Intracellular:* represented by reserve polysaccharides
2. *Structural:* represented by cell wall polysaccharides
3. *Exopolysaccharides* (EPS): represented by capsule- and slime polysaccharides

19.1.1 EXOPOLYSACCHARIDES (EPS)

Because of relevance, only EPS will be described here. EPS are basically water-soluble gums of microorganisms. EPS in microorganisms occur in the form of capsules or slimes. Commercially important EPS are those that readily diffuse in fermentation broth and can be subsequently recovered. EPS have great commercial value because of their novel and unique physicochemical properties. They have found a wide range of applications in the food, pharmaceutical, and other industries. Some of the more important EPS are xanthan gum, alginates, and dextran.

19.2 GENERAL PROCESS FOR MICROBIAL EPS PRODUCTION

Processes for EPS production are characterized by extreme rheological changes of the fermentation broth, low product concentration, and the diversity of subtle structural changes which occur throughout fermentation and the marked effect these changes can have on the product's end application performance. The success of EPS production therefore depends on controls at two levels: (i) environmental, and (ii) equipment design and operation. It is difficult to generalize EPS production process but the more general aspects of a typical EPS production process can be outlined as:

- *Fermentation medium:* defined glucose salts or sucrose, inorganic nitrogen supplement, and minerals
- *Duration of fermentation:* 40 hrs
- *Temperature:* 30°C
- *Process:* batch
- *Yield:* 0.7 kg dried product/kg of glucose
- *Moisture:* 5%
- *Product loss:* 5%

19.2.1 GENERAL RECOVERY PROCESS

As with many microbial metabolites, the recovery process entails:

1. Concentration
2. Purification
3. Deactivation of contaminating enzymes (cellulase, pectinase, etc.)
4. Modification (to alter functional properties such as handling and dispersion characteristics)

The steps involved, in general, are:

1. Cell removal (dilution followed by centrifugation/ filtration)
2. Isolation (precipitation, liquid-liquid partitioning, etc.)
3. Dewatering (drying in forced air or vacuum dryers)
4. Milling (to regulate size for dispersibility, flowability, etc.)
5. Additional processing (physical- and chemical treatment to affect purity, cosmetic appearance, moisture pick-up and clumping, handling characteristics, etc., depending on the intended end application)
6. Packaging

19.3 GENERAL USES OF EPS

EPS are generally used as emulsifiers, stabilizers, binders, gelling agents, coagulants, lubricants, film formers, thickening agents, molecular sieves, etc. In the following sections, some EPS and their uses will be discussed.

19.4 XANTHAN GUM

Xanthan gum is an anionic, branched heteropolysaccharide. Its molecular weight is greater than 10^6. This macromolecule has a cellulose backbone linked to trisaccharide residue, *viz*., β-D-glucose, α-D-mannose, β-D-glucuronate and variable amounts of acetate and pyruvate (see Fig. 19.1).

The gum has very interesting properties in aqueous solution. Its viscosity is independent of temperature over the range 10-70°C and is fairly constant over the pH range of 6-9.

Xanthan gum is commercially produced from mutant strains of *Xanthomonas campestris*. Xanthan gum is the only microbial EPS which constitutes an important component of total world polysaccharide market.

19.4.1 PRODUCTION OF XANTHAN GUM

The medium for the production of xanthan gum from *Xanthomonas campestris* consists of dextrose, sucrose and cruder forms of carbohydrates, the choice being dependent on the type of end product desired. Nitrogen, phosphate and magnesium are also supplied.

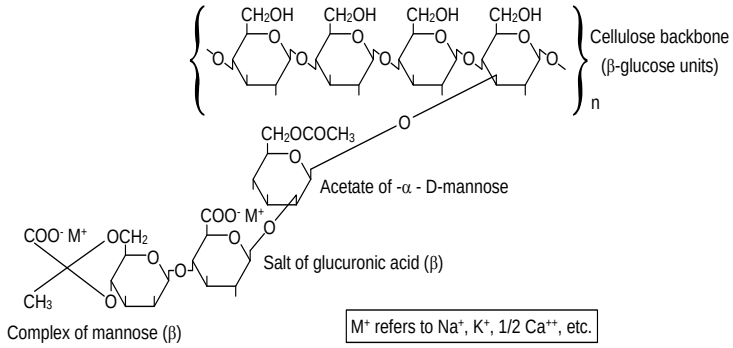


Fig.19.1 Molecular structure of xanthan gum

The inoculum is built up from lyophilized culture. Series of serial transfers and tests are needed before the culture is transferred to shaker flask. The contents of the shaker flask are in turn transferred to a seed tank for the final inoculum. The growth is highly aerobic. The seed tank has provisions for aeration. The main fermentation medium contains glucose, distiller's solubles, KH_2PO_4, and trace elements. Because of the high viscosity attained by the culture fluids, only about 3-5% concentration of glucose can be utilized efficiently. Fermentation is carried out at 28-32°C. The pH is controlled at 6-7.5 using suitable neutralizing chemicals. The fermentation is controlled by frequent checks for contamination and polysaccharide production (colorimetric). The duration of fermentation is about 2 days. At the end of the fermentation, the culture fluids attain a viscosity of as high as 7000 cps.

After the fermentation has completed, the broth is heated to kill the cells. Water is added to dilute the broth. The purification/concentration of xanthan gum is carried out by precipitation with alcohol. Dewatering, drying and milling can be done by any of the conventional methods. See Fig. 19.2 for the flow diagram.

19.4.2 USES OF XANTHAN GUM

Xanthan gum finds both food- and non-food uses:

1. Food applications
    - Dressings (high oil, low oil, no oil), relishes and sauces, syrups and toppings, starch-based products (canned desserts, sauces, fillings, retort pouches), dry mix products (desserts, gravies, beverages, sauces, dressings), farinaceous foods (cakes), dairy products (ice cream, cheese), and confectionary. Deacylated polysaccharide has an excellent film-forming property. The derivative is prepared by controlled treatment with alkali
2. Non-food applications
    - Flowable pesticides, liquid feed supplements, cleaners, abrasives, polishes, metal workings, ceramics, foundry coatings, texturized coatings, slurry explosives, dye- and pigment suspensions
    - Oil field application

- Drilling fluids, polymer flooding, work-over and completion fluid

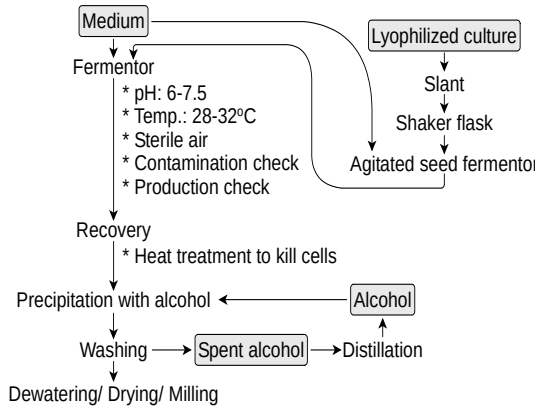


Fig. 19.2 Outline of microbial production of xanthan gum

19.5 DEXTRAN

Dextrans are branched, neutral homopolysaccharides composed exclusively of α-D-glucose residues. 95% of these residues are linked through carbon 1 and 6. About 5% are linked by 1-3 bonded side chain containing about 3 units. The molecular weight is between 30×10^6 and 39×10^6. See Fig. 19.3 for the structure of dextran.

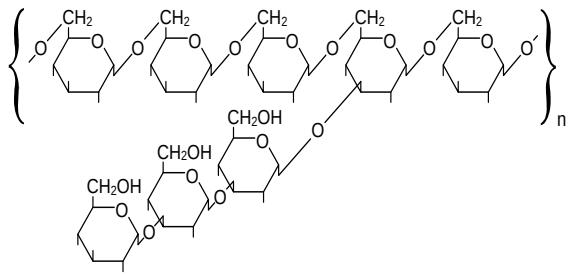


Fig. 19.3 Molecular structure of dextran gum

19.5.1 PRODUCTION OF DEXTRAN

Dextran is produced by two methods, viz., (i) *whole culture method*, and (ii) *enzymatic method*. Two species of bacteria are used for the fermentation: they are *Leuconostoc mesenteroides* and *Leuconostoc dextranicum*.

19.5.1.1 The Whole Culture Method

The main substrate is sucrose. A typical composition of the fermentation medium is: 10% sucrose, 0.5% KH_2PO_4, 0.25% yeast extract, 0.1% NaCl, 0.06% $(NH_4)_2SO_4$, and 0.02% $MgSO_4.7H_2O$.

The medium is taken to a slurry tank (1,500 gallon). The medium is heated (with agitation) to 60°C, and finally pasteurized in plate heat exchanger at 142°C. Thereafter the medium is cooled instantly to 25°C. This medium is used for the main fermentation as well as the seed vessels. The inoculum is built up in several stages at 25±0.5°C with an aeration of 0.5 vol/vol/min. The final inoculum tank, called *bazooka* has 10 gallon capacity. The seed tank has 206 gallon capacity. The main fermenter has 1,300 gallon capacity. Fermentation is done at 25°C. The initial pH is kept at 7 but is allowed to fall to 4.5 during fermentation. This final pH is also an indicator to the completion of fermentation. No sucrose will be left in the medium at this stage. Fermentation is usually carried out until the viscosity of the culture fluid increases to 400-700 cp.

19.5.1.2 The Enzymatic Method

The enzyme responsible for the production of dextran is called *dextran sucrase*. It is elaborated by strains of *Leuconostoc mesenteroides*. The enzyme helps polymerize the glucose fraction of the sucrose molecule that has been supplied as the substrate.

The production is carried out in two stages: (i) enzyme production by growing the microorganism, and (ii) enzymatic conversion with the enzyme thus formed. The initial fermentation is for enzyme production and therefore requires, in addition to sucrose, a balanced medium that contains nutrients adequate enough to support the growth of the enzyme producer. Typically, the growth medium contains 2% CSL, 2% sucrose, 0.1% KH_2PO_4, and trace amounts of inorganic salts. Fermentation is carried out at 24°C. The pH is kept at 6.7 and the fermentation carried out for 6 hrs. The enzyme produced in the broth is recovered by centrifugation and the crude extract used for the second stage fermentation (enzymatic reaction). See Fig. 19.4 for the outline of dextran production.

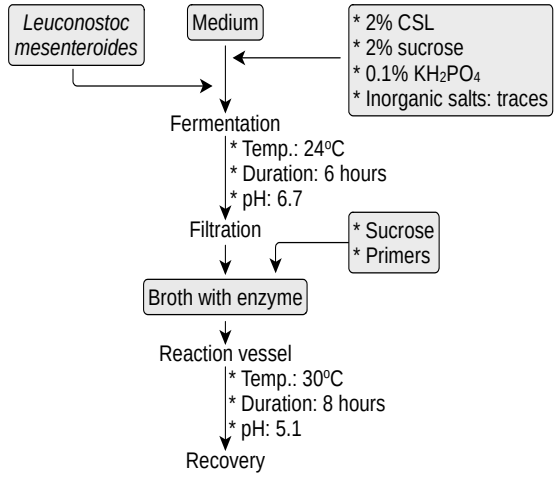


Fig. 19.4 Production of dextran by enzymatic method

The second stage reaction is carried out at 30°C, at a pH of 5.1, using 5-10% sucrose solution as the substrate. Manipulation can be done here to regulate the molecular

weight of dextran. Increasing the concentration of sucrose will produce low-molecular weight dextran. *Primers* are universally used to initiate the reaction. Primers, as mentioned here, refer to hydrolyzed dextran at a concentration of about 2%. Primers come mainly from methanol taken away for recovery (see later). The reaction proceeds for 8 hrs. Stirring can be done to bring about uniformity in reaction. Dextran sucrase uses only the glucose portion of sucrose for polymerization. Thus, approximately 50% of the original weight of sucrose solution remains as D-fructose at the end of the reaction.

19.5.2 RECOVERY OF DEXTRAN

Dextran broth is first of all precipitated in tanks by adding an equal volume of methanol. The supernatant contains low molecular weight dextrans and this is taken away for methanol recovery while the precipitate is dissolved in double-distilled, pyrogen-free water at 60-70°C. It is then hydrolyzed with HCl at 100-105°C to produce *clinical dextran* (average mol wt 75,000 ± 25,000). Vacuum is applied at this stage to remove residual methanol. The liquor is then mixed with diatomaceous earth and polish-filtered in a plate-and-frame filter. The filtrate undergoes several purification stages to obtain clinical dextran. That is, the liquor is again precipitated with methanol. This time, methanol is added in calculated amount so that the clinical dextran is selectively precipitated while the low molecular weight dextrans remain in the solution. The temperature, pH, etc., must be closely controlled. The precipitate is again purified, concentrated, and spray-dried.

19.5.3 USES OF DEXTRAN

Some of the varied uses of dextran are listed as follows:

- Oil well drilling: It was used as oil drilling fluid additive until 1950's. It is now considered uneconomical
- Blood plasma extender: Sterile, pyrogens-free, approx. 6% solution of dextran having molecular weight in the range 50,000-100,000 (clinical dextran) can be used in emergency to restore blood volume in cases of shock due to blood loss
- Iron-dextran complex, which can be used as a source of nutritional iron
- Molecular sieves: Molecular sieves are prepared by cross-linking dextran with epoxy compounds and NaOH. The degree of cross-linking determines the pore size and water regain value of the molecular sieves (and thus their molecular exclusion characteristics)

19.6 ALGINIC ACID

Although most of the commercial alginate produced today is derived primarily from the sea kelp *Macrocystis pyrifera*, microbiologically derived alginates are under development and their future is very promising. The wide variety of products obtained from *Azotobacter vinelandii* have been found to possess physical properties similar to the alginates derived from marine algae. The biopolymers derived from this organism have a wide range of molecular weights and it has been postulated that

the extracellular enzyme *alginate lyase* may play an important role in the molecular weight of alginates.

19.6.1 CHEMISTRY OF ALGINATE

Alginate is a general term used to describe the salts of alginic acid, the most notable of which is sodium alginate. Alginic acid is a weak organic acid, which readily forms salt with different bases. The acid is a linear polysaccharide composed of varying proportions of β(1→4) linked D-mannuronic acid and α(1→4) linked L-guluronic acid residues in blocks and alternating sequences in their linear chain. The presence of L-guluronic acid in alginic acid is important because increasing its content improves gelling characteristics of alginate in the presence of calcium ions. See Fig. 19.5 for the chemical structure.

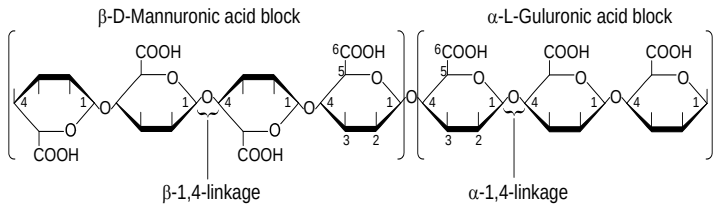


Fig. 19.5 Partial structure of alginate (note the orientation of bonds at C1 and C4)

19.6.2 USES OF ALGINIC ACID

Sodium alginate is widely used in research as a gelling agent to immobilize a wide variety of cells, such as microbial cells, and plant and mammalian cells. Alginates have ion exchange properties similar to ion exchange resins. The relative affinity of divalent metal ions depends on the relative amounts of D-mannuronic and L-guluronic acid units that are present in the macromolecule. The gum also finds use as film former, emulsifier, restructuring agent (in fruit gels), and stabilizer (in ice cream).

19.6.3 FERMENTATION

Some of the bacteria capable of producing alginate are *Azotobacter vinelandii*, *Pseudomonas aeruginosa*, etc. *Pseudomonas aeruginosa* is not preferred because it has association with pathogenic infection in humans.

The fermentation is carried out in continuous fermenters. Since the bacterium is fastidious the medium is probably very complex. Oxygen is closely monitored. Too high levels of aeration increase respiration, leading to conversion of substrate to CO_2. Too low levels of aeration, on the other hand, lead to accumulation of poly-β-hydroxybutyrate (reserve lipid).

There is not much detail about the recovery of bacterial alginate. The recovery method of algal alginate is probably used here also. The method, in essence, entails precipitation with $CaCl_2$, regeneration with acid-water, and neutralization with

sodium carbonate to obtain alginic acid in the form of sodium salt. See Fig. 19.6 for an outline of the fermentation and recovery.

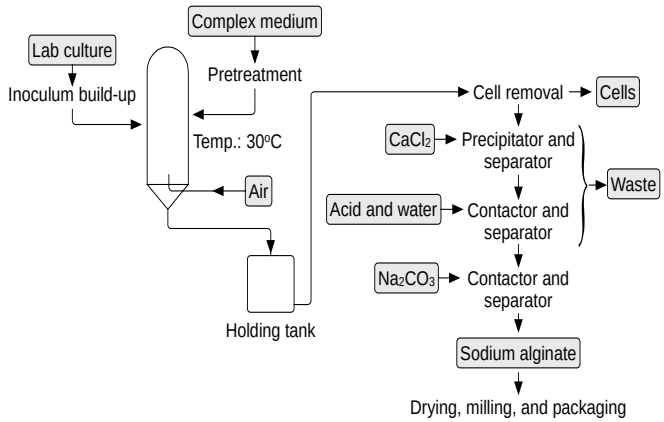


Fig. 19.6 Production and purification of alginate

19.6.4 MICROBIAL BIOSYNTHESIS OF EPS

Very little is known about the biochemical pathways involved in the biosynthesis of different EPSs. The majority of the EPSs are assumed to be synthesized within the cell in a mechanism analogous to that involved in cell wall synthesis. Very few EPSs have been reported to be synthesized outside the cell. A simple sequence for the synthesis of a homopolysaccharides of glucose units can be shown as in Fig. 19.7.

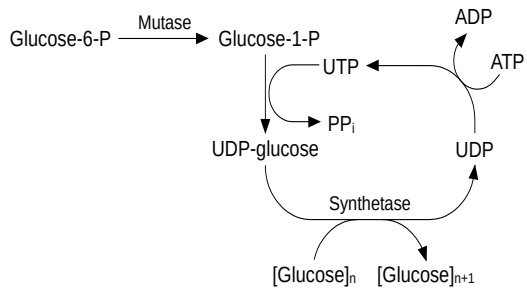


Fig. 19.7 Biosynthesis of glucose polymer

Branches in the linear chain occur under the influence of *branching enzymes.*

CHAPTER 20

MICROBIAL PRODUCTION OF FATS

20.1 INTRODUCTION

Microorganisms have long been known to produce lipids and therefore to be potentially useful for the production of oils and fats. Such organisms may be termed *oleaginous* in keeping with the terminology used for oil-bearing plant seeds. For the most part, oils produced by oleaginous strains of eukaryotic microorganisms approximate to that of conventional oilseeds (with respect to physicochemical properties).

A definition of what constitutes an oleaginous organism poses some difficulty. A pragmatic definition would suggest that a microorganism containing more than 20-25% oil would be deemed a suitable candidate for commercial consideration.

The lipid-containing microorganisms include bacteria, algae, and fungi. The range of fatty materials produced by the microorganisms can be conveniently classified as:

1. *Simple lipids:* represented by triglycerides
2. *Compound lipids:* represented by phospholipids, glycolipids, etc.
3. *Miscellaneous:* represented by vitamin A, sterols, carotenoids, etc.

A short list of microbial lipids, the organism involved, and the substrate used is given in Table 20.1.

Table20.1 Representative groups of lipid producing microorganisms

Representative organism	Substrate	Product (lipid)
Chlorella pyrenoidosa (algae)	Carbohydrate	Triglyceride
Blakeslea trispora (algae)	Carbohydrate	β-carotene
Saccharomyces cerevisiae (yeast)	Wort	Ergosterol
Acinetobacter sp HO1-N (bacteria)	Hydrocarbon	Waxes
Alcaligenes eutrophus (bacteria)	Glucose	Poly-β-hydroxybutyrate

A brief description of each of the above lipid types is given in the following paragraphs:

20.1.1 TRIGLYCERIDES

These are natural oils, the hydrolysis of which gives fatty acids and glycerol only. The general structure is shown in Fig. 20.1.

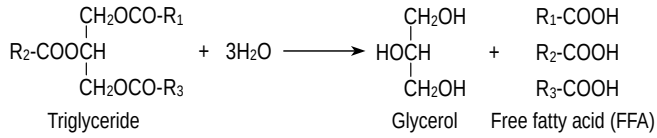


Fig. 20.1 Triglyceride and its hydrolysis

20.1.2 POLY-β-HYDROXYBUTYRATE

Poly-β-hydroxybutyrate (PHB) is a polyhydroxyalkanoate (PHA) that consists of repeating units of -CH(CH_3)-CH_2-COO- (Fig. 20.2). PHB is an energy storage material produced by a variety of microorganisms in response to environmental stress (limitation of nitrogen or phosphorus in the growth medium). Although many bacteria are capable of producing PHB, *Alcaligenes eutrophus* strains have remained the organisms of choice for industrial production. They can produce up to 96% PHB (based on dry cell mass). Commercial PHB is sold under the name Biopol®.

Because PHB is biodegradable, there is considerable interest in using PHB for packaging purposes (such as edible packaging) in order to reduce impact of human garbage on environment. PHB has application in antibiotics, drugs delivery, medical suture, and bone replacement.

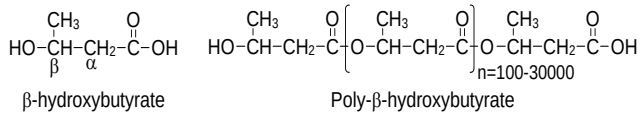


Fig. 20.2 Structure of poly-β-hydroxybutyrate

20.1.2.1 Biosynthesis of PHB

Poly-β-hydroxybutyrate is synthesized by the bacterium (*Alcaligenes eutrophus*) utilizing three enzyme systems, viz., (i) β-keto thiolase, (ii) reductase, and (iii) PHA synthase. The natural producers also have PHA depolymerase that degrades the polymer and uses the breakdown metabolites for cell growth. A summary of PHB synthesis is given in Fig. 20.3.

PHB is insoluble in water and is relatively resistant to hydrolysis. It sinks in water, has good oxygen permeability, is non-toxic, and is biodegradable. Despite these interesting properties, PHB is used only in limited areas because of two fundamental reasons, viz., (i) prohibitively high cost of PHB, and (ii) brittleness. Researches are being done to overcome these limitations. The cost can be brought down by large-scale production, strain improvement, and improvement in the fermentation process. The brittleness can be reduced by incorporating other alkanoates in the material to form copolymers with a wide range of plasticity and other physicochemical properties.

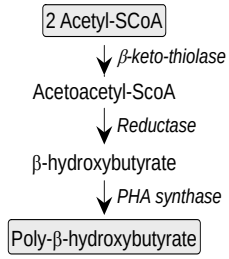


Fig. 20.3 Biosynthesis of poly-β-hydroxybutyrate by *Alcaligenes eutrophus*

20.1.2.2 Microbial production

Using *Alcaligenes eutrophus*, PHB can be produced by two general methods, namely, (i) Parallel process, and (ii) Serial process. In the Parallel process, cell growth and PHB formation occurs together in a single fermenter. In the Serial process (which is more common), microorganisms are first grown on carbon source (usually glucose) to obtain large biomass. Then the medium is depleted of an essential nutrient (nitrogen) and polymer-forming substrate is added (Fig. 20.5). This is converted directly to polymers and essentially only little growth occurs. An outline of a typical PHB production is given in Fig. 20.4.

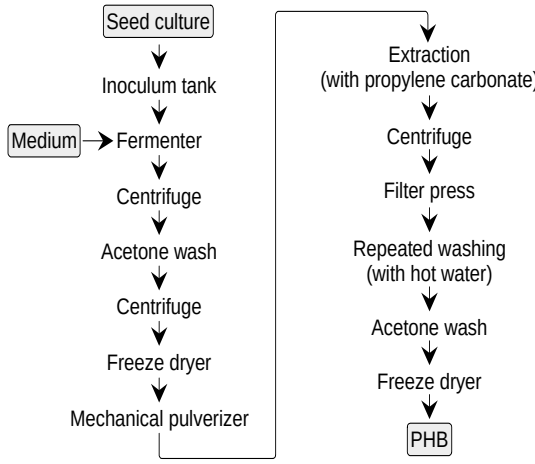


Fig. 20.4 A typical outline of microbial production of PHB

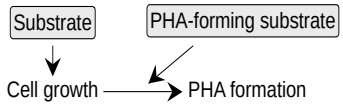


Fig. 20.5 Serial process of PHB production

20.1.3 WAXES

The composition of wax is of a simple ester of a fatty acid with a fatty alcohol:

$$CH_3(CH_2)_xCOOH + HOCH_2(CH_2)_yCH_3 \rightarrow CH_3(CH_2)_xCOOCH_2(CH_2)_yCH_3 + H_2O,$$

where *x* and *y* are usually either 14 or 16, although shorter chain alcohol with *y* =1 to 3 have been reported.

20.1.4 BETA CAROTENE

It is a precursor of vitamin A. It contains 40 carbon atoms (or 8 *isoprenoid* units). The simplified structure is given in Fig. 20.6. For microbial production, see page 391.

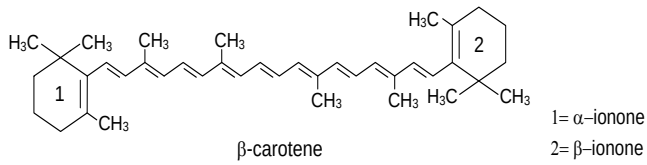


Fig. 20.6 Simplified structure of β-carotene

20.1.5 ERGOSTEROL

Ergosterol occurs in yeast. Yeast requires this sterol for the synthesis of essential membrane components. It is also regarded as an *anaerobic growth factor* for yeast. The chemical structure of the compound is given in Fig. 20.7.

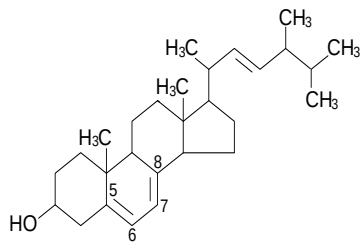


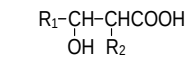
Fig. 20.7 Structure of ergosterol

20.2 SOME MICROORGANISMS CAPABLE OF PRODUCING LIPIDS

20.2.1 BACTERIA

Only a few bacterial species are known which can produce appreciable amounts of extractable neutral lipids (the triglycerides). The *Mycobacteria-Nocardia* group of organisms is well known for cellular lipid contents but these lipids are complex-structured, often occurring in a bound form as part of the cell envelope structure. Some of these species do contain triglycerols but their exploitation is not sensible as the co-extraction of toxic or allergenic substance from them is highly likely. For

example, cell walls of *Corynebacterium*, *Mycobacterium*, and some other nocardioforms contain mycolic acid. Mycolic acid is a high molecular weight α-branched β-hydroxy fatty acid of the general formula:



The mycolic acid derivative, called *cord factor* (= Trehalose dimycolate) is toxic and plays an important role in the diseases caused by *Corynebacterium diptheriae* and *Mycobacterium tuberculosis*. The cord factor inactivates the mitochondrial membranes of the phagocytes.

The only bacterium which has been reported as producing significant amounts of triglycerol is *Arthrobacter* AK-19. This organism is unlike any other bacteria in that it can contain up to 80% of its biomass as lipid; this lipid, moreover, is predominantly composed of triglycerols and would thus seem an excellent candidate for commercial exploitation. The only drawback in this organism is its slow growth rate. However, there is possibility of growing the bacterium as a symbiont along with an algal culture. The provision of an external carbon source, other than CO_2, would then be obviated.

20.2.2 ALGAE

Notable oleaginous algae, (fat content in parenthesis) are *Chlorella pyrenoidosa* (70%), *Botryococcus braunii* (53%), *Dunaleilla salina* (47%), *Monalanthus salina* (70%), etc. The major limitations in their use are production of lipids other than triglycerol type and markedly slow growth rate.

Chlorella pyrenoidosa and *Chlorella vulgaris* appear worth investigating among the oleaginous algae. The algal lipids are marked by their exceptionally high proportions of *polyunsaturated fatty acids* (PUFA). Unfortunately, as these are the types found in fish oil, the frequent complaint against algal lipids is about their unpleasant *fishy* odor. The desirability of including polyunsaturated fatty acids in the diet might suggest that a proportion of algal oils could be mixed with a more saturated or monounsaturated oil, *viz*., palm oil or rapeseed oil, to give nutritionally acceptable blend.

20.2.3 YEASTS AND MOLDS

The number of oleaginous microorganisms in this class is not very large. Some 16 classified species have been reported as producing better than 25% lipid. The prerequisite for lipid accumulation in these microorganisms is the possession of ATP:citrate lyase; this enzyme is present only in lipid-producing yeasts and therefore is an extremely powerful determinant for lipid production. Some examples of oleaginous yeasts (lipid content in parenthesis) are: *Cryptococcus terricolus* (55-65%), *Rhodotorula glutinis* (syn. *gracilis*) (74%), and *Candida curvata* (51-58%).

The major accumulating lipid of yeasts and fungi is the triglycerol fraction, which accounts for up to 92% of the total lipid of the cell. The fatty acids of yeasts are usually in the approximate order of abundance: oleic > palmitic > linoleic > stearic

acid. Modifying the cultural conditions, however, can vary the order of abundance of fatty acids. Such conditions would include variation in O_2 tension, choice of growth substrate, growth temperature, as well as the growth rate of the organism itself.

The fatty acids from molds show a greater range and diversity than those from yeasts. Members of *Entomopthoraceae* are characterized by the presence, often in substantial amounts, of short-chain fatty acids (C_{10}-C_{14}).

20.3 GENERAL CULTURAL CONDITIONS

A wide range of substrates has from time to time been considered for the production of oils and fats. These include various starchy crops and wastes, molasses, whey, peat (compost) hydrolysates, and ethanol. The use of hydrocarbons, e.g., methanol, has fallen out of favor.

As lipid represents a reserve storage material, the medium should have a high *carbon-to-nitrogen* ratio (usually, 50:1). In a batch culture the microorganism grows until the nitrogen is consumed but thereafter it continues to take up the excess carbon and convert this to lipid. Thus, a biphasic growth pattern can be expected.

With some of the slower growing molds, the rate of lipid accumulation appears to coincide with the growth rate. Although this is probably fortuitous, the result is that the lipid content of the cells increases at the same rate as growth proceeds.

In a continuous culture, lipid accumulation is achieved by growing microorganisms under *nitrogen-limiting* conditions at a dilution rate of about 30% of maximum specific growth rate. The build-up of lipid is dependent on the correct balance between growth rate and the specific rate of lipid biosynthesis so that the optimum amount of carbon can be diverted into lipid and the minimum into other cell components.

A conversion of carbohydrate to lipid of 20% would appear near to possible practical limit because the theoretical maximum is about 33 g triglycerol from 100 g of glucose, assuming that all the carbon of the medium is converted into lipid without the synthesis of any other cell components. The low conversion is apparent from the following fact: synthesis of one mole of palmitic acid in a eukaryotic cell such as yeast needs, theoretically, 145 ATP as against 130 ATP obtained by β-oxidation of the same. A total of 8 acetyl-ScoAs are diverted for the biosynthesis. Assuming that all the acetyl-ScoA come from carbohydrate, 4 moles of glucose are wasted for energy production alone. Since there are 3 fatty acids plus a glycerol moiety in a triglyceride, the energy requirement in terms of glucose is very high. Only after this energy has been furnished can the conversion of carbohydrate to fat occur.

As far as lipid production from yeast is concerned, the only current commercial enterprise utilizing non-carbohydrate carbon source is that using gas oil to yield the protein called *Fermosin*. This biolipid is produced as a byproduct.

20.4. MICROBIAL PRODUCTION OF PUFA-RICH OIL

This discussion relates to production of high-value product, namely essential fatty acid, using microbial technique. Reference is given to the patent filed by Streekstra and Brocken (2005). These workers have described a method for the production of microbial oil rich in polyunsaturated fatty acids (PUFA), arachidonic acid (which is an *essential fatly acid*) in particular. The process involves a two-stage fermentation using *Mortierella alpina* (a fungus). In the first stage, the organism is provided with excess of carbon and nutrients so that lipids (including arachidonic acid) are accumulated in large amounts. The second stage of fermentation involves restricted feeding of the carbon source, with the result that lipids except arachidonic acid are preferentially utilized to meet the metabolic need. This event leads to increase in the proportion (35-40%) of arachidonic acid in the lipid bulk. The fatty acid is predominantly in the triglyceride form (about 99.5%).

Briefly, the production process is as follows:

The organism is propagated in the order: 100 ml $\rightarrow$ 500 ml $\rightarrow$ 2400 liter by aerobically growing it at 25°C for 24-48 hrs in each stage. The culture medium consists of glucose (2%), yeast extract (1.2%), and silicone antifoam (0.02%). The medium is sterilized after adjusting the pH to 7. Aeration in the propagators is achieved by bubbling air and agitation.

The first-stage fermentation medium contains 3.5% glucose, 0.5% yeast extract, 0.03% antifoam, 0.1% $NaH_2PO_4.2H_2O$, 0.2% $KH_2PO_4.2H_2O$, 0.05% $MgSO4.7H_2O$, 0.06% citric acid monohydrate, and 0.0001% $ZnCl_2$. The pH is maintained at 6 ± 0.1 before fermentation. Aeration is carried out at a rate of 0.5 vol/vol/min. Fermentation is carried out for 170 hrs at 25°C. This phase is characterized by accumulation of lipids by the organism.

The second-stage fermentation starts 5-6 hrs before the termination of the fermentation (the total fermentation time for both the stages being about 175 hrs). In this stage, a 50% aqueous solution of sterile glucose is fed to maintain glucose content at about 0.5 g/kg of medium/hr. A 25% solution of yeast extract is also fed to maintain ammonia level at around 30 mg/liter. During this stage, the microorganism experiences starvation because of carbon source restriction and to overcome this state it preferentially falls back on lipids other than arachidonic acid.

After the fermentation has completed, the microbial biomass is harvested by filtration. The filtered cake is pasteurized (to kill the cells and inactivate lipid-degrading enzymes), extruded/crumbled/kneaded, and dried for extraction of oil using organic solvents (e.g., hexane) or supercritical fluid (liquid CO_2).

Although the oil can be used as such, it can also be further purified to meet the exacting criteria by refining, bleaching, deodorization and polish filtration.

This microbial oil (which is rich in essential fatty acid, arachidonic acid) is suitable for including in infant formula, human foodstuffs, feed supplement, and pharmaceutical preparations.

20.5 OUTLINE OF BIOSYNTHESIS OF SIMPLE LIPID IN EUKARYOTES

Acetyl-ScoA is the precursor of fatty acids. However, it does not spontaneously polymerize; it must first be activated to a highly reactive form, malonyl-ScoA, (a three-carbon unit). The carboxylation requires biotin-dependent enzyme and so the requirement of biotin for oleaginous microorganisms is obvious. The biosynthesis takes place in somewhat complex manner, but the essential steps for one cycle that allows lengthening of the existing chain of fatty acid by 2 carbon units are: *Activation*, *Attachment to binding sites*, *Condensation*, *Dehydration*, and *Reduction* (see Fig. 20.8).

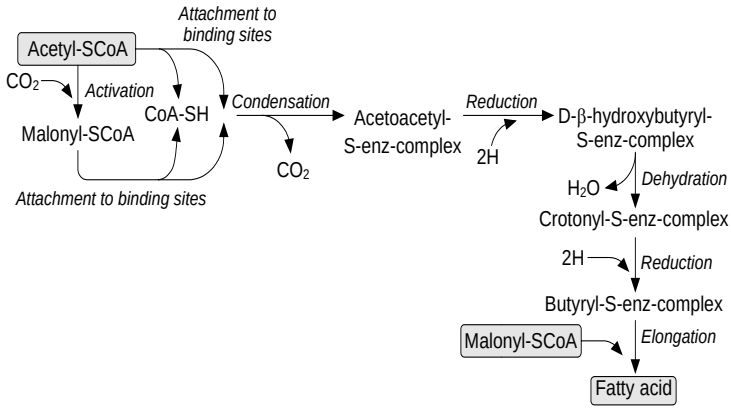


Fig. 20.8 Biosynthesis of fatty acid in eukaryotes

20.6 FUTURE PROSPECTS

The future prospects for microbial oils might be seen to lie in three possible areas:

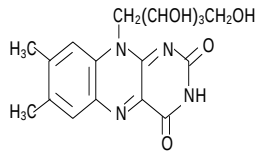
1. As a substitute for high-value plant oils
2. As novel material not available from other sources
3. As an alternative to SCP production in waste-processing systems

CHAPTER 21

RIBOFLAVIN PRODUCTION BY YEAST

21.1 INTRODUCTION

Riboflavin (= vitamin B_2, or vitamin G, or lactoflavin) has the empirical formula $C_{17}H_{20}N_4O_6$ and the structural formula is:



Riboflavin (oxidized state)

It is a crystalline yellow-orange powder. It is bitter in taste and practically odorless. It is sensitive to alkalis and is decomposed by UV radiations. This vitamin is an essential growth factor for animals and humans.

Riboflavin can be produced by two methods, *viz*., (i) Chemical, and (ii) Microbiological. The chemical synthesis is carried out by Kuhn's process (Fig. 21.1) or modification of it. The basic material needed for chemical synthesis are D-ribose, 6-nitro-3,4-xylidine, and alloxan. Condensation of D-ribose and 6-nitro-3,4-xylidine followed by catalytic reduction gives *phenylenediamine*. The latter reacts with alloxan to give riboflavin.

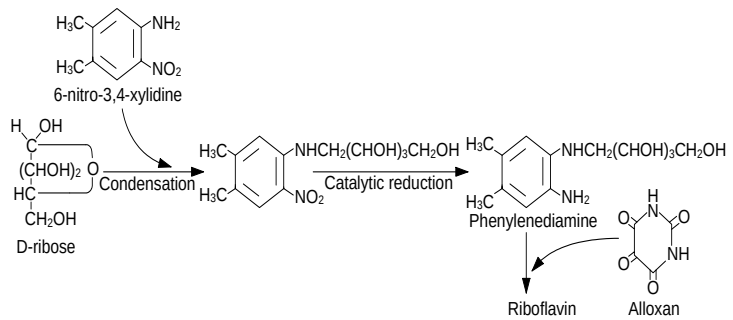


Fig. 21.1 Chemical synthesis of riboflavin

Since the purest form of riboflavin is produced by chemical means it is this type that is valued therapeutically.

- Therapeutic purposes
- Fortification of cereal products (e.g., enriched bread, flour, etc)
- In animal feed (2-8 g/metric ton)

21.2 MICROBIAL PRODUCTION OF RIBOFLAVIN

Riboflavin can be produced by a number of microorganisms, including bacteria, yeasts, and yeast-like microorganisms. The most important (commercially) of these are *Eremothecium ashbyii*, *Ashbya gossypii*, and certain *Clostridium* species. A short list of the microorganisms of potential or realized value is given in Table 21.1.

Table 21.1 Some noted producer microorganisms of riboflavin

Bacteria	Yeast	Yeast-like
Clostridium butyricum	*Candida flareri*	*Ashbya gossypii*
Clostridium acetobutylicum	*Candida guilliermondia*	*Eremothecium ashbyii*

The bacteria *Clostridium butyricum* and *Clostridium acetobutylicum* are commercially used for acetone-butanol fermentation but riboflavin can be recovered as a byproduct of the process.

The yeast-like organisms, *Ashbya gossypii* and *Eremothecium ashbyii*, are plant pathogens that cause disease in cotton and other plants. This attribute makes it mandatory that sterilization be carried out before discarding the cultures and fermentation residues. Of the two microorganisms mentioned above, *Ashbya gossypii* has greater stability with respect to riboflavin-producing capacity: it does not degenerate as readily as *Eremothecium ashbyii*.

21.2.1 TRADE FERMENTATION USING *Ashbya gossypii*

21.2.1.1 Stock culture

The culture may be transferred at weekly intervals on a medium containing peptone (0.5%), yeast extract (0.3%), malt extract (0.3%), commercial glucose (1%), and agar (2%). Incubation may be done at 27-30°C.

21.2.1.2 Inoculum development

A loopful of 24-hr old culture of *Ashbya gossypii* NRRL-Y-1056 is placed in 100 ml of the following medium in 500 ml flask and incubated for 24 hrs in a reciprocating shaker at 26-30°C: glucose 2%, peptone 0.5%, cornsteep liquor 1%, and water to make 100 ml. The pH of the medium is adjusted to 6.5 before sterilization.

The contents of the flask are used to seed 6 liters (in a 9-liter flask) of medium having following composition: glucose 2%, cornsteep liquor 1%, animal-stick liquor

0.5%, and water to make 6 liters. The pH of the medium is adjusted to 6.5 before sterilization.

The organism is grown for 24 hrs with aeration provided by passing sterile air. The culture can now be inoculated in 1000-1500 liters of medium for the main fermentation.

21.2.1.3 Main fermentation

The medium for main fermentation can be semi-purified sugar, glucose, plus additional crude organic nutrients such as peptone, cornsteep liquor, etc. In certain instances, however, glucose may be totally replaced by lipid such as corn oil. The medium can be sterilized (continuous sterilization) at pH 4.5 and 135°C for 5 min. In batch sterilization, 15 psig can be used for 3 hrs.

Iron content of the medium above 5 ppm is detrimental. To regulate it to 1-3 ppm, which is the optimum, iron or steel fermentation equipment is avoided. Plastic or cobalt-coated fermenters can be used instead.

The temperature is maintained at 28-30°C. The initial pH is 6-7.5. The process is a submerged aerated fermentation but excess air inhibits mycelial production and reduces the riboflavin yield. An aeration rate of about 0.25 vol/vol/min is satisfactory. If foaming becomes excessive, it can be controlled by the initial addition of emulsified silicone antifoam and later by soybean oil. During the fermentation, the riboflavin content must be periodically determined (by fluorimetry). Contamination checks are made by growing the samples in malt-yeast extract agar. The fermentation lasts for 4-7 days.

21.2.1.4 Recovery

The final beer is heated for an hour at 60-120°C to free riboflavin from the mycelium. The solids may be dried to a crude product for animal feed supplementation, or processed into a refined grade. In either case the pH is adjusted to 4.5. For feed-grade product, the broth is concentrated to about 30% solids and dried on double drum driers.

When a crystalline product is desired, the broth is heated for an hour at 121°C to solubilize riboflavin. Insoluble matter is removed by centrifugation and the broth treated to recover the vitamin. The broth is reduced by bacterial or chemical means to precipitate riboflavin from the broth. The precipitated riboflavin is then dissolved in water or polar solvents, or an alkaline solution, oxidized by aeration, and recovered by crystallization from the aqueous or polar solvent solution or by acidification of the alkaline solution.

21.2.1.5 Changes occurring during the fermentation

The fermentation progresses through 3 phases as described in the following paragraphs:

Phase-I

Rapid growth, little production of riboflavin, rapid utilization of glucose, lowering of pH due to pyruvate accumulation

Phase-II

Glucose depletes, growth ceases, sporulation starts, pyruvate decreases, ammonia increases due to increase in deaminase activity, pH value increases, rapid synthesis of cell-bound riboflavin (as FAD and FMN) occurs, rapid increase in catalase activity and a disappearance of cytochromes

Phase-III

Cellular regulatory mechanisms for FAD synthesis break down. Autolysis occurs, releasing free riboflavin into the medium as well as some riboflavin in the nucleotide form.

From these observations, it can be concluded that at about the same time of sporulation there is a shift from the initial cytochrome-type terminal respiration to a terminal respiration utilizing flavoproteins, and that this flavoprotein respiration is accompanied by an overproduction of the flavin prosthetic group.

Certain purines, but no pyrimidines, stimulate riboflavin production without simultaneously stimulating growth. This is because purine or purine precursors are used by the microorganisms to construct the middle and right hand rings of the riboflavin molecule.

21.3 BIOSYNTHESIS

The starting material is the guanosine derivative (Fig. 21.2). This is transformed to *6-hydroxy-2,4,5-triaminopyrimidine* derivative. The nature of R_1 and R_2 is unknown.

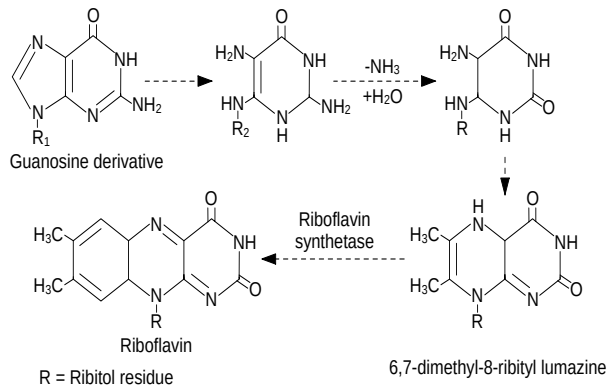


Fig. 21.2 Microbial synthesis of riboflavin

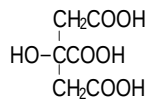
The latter derivative is reduced to corresponding *ribityl* derivative. Replacement of an amino group by a keto group yields *5-amino-2,6-dioxy-4-(1'-D-ribityl amino) pyrimidines*. The steps leading to *6,7-dimethyl-8-ribityl lumazine* are unknown. Formation of riboflavin is completed by riboflavin synthetase.

CHAPTER 22

MICROBIAL PRODUCTION OF ORGANIC ACIDS

22.1 CITRIC ACID

Citric acid is a tricarboxylic acid with the molecular formula:



It was first isolated by Scheele from lemon juice in 1784. Microbial production of citric acid started in USA in 1923. In 2000, the annual production of citric acid worldwide was 736,000 MT.

22.1.1 PRODUCTION METHODS

Citric acid can be produced using molds, yeasts, or bacteria. Except for patents and few research articles, literature details on yeast- and bacterial processes are still scarce. *Candida*, *Pichia*, etc., are the main organisms studied in the yeast process. In the bacterial process, mutated strains of *Corynebacterium* species have been reportedly used. The mold process is by far the most important from trade fermentation point of view. Today, improved strains of *Aspergillus niger* (mold) are universally used for citric acid production.

22.1.1.1 The mold (fungal) process

Based on the fermentation differences, there are three main types of mold process for citric acid production: (i) solid substrate fermentation (*koji* process), (ii) surface culture, and (iii) submerged culture. *Aspergillus niger* strains used for the commercial processes are very efficient, producing above 80 g citric acid per 100 g glucose. The organism in general exhibits marked sensitivity to iron and zinc, particularly to iron, in the medium. These elements promote growth of the organism but at the cost of citric acid.

1. The koji process

This method is widely used in Japan. The method accounts for 1/5th of total citric acid produced in Japan. The fermentation is carried out in moist wheat bran. Since wheat bran is rich in minerals, only special, iron-tolerant strains of *Aspergillus* can be used. Although wheat bran contains ~ 66% carbohydrate, it is probably supplemented with suitable amounts of sugar for the fermentation. Ferrocyanides or copper may also be supplemented. After sterilization of the substrate (which is in the

paste form with ~ 70% moisture content), the fermentation is carried out in batches, in shallow trays or rotolouver-type drum fermenters, at a pH of 5.5. Temperature, humidity, and air supply can be maintained in a manner similar to that for other koji processes. Inoculation is done with pure mold spores. Mold spores are usually suspended in 0.1% Tween 80 (a wetting agent) before mixing with the substrate. The concentration of the spore is, of course, of prime importance, and is typically maintained at 10^7 spores per ml of suspension. Fermentation is carried out at around 30-32°C until the pH of the bran extract falls to 1.8-2.0, which corresponds to about 6 days. Aeration rate is optimally maintained at 0.8 vol/vol/min. After fermentation is over, the extract is recovered by maceration in water followed by filtration. The liquor is purified by precipitation with lime followed by regeneration with H_2SO_4. The final liquor is treated with activated carbon, concentrated, and crystallized. The yield is low because of the difficulty in controlling trace metals and process parameters.

2. Surface culture method

Production

The medium can be either synthetic (refined sugars) or complex (cane molasses). When cane molasses is used, the excess minerals must be removed prior to fermentation. Several treatment options are available for the same, for example, deionizing, use of sequestrants, etc. In trade fermentation, the deleterious effect of iron is counteracted by *dosing* alkali ferrocyanide (e.g., $K_4Fe[CN]_6$) as iron-chelating agent. This compound becomes toxic if present in the medium in excess. It is therefore customary to keep the free $K_4Fe[CN]_6$ below 20 µg/ml in the medium. Molasses medium should be adjusted to a pH of 5-6 (usually with H_2SO_4) although this is not the optimum pH for the mold. The main reason behind this is the presence of acetic acid in molasses. Unionized acetate (which occurs at lower pH values) represses spore germination. The pH is therefore brought to 5-7 to ionize acetic acid and make germination of spores favorable. This is not a problem when synthetic media are used. A pH as low as 2.5-3.0 can be used in the case of refined media. The sugar concentration of the medium is maintained at 15%. This level represents a good compromise. Sugar contents above 15% lead to increased residual sugar and accumulation of oxalic acid in the beer while sugar contents below 15% result in reduced yield.

Additional nitrogen requirement for the fermentation can be met by supplying $(NH_4)_2SO_4$. After normal sterilization procedure the medium is inoculated with spores of *Aspergillus niger* grown separately. The concentration of spore is typically 10^5-10^7 per ml of medium.

The spores of the culture are prepared in a stepwise manner from lyophil vials. The first step is to grow the organism in special *sporulation* agar. Thereafter it is transferred to other suitable medium for either mycelium build up or spore production. Both the forms (spores and mycelia) can be used for the main fermentation. When spores are to be used, they are usually suspended in suitable wetting agents, such as Tween 80, as the carrier.

Fermentation is carried out in very high-purity shallow aluminum trays. The ratio of medium volume to surface is maintained 1.22 ml/cm^2 for maximum performance. The organism is highly aerobic and hence must by supplied with adequate amount of oxygen. This is met by supplying air at the rate of 0.5-1.5 vol/vol/min. The inoculated trays are stacked in racks kept in rooms or compartment with provision for ventilation and continuous aeration. The temperature is maintained at around 30°C and the duration of fermentation ranges from 7 to 14 days. The final broth contains about 7% citric acid.

The fermentation must be continuously controlled. Laboratory analysis must be periodically carried out for citric acid and residual sugar contents.

Recovery

The first step in the recovery is filtration of mycelia in rotary vacuum filter (Fig. 17.10a and 17.10b). The mycelia must be repeatedly washed with water but without bringing about excess dilution. The citric acid in the liquor is precipitated out as calcium citrate followed by regeneration with H_2SO_4. For detail of recovery, see submerged fermentation described shortly.

3. Submerged culture method

Production

The preparation of medium is the same as that for surface culture. The inoculum is normally in the mycelial form.

Fermentation is carried out in very high-grade stainless steel fermenters. The fermenters can be of two main types, (i) *stirred, aerated, baffled tank*, and (ii) *aerated tower tank*. The cultural condition is also similar to that of surface culture. Aeration is done at a rate of 3×10^{-6} gram mole O_2/ml/min. Aeration is very critical during the final stage of fermentation: lack of O_2 leads to *remetabolism* of citric acid. The temperature is maintained at 30°C and the fermentation usually lasts for 4-5 days.

Stimulants are universally added in submerged fermentation. One of the most important stimulants used in citric acid fermentation is methanol. It is added at the rate of 3-4 % during fermentation. Methanol has been assumed to delay spore formation and alter cell wall permeability in *Aspergillus niger*, thereby increasing the yield. Antifoams (such as silicone oil, octadecanol, etc.) are also added during the fermentation.

There are some variations in the submerged culture process. In one variation, the fermentation is carried out in two stages, *viz*., *growth stage* and *production stage* in separate vessels. In another variation, the mycelium is reused up to 3 times: this eliminates the costly inoculum build-up stage.

Recovery

The beer is filtered in rotary vacuum filter to remove mycelia. The liquor can be treated by any of the two different methods, *viz*., (i) *classical method* and (ii) *solvent extraction method*, for refining citric acid. There are many other patented methods also.

The classical method, which entails precipitation of citric acid with lime and regeneration with H_2SO_4, is probably the most widely used method. The solvent extraction method uses combination of solvents for extraction of citric acid and back-extraction in alkaline aqueous phase as calcium citrate. The most commonly used solvents are mixtures of *tributyl phosphate* plus *kerosene*, and *tributyl phosphate* plus *butyl acetate* (100: 5-30).

The classical method of recovery

The filtered liquor is first treated with lime to selectively precipitate out oxalate. As a rule, lime is added to the liquor in calculated amount to allow spontaneous rise in temperature to 80-90°C. The precipitated calcium oxalate is removed by filtration and the liquor treated again with hydrated lime. Hydrated lime is added to the liquor at controlled rate (1 part hydrated lime per 2 parts liquor) over a period of 1 hr until the temperature of the liquor reaches ~ 95°C. This event precipitates out citric acid as calcium citrate. The insoluble calcium citrate is separated from the liquid portion by filtration and the precipitate treated with equivalent amount of H_2SO_4 to regenerate citric acid. The gypsum (calcium sulfate) resulting from this treatment is thrown away. The final steps of purification consist of decolorization, crystallization, drying, and packing. See Fig. 22.1 for an outline of recovery by classical process.

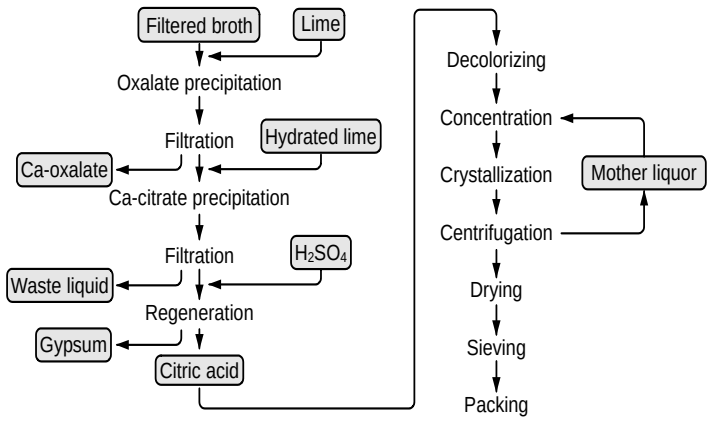


Fig. 22.1 Recovery of citric acid by precipitation-regeneration method

At 40°C, citric acid crystallizes out as the anhydrous acid, and below 36.5°C as the monohydrate.

Yield of citric acid

Under practical condition, the yield of citric acid is 70-90 g per 100 g of glucose. The theoretical yields from sucrose and glucose anhydrous are 123% and 117% respectively.

22.1.2 BIOSYNTHESIS OF CITRIC ACID

Citric acid produced by commercial strains of *Aspergillus niger* is typically an overflow product due to faulty operation of citric acid cycle. The major route of formation is the condensation of acetyl-ScoA and oxaloacetate. *Aspergillus niger* uses 78% of the sugar via EMP (Embden-Meyerhof-Parna) pathway. Oxaloacetate, which is constantly needed in the synthetic step, is regenerated by carboxylation of *triose* compounds or by *glyoxylate* cycle. It has been found that the enzymes leading to the synthesis of *cis* aconitate and isocitrate is rather inefficient in citric acid producing strains. This could be one of the reasons for overproduction of citric acid.

22.1.3 USES OF CITRIC ACID

Citric acid is used in food, confectionery and beverages, in pharmaceuticals and in industrial fields. Its use depends on three properties: acidity, flavor, and salt formation. A summary of the uses of citric acid is given in Table 22.1.

Citric acid forms a wide range of metallic salts including complexes with copper, iron, manganese, magnesium, and calcium. These salts are used as sequestering agents in industrial processes and as anticoagulant blood preservative. Citric acid also exhibits antioxidant properties in fats and oils where it reduces metal-catalyzed oxidation by clelating traces of metals such as iron. There are two components to its use as a flavoring: the first is due its acidity, which has little aftertaste; the second to its ability to enhance other flavors.

Table 22.1 Application of citric acid

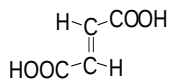
Industry	Property	Market share
Food		About 75%
Beverages	Acidulant	
Jellies, jams, etc.	Flavoring	
Fats and oils	Antioxidant	
Frozen foods	Antioxidant	
Pharmaceuticals		About 10%
Effervescent	Acid	
Vitamins	Antioxidant	
Anticoagulants	Sequestering	
Iron preparations	Salt formation	
Cosmetics	Buffering	
Industrial		About 15%
Cleaning (metals)	Sequestering	
Detergents	Buffering	
Photographic	Buffering	
Primer binding	Sequestering	
Polymerizations	Sequestering	

A process to remove sulfur dioxide from flue gases has been developed where citric acid is used as a scrubber, forming a complex ion which then reacts with H_2S to give elemental sulfur, regenerating citrate. This may become more important with increased environmental pressures.

Citric acid esters of a range of alcohols are known: the triethyl-, butyl- and acetyltributyl- esters are used as plasticizers in plastic films and monostyryl citrate is used instead of citric acid as an antioxidant in oils and fats.

22.2 FUMARIC ACID

Fumaric acid was formerly produced by fermentation, but now it is produced by hydrocarbon oxidation. The microbial process has fallen out of favor. The molecular structure of the acid is:



Fumaric acid is crystalline in nature. It is sparingly soluble in water (0.7 g/100 ml). Sodium- and potassium salts are readily soluble in water. The acid finds use in acidification of beverages, manufacture of aspartic acid, aspartame (a dipeptide artificial sweetener composed of aspartic acid and phenylalanine), and polyester fabrics. Trade fermentation utilizes improved strains of *Rhizopus nigricans*.

The acid is biosynthesized in the TCA cycle but can also be formed via glyoxylate cycle. Since ethanol can also serve as a carbon source, the pathway appears to be more than one.

22.2.1 MICROBIAL PRODUCTION

22.2.1.1 Cultural condition

Hexose sugars are the raw materials of choice. Molasses can also be used but the invertase activity is not possessed by all strains. The most common nitrogen source is ammonia or urea. Minerals play a very important role in fumaric acid fermentation. Zinc in particular should be kept at suboptimal level. Excess zinc allows the formation of acids other than fumaric acid. Methanol is generally used as a stimulant but the effect of methanol is quite different from that in citric acid production: the methanol molecule gets incorporated in the fumaric acid molecule.

22.2.1.2 Fermentation

No details are available for the fermentative production of fumarate. The inoculum is probably prepared as in the case of citric acid. The fermentation is carried out either as submerged- or surface culture, at 28-33°C. The pH is maintained at around 5-6 with constant addition of Na_2CO_3 or K_2CO_3. Continuous neutralization is essential because the organism is very sensitive to acidity resulting from fumaric acid accumulation. The acid being sparingly soluble in water tends to crystallize out in

mycelium. Addition of Na_2CO_3 or K_2CO_3 makes it soluble. $CaCO_3$ is not used for the neutralization because it also crystallizes. Aeration is a crucial aspect of fumaric acid fermentation. Deficiency of O_2 supply leads to accumulation of ethanol in the medium, which is undesirable.

22.2.1.3 Recovery

The first step is the familiar rotary vacuum filtration (Fig. 17.10a and 17.10b). The broth that contains the salt of fumaric acid is acidified thereby rendering the acid insoluble in water. The final step entails crystallization of the acid from hot water.

22.3 GLUCONIC ACID

D-gluconic acid or more correctly, pentahydroxy (α, β, γ, δ, ε) caproic acid, is an oxidation product of glucose. Its molecular formula is:

$$HOOC\text{-}(CHOH)_4\text{-}CH_2OH$$

Gluconic acid can be produced by microbial as well as chemical means. Both the methods are fully competitive.

22.3.1 USES OF GLUCONIC ACID

Gluconic acid finds wide use in food as well as non-food items, for instance:

1. Glucono-δ-lactone is used as a latent acid in baking powders, and in instant, chemically leavened breads
2. Sodium gluconate is used as calcium- and iron sequestrants
3. Gluconic acid is used as an ingredient in chemicals for removing *milk stone* in glass bottles
4. It can be used for the treatment of calcium deficiency diseases. Gluconic acid being non-toxic and metabolizable, it can be used for introducing cations like Ca^{++} into the body
5. Gluconic acid finds use as an additive in cement mixes
6. It is used in foliar formulation (for supplying trace minerals)
7. It is also used for the preparation of *chlorheximide* (a disinfectant)

22.3.2 BIOSYNTHETIC PATHWAY

The organism uses a mixture of pathways. The main pathway, which directly leads to gluconic acid, utilizes *glucose oxidase*, an FAD-dependent enzyme:

$$\beta\text{-glucose} \xrightarrow[\text{oxidase}]{O_2} \text{Glucono-}\delta\text{-lactone} + H_2O_2$$

Gluconic acid tends to form internal linkages to form δ, and γ *lactones*. All the three species exist in equilibrium. See Fig. 22.2 for the outline of biosynthesis.

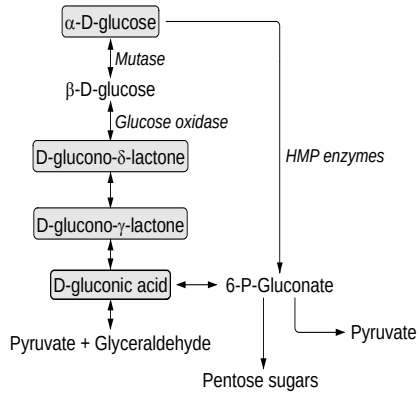


Fig. 22.2 Biosynthetic pathway of gluconic acid

22.3.3 MICROBIAL PRODUCTION OF GLUCONIC ACID

Commercial production of gluconic acid utilizes mold (*Aspergillus niger*) or bacteria (*Gluconobacter suboxydans*).

22.3.3.1 Cultural condition

Glucose is the main carbon source in the mold process. CSL, ammonium salts and urea are used for nitrogen source. Minerals must also be balanced. An outline of the production process (mold) is given in Fig. 22.3. The glucose concentration is kept at around 22%. The inoculum build-up process starts by sporulation in solid agar medium. The main inoculum is in the form of mycelia. About 10% by volume is used for the final inoculation. The pH is maintained at 6.5 with NaOH. Fermentation is carried out in stirred, baffled tanks at 30-33°C for a period of about 19 hrs. Aeration is done at the rate of 1.5 vol/vol/min (a back- pressure of 2 bars). NaOH is continuously added to maintain the pH: this also prevents the crystallization of gluconic acid in the pipelines.

Harvesting is preceded by a short rest period (30 min) to allow the mycelia to settle at the bottom of the fermenter. Later on the mycelia is forced out along with about 20% of the broth. This fraction is used again as inoculum, thereby reducing the *downtime*. After certain cycles, the mycelia is separated in a rotary vacuum filter and used as a source of glucose oxidase.

The broth contains all the three species of gluconic acid, *viz*., gluconic acid, glucono-δ-lactone, and glucono-γ-lactone. These species are separated by selective crystallization by preparing supersaturated solution and seeding with appropriate species. For example, gluconic acid can be crystallized out at temperatures below 30°C, glucono-δ-lactone at 36-57°C, and glucono-γ-lactone at temperatures above 70°C. Pure gluconic acid can be produced by full neutralization with $Ca(OH)_2$, followed by regeneration with H_2SO_4. The final step entails removal of residual calcium ion by ion-exchange resin.

The theoretical yield of gluconic acid from anhydrous glucose is 109% (m/m). Under practical conditions, the yield is 90% for a good fermentation.

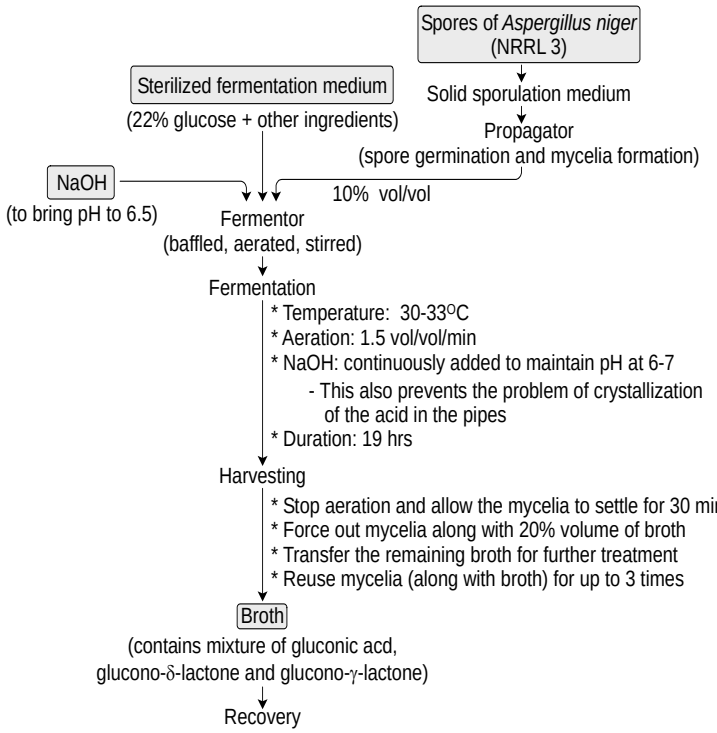


Fig. 22.3 Mold process for gluconic acid production

Gluconic acid can also be produced by bacterial fermentation. See Fig. 22.4.

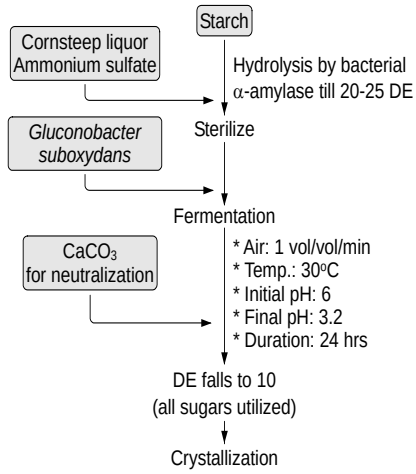


Fig. 22.4 Bacterial process for gluconic acid production

The basic raw material is starch. Cornsteep liquor and ammonium phosphate are added to meet nitrogen- and growth factor requirements. $CaCO_3$ is used during the fermentation for neutralization of the acid. The duration of fermentation is about 24 hrs.

CHAPTER 23

MICROBIAL PRODUCTION OF AMINO ACIDS

23.1 INTRODUCTION

Amino acids are building blocks of proteins. In mammals, and especially in man, a number of amino acids cannot be formed by generally known biosynthetic mechanism. This is basically because man cannot synthesize the *a-keto* acids needed for the synthesis of corresponding amino acids. Such amino acids are called *essential* amino acids (it is more correct to call them *indispensable* amino acids), and they must be supplied externally, for instance, through diet.

23.2 PRODUCTION ASPECT

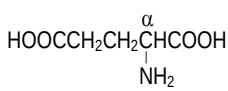
Amino acid can be synthesized quite economically by chemical means. Chemically synthesized amino acids are usually *racemic* mixtures of D- and L-isomers. It is to be noted, only the L-isomer is of value for flavor application or for food/feed supplement. The D-isomers are biologically inactive. To date, chemical resolution of DL-racemic mixtures has been relatively expensive, although a Japanese process is apparently in commercial use.

Microbiological production of amino acids on the other hand does not involve operational difficulties of high temperature and pressure often encountered in chemical catalytic processes. The microbial process can be carried out under ambient conditions. Besides, the end products produced by them can be obtained in a pure form because the enzyme systems in an organism are known for high selectivity.

23.3 PRODUCTION OF L-GLUTAMIC ACID

In 1995, the annual worldwide production of glutamic acid was 370,000 MT. By 2002, the production reached 1 million MT. The main producers of glutamic acid are Japanese companies: Ajinomoto Co., and Kyowa Hakko Kogyo Co.

Glutamic acid is a negatively charged dicarboxylic acid having the structure:



23.3.1 USES

Monosodium salt of glutamic acid (MSG) is used as flavor enhancer. It enhances the flavor of meat and meat products. Glutamic acid is also the starting material for a variety of specialty chemicals. *N-acyl glutamate* is used in cosmetics, soaps, and

shampoos. *Oxypyrrolidone carboxylic acid* is used as a natural moisturizer. Amides of glutamates can be used as gelatinizing agents: it can gelatinize mineral oil spilled in the ocean. In particular, this property can be gainfully utilized for marine antipollution purposes.

23.3.2 MICROORGANISMS

Most glutamic acid producing bacteria are Gram-positive, non-spore forming, non-motile, and biotin dependent. Examples of some of the more important glutamic acid bacteria are given in Table 23.1. Overproduction of glutamic acid is possible through the use of organisms dependent on biotin, oleic acid, or glycerol (they are auxotrophic mutants).

Table 23.1 Examples of commercially employed glutamic acid bacteria

Genus	Representative organism
Brevibacterium	*B. divericatum, B. flavum*
Corynebacterium	*C. glutamicum, C. lilium*
Microbacterium	*M. flavum* var *glutamicum*
Arthrobacter	*A. globiformis*

23.3.3 BIOSYNTHESIS OF GLUTAMIC ACID

Glutamic acid is synthesized through (i) *glyoxylate cycle* as an oxaloacetate generating system (without CO_2 fixation), and (ii) through *Phosphoenol pyruvate* (PEP) to form oxaloacetate with CO_2 fixation. See Fig. 23.1 biosynthetic pathway.

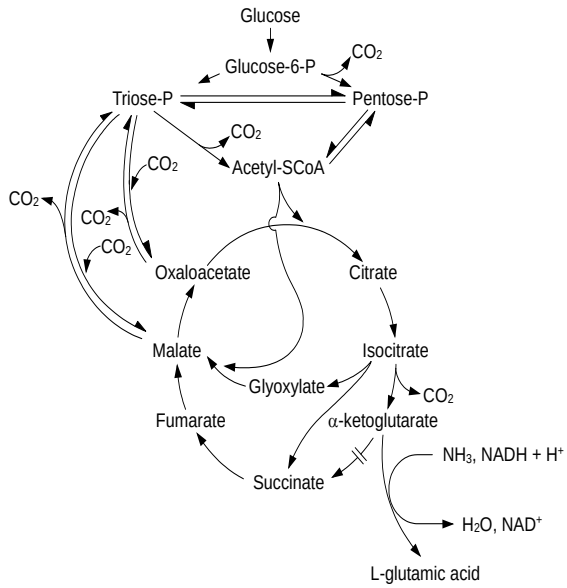


Fig. 23.1 Biosynthesis of glutamic acid

The bacteria use both *EMP* and *HMP* pathways. Compounds from these pathways are fed into *TCA cycle*. In all, the bacteria use about 16 enzymatic steps. The final product is formed by reductive amination of α-ketoglutarate.

Two enzymes play very important role in the biosynthesis of glutamic acid. They are (i) *PEP carboxylase*, and (ii) *α-ketoglutarate dehydrogenase*. The efficiency of CO_2- fixation depends on PEP carboxylase activity. α-ketoglutarate dehydrogenase can transform α-ketoglutarate to glutamic acid as well as CO_2 + water via succinyl-ScoA. Bacterial α-ketoglutarate dehydrogenase is such that it carries out the preferential synthesis of glutamic acid at a rate several times faster than that for the oxidation.

23.3.4 GLUTAMIC ACID EXCRETION AND CELL WALL PERMEABILITY

The overproduction of glutamic acid is in fact a *function of cell wall permeability* of the bacterium. Under normal condition, the cell wall is impervious enough to block the flow of glutamic acid that has been synthesized inside the cell. Accumulation of glutamic acid inside the cell soon exerts *product inhibition* and the organism, in response to this, stops the synthetic reaction. This is an undesirable aspect when it comes to overproduction of glutamic acid. The elucidation of biochemistry of glutamic acid biosynthesis has made it possible to overcome this effect. The basic strategy used in this case is to weaken the cell wall of the bacterium. This can be achieved by adding, during the growth phase, agents capable of inhibiting cell wall synthesis, e.g., penicillin, cephalosporin, detergents, etc. An equivalent effect can be achieved by limiting biotin content in the medium or by supplying saturated C_{16} and C_{18} fatty acids. The explanation for the last sentence runs as follows: biotin is a cofactor of acetyl-ScoA carboxylase, an enzyme responsible for the conversion of acetyl-ScoA to malonyl-ScoA (the starting compound for the synthesis of fatty acid, *viz*., oleic acid). Good supply of biotin in the medium leads to normal production of oleic acid. Oleic acid in turn combines with inositol, mannose, etc., to form phospholipid, which is the component of cell membrane (see Fig. 23.2). Limiting biotin in the medium leads to synthesis of weak cell membrane, thus producing *leaky cells*. Leaky cells cannot withhold the glutamic acid synthesized inside the cell. The addition of saturated fatty acids also has similar function. They repress the synthesis of oleic acid.

The elucidation of biochemistry of glutamic acid biosynthesis has been a turning point. It has made possible to use molasses for glutamic acid production. The initial failure was, of course, due to high biotin content in the medium.

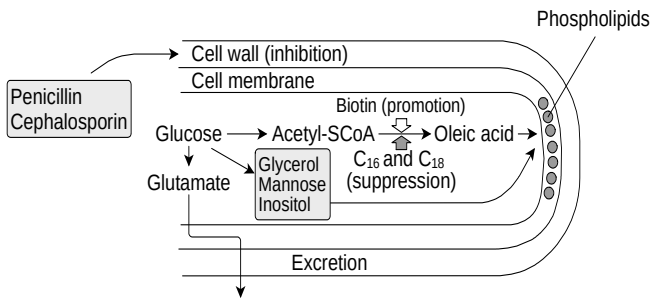


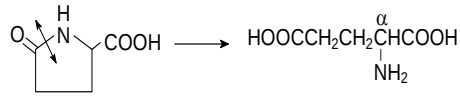
Fig. 23.2 Relation between cell wall and excretion in glutamic acid bacteria

23.3.5 PRODUCTION METHODS

Glutamic acid can be produced by four methods:

1. By the hydrolysis of wheat gluten, soybean cake, or other proteinaceous materials

2. By cleavage of pyrrolidone carboxylic acid (5-oxoproline)



5-oxoproline Glutamic acid

3. By one-stage fermentation (involving one organism)

4. By two-stage fermentation process (one organism produces α-ketoglutarate and another organism produces glutamic acid from α-ketoglutarate)

Because one-stage fermentation is universally used, discussion on the glutamic acid production will be limited to one-stage fermentation, which may be of batch-, fed-batch-, or continuous type.

23.3.5.1 One stage fermentation process

The most widely used carbon and energy source is the carbohydrate, such as molasses and starch hydrolysates. Certain strains can also utilize non-carbohydrate materials such as acetic acid. When molasses is used, most of the growth requirements are met. The nitrogen requirement is met normally by supplying gaseous ammonia. Gaseous ammonia fulfils several objectives: (i) supplies nitrogen, (ii) maintains pH by neutralizing the accumulated acid, and (ii) avoids unwanted dilution of the medium.

Whatever the mode of operation (batch, fed-batch, or continuous), the basic strategy during the production phase (which is different from the active growth phase) is the creation of a condition unfavorable for further growth of the organism (either by limiting key components or by adding agents that interfere with their growth).

The fermentation is a highly aerobic one. Air pressure in the fermentation vessel is critically maintained slightly above the actual requirement for cellular respiration. Inadequate air supply leads to accumulation of lactic and succinic acid while excess air supply promotes α-ketoglutarate accumulation.

Batch process

In the batch process, the sugar concentration is maintained at 10% glucose equivalent. pH is maintained near neutrality with ammonia gas. The biotin content in the medium should be suboptimal: it has been worked out that biotin should be 1-5 μg/liter of medium. Penicillin and similar other agents are added (to weaken the cell wall) during the growth phase. The duration of fermentation is around 3 days at 30-

35°C. Glutamic acid excretion starts after the intracellular concentration of glutamic acid has reached 50 mg/g of dry cell.

Nakashi and coworkers (1981) have patented a method which uses *Corynebacterium glutamicum* that has been mutated to acquire temperature-sensitivity remediable with an unsaturated higher fatty acid. The organism can readily overproduce glutamic acid even in biotin-rich medium. Additionally, the process does not require addition of agents for counteracting effects of biotin.

In this method, the mutant is initially cultured at a low temperature (28°C) environment wherein the strain shows adequate growth until multiplication proceeds to a certain desired extent. The temperature is then elevated to 40°C wherein growth is reduced without an unsaturated fatty acid. This near-starving condition leads to overproduction of glutamic acid whether the medium contains unsaturated fatty acids or not.

The pH preferred for the fermentation is 6-9. A duration of about 72 hrs appears to be sufficient.

Fed-batch fermentation process

The medium composition is basically similar to that for a batch process. At the beginning of the fermentation, 0.65 ml oleic acid/liter of medium is added. The pH is set at 8.5 with ammonia and automatically maintained at 7.8 during the course of fermentation. After about 14 hrs, the temperature is increased from an initial 32-33°C to 38°C due to growth. After the initial glucose is metabolized down to the level of 0.5-2% (from an initial of about 12% glucose equivalent in the molasses medium) glucose feeding is done until the fermentation is complete. On an average, 160 g of glucose is fed per liter of medium. Glutamic acid content is analyzed hourly. Aeration is controlled in such a way that CO_2 of the exhaust gas does not exceed 4.5% by volume. Fermentation is stopped after 30-35 hrs after the glutamic acid production reaches about 100 g/liter. In general, the fermentation titer of glutamic acid in industrial production is about 88 g/ liter.

Continuous process

Reference will be made here to the invention made by Tatsuya and coworkers (1999), the work being assigned by Ajinomoto Co., Japan. Their system of continuous fermentation allows simultaneous growth (of glutamic acid bacteria) and accumulation of glutamic acid. Fermentation is carried out in a single fermenter. There is no provision for cell recycle. Unlike in fed-batch or batch process, there is no requirement for terminating the cell growth for inducing glutamic acid accumulation. The schematic of the system published by the investigators is given in Fig 23.3.

In this system, medium containing adequate nutrients for growth of the bacteria is continuously fed but the growth is controlled through temperature, surfactants, antibiotics, biotin concentration, and the like. After addition of the inoculum to the fermenter, feeding and extraction of the medium is started from an appropriate stage, intermittently or continuously. An example of the experimentation published by the investigators is as follows:

The organism used for the study was glutamic acid producing bacteria *Brevibacterium lactofermentum* ATCC 13869. The organism was grown in a shaker flask containing sterilized (115°C for 15 min) medium of following composition at 30°C for 24 hrs: 3% glucose, 0.01% KH_2PO_4, 0.004% $MgSO_4.7H_2O$, 0.4% urea, 0.002% $FeSO_4.7H_2O$, 0.002% $MnSO_4.4H_2O$, 1.5% liquid soy protein hydrolysate, and 300 μg biotin/liter.

The seed culture was transferred aseptically to a small fermenter containing following medium composition and having provision for agitation, aeration, feeding, extraction, etc., needed for the continuous fermentation: 6% glucose, 0.01% KH_2PO_4, 0.01% $MgSO_4.7H_2O$, 1.5% liquid soy protein hydrolysate and 300 μg biotin per liter of medium. The pH was adjusted to 7.5 with NH_3 and aeration done at the rate of 1.1 vol/vol/min. Fermentation was carried out at 30°C.

After 5 hrs of culturing, polyoxyethylene sorbitan monopalmitate (a surfactant) was added to a final of 500 mg/liter. To the fermenter, a feeding solution containing 18% glucose, 0.01% KH_2PO_4, 0.01% $MgSO_4.7H_2O$, 1.5% soy protein hydrolysate and 500 mg/liter of polyoxyethylene sorbitan monopalmitate was continuously added at the dilution rate of 0.11 per hour (that is, the same volume was continuously extracted). The sugar concentration of the extracted culture was maintained at 5 g/liter or less.

The outcome of the cultivation for 40 hrs was such that the yield of glutamic acid was 56% and the productivity 5 g/liter/hr. Compared to fed-batch method (control used in this experiment), the productivity in the continuous culture was found to be 2-fold higher. The productivity was calculated as follows:

$$\text{Productivity (g/liter/hr)} = \frac{\left(\begin{array}{c}\text{Glutamic acid concentration}\\ \text{in drawn-out solution (g/liter)}\end{array}\right) \times \left(\begin{array}{c}\text{Flow rate of drawn-out}\\ \text{solution (liter/hr)}\end{array}\right)}{\text{Working volume in fermenter (liter)}}$$

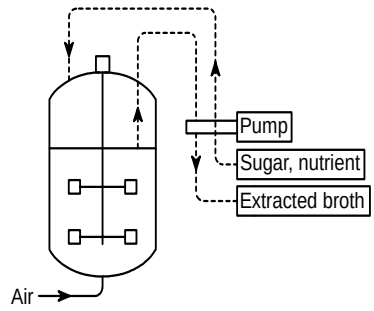


Fig. 23.3 Schematic of continuous fermentation

23.3.6 COMMERCIAL YIELD OF GLUTAMIC ACID

After the growth phase, an ideal fermentation should proceed as:

$$C_6H_{12}O_6 + NH_3 + 1.5\ O_2 \rightarrow C_5H_9O_4N + 3\ H_2O$$

This represents a 100% molar conversion, or 81.7% weight conversion of glucose anhydrous to glutamic acid. Under practical condition, the molar conversion is 50 to 75% by *resting* cells.

23.3.7 PURIFICATION OF GLUTAMIC ACID

Before commercial methods of glutamic acid purification are discussed, a brief mention of the polymorphism exhibited by the acid will be made here. Polymorphism, contextually, is the existence of a chemical compound to adopt different crystalline arrangements. Although chemically identical, different polymorphs display a variation in physical properties (e.g., crystal morphology, density, solubility and color) which exert an influence on the performance of the product, for example, the bioavailability and shelf-life of pharmaceutical compounds. L-glutamic acid exhibits two polymorphs, viz., (i) the metastable α-form that is prismatic or granular in nature, and (ii) the stable β-form that is needle-shaped. When a saturated solution of glutamic acid is heated to 70°C and cooled rapidly, α-crystals are formed. When the saturated solution at 90°C is cooled gradually to 40-50°C, β-crystals are formed. The β-form is not preferred because it hinders subsequent filtration or centrifugation associated with the purification steps.

In the industrial processes, the α-form of glutamic acid is generally preferred because of the ease in subsequent downstream processing. However, because of its metastable nature, the crystals will slowly transform into the stable β-form at elevated temperatures (above 55°C). During the process, dehydration of glutamic acid inevitably takes place thereby leading to the formation of appreciable amounts of pyrrolidone carboxylic acid. Various methods have been developed to increase the purification rate and minimize loss. These developments have largely been based on the manipulation of temperature-, solvent addition-, and solution concentration regimes. A method developed by Gallagher (1976) is as follows:

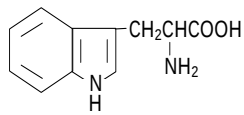
The fermentation broth containing glutamic acid not less than 60 g/liter (if it is less, evaporation can be carried out) is adjusted to pH 4.5 with H_2SO_4 at room temperature (25°C). If the pH is already low, it is adjusted with NH_3. The broth is agitated constantly for about 20 min until seed crystals begin to appear. Thereafter, the broth is acidified to pH 3.2 (isoelectric pH of glutamic acid) and heated to 50-55°C (heating above 55°C is avoided because this leads to formation of β-crystals). Next, continuing agitation, the temperature of the mixture is brought down to 20°C. Crystals that consist of 70-80% α-form will now be formed. The crystals are recovered by centrifugation or filtration. The product can now be further purified to meet the exacting criteria (e.g., recrystallization, ion exchange, decolorizing, etc). If needed, the acid can be neutralized with NaOH to obtain monosodium glutamate.

Recently, Yoshiki and coworkers (2005), have filed a patent (assignee: Ajinomoto Co., Japan) of glutamic acid purification. The method involves rapid transformation of primary crystals into β-form (which is later recovered) by the application of activated carbon. The principle of the method is as follows:

Broth containing about 150 g glutamic acid per liter is mixed with 1% activated carbon (w/w, based on the amount of glutamic acid) and heated to 90°C. The mixture is stirred at this temperature for sufficient time interval until at least 30% of the α-crystals of glutamic acid are converted into the β-form. The addition of activated carbon has a very prominent effect in increasing the rate of transformation. The experimental finding showed that the transformation time for carbon-treated glutamic acid broth was 75 min against a control (containing no carbon), which was 588 min. Similarly, the level of pyrrolidone carboxylic acid formed was 4 mole% for the treatment and 28 mole% for the control.

23.4 MICROBIAL PRODUCTION OF L-TRYPTOPHAN

L-tryptophan is an aromatic, indispensable amino acid. Its chemical name is α-amino-β-indole propionic acid. The condensed structure is:



This amino acid is produced by Japanese companies, *viz*., Showa Denko, Ajinomoto Co., and Tanabe Seiyaku. The annual world production in 1995 was 400 MT. By 2002, the production volume reached 600 MT.

23.4.1 USES

Therapeutically, it is used as a component solution for transfusion. Since it is an indispensable amino acid, it can also be used for the fortification of food commodities like corn (maize) that contain limiting amounts of tryptophan.

23.4.2 MICROORGANISMS USED

Microorganisms used for the production of tryptophan are highly improved strains of bacteria. Some of the industrially exploited or tested microorganisms are: *Corynebacterium glutamicum*, *Brevibacterium flavum*, *Bacillus subtilis*, *Candida fumicola*, *Achromobacter liquidium*, *Pseudomonas putida*, etc.

23.4.3 BIOSYNTHETIC PATHWAY

The microorganism uses *chorismic* acid pathway for the synthesis of tryptophan. Since the synthesis of phenylalanine and tyrosine also share the same pathway, it is obvious that tryptophan-producing strains are auxotrophic mutants. The outline of the biosynthetic pathway followed by *Corynebacterium glutamicum* strain is shown in Fig. 23.4.

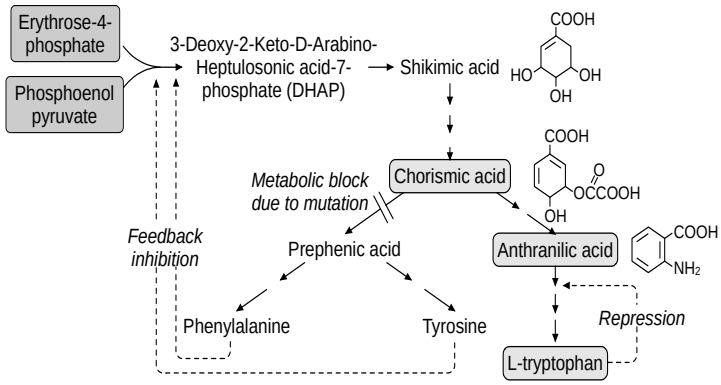


Fig. 23.4 Biosynthetic pathway of tryptophan in *C. glutamicum*

23.4.4 PRODUCTION METHODS

There are three main methods for the production of tryptophan:

1. *Production by fermentation*
2. *Production by microbial conversion*
3. *Production by enzymatic method*

Detailed information on any of the above methods is not available. Literatures are therefore based only on classical researches and patents filed for the method. Because of relevance, only two of the above methods will be described here.

23.4.4.1 Production by fermentation

In the overproduction of tryptophan by fermentation, the basic strategy is to obtain auxotrophs and/or analog resistant strains by mutation. Mutants carrying multiple markers are more suitable as they are more stable. The classical work carried out by Nakayama (1976) is used here as an example. He used *Corynebacterium glutamicum* KY 9456, a double auxotroph of phenylalanine and tyrosine for further mutation. A stepwise mutation finally produced a strain called Px-115-97 that produced significantly higher amounts of tryptophan. The parent strain was mutated in a stepwise manner to develop resistance to 5-methyl tryptophan ($5MT^r$), tryptophan hydroxamate ($TrpHx^r$), 6-fluoro tryptophan ($6FT^r$), 4-methyl tryptophan ($4MT^r$), parafluoro phenylalanine (PFP^r), paraamino phenylalanine (PAP^r), tyrosine hydroxamate ($TyrHx^r$), and phenylalanine hydroxamate ($PheHx^r$). The genealogy of the bacterium used in the study appears in Table 23.2.

The auxotrophy produced metabolic block while the analog resistance released the bacterium from repression by tryptophan. The yield gradually increased from a mere 0.15 g/L to final of 12 g/L in a nutritionally balanced cane molasses medium of following composition: cane molasses (10% glucose equivalent), $MgSO_4.7H_2O$ (0.025%), KH_2PO_4 (0.05%), K_2HPO_4 (0.05%), $(NH_4)_2SO_4$ (2%), $CaCO_3$ (2%), and Cornsteep liquor (2%). The pH was kept at 7.2. The organism was still sensitive to

phenylalanine and tyrosine, which implied that there was further scope for the development by building multiple analog resistances.

Table 23.2 Genealogy of *C. glutamicum* mutated for tryptophan production

Genealogy/mutation	Production (g/liter)
KY 9456 Phe^{-},Ty^{-}	0.15
↓ $5MT^{r}$, $TrpHx^{r}$, $6FT^{r}$, $4MT^{r}$	↓
4MT-11	4.9
↓ PFP^{r}	↓
PFP-2-32	5.7
↓ PAP^{r}	↓
PAP-126-50	7.1
↓ $TyrHx^{r}$	↓
Tx-49	10
↓ $PheHx^{r}$	↓
Px-115-97	12

Following Nakayama's work, several workers (Kino and coworkers, 1988; Ozaki and coworkers, 1989; Ishida and coworkers, 1989, etc.) have patented improved methods for the microbial production of tryptophan. Most of the works are based on genetically engineered strains of *Corynebacterium glutamicum*. In the commercial fermentation, the fermentation titer is about 58 g/liter.

23.4.4.2 Production by microbial conversion

Various microorganisms including auxotrophic- and regulatory mutants were selected by different workers. *Candida fumicola*, *Corynebacterium glutamicum*, *Bacillus subtilis*, and *E. coli* were studied. In this method, precursor of tryptophan such as anthranilic acid or indole (which is toxic to microorganisms at higher concentrations) is used in the medium. These precursors are chemically synthesized. The conversion is catalyzed by tryptophanase (= tryptophan synthetase). A bioconversion method developed by Stephen and coworkers (1990) utilizing recombinant *E. coli* will be described next.

These workers have described a multi-stage microbial process for the production of tryptophan where biocatalyst and bioconversion stages are segregated. In the biocatalyst production stage bacterial host cells are transformed with a vector containing a DNA sequence coding for tryptophanase wherein the expression of tryptophanase gene is directly controllable; the transformed cells are induced to synthesize tryptophanase; and then in a subsequent bioconversion stage the reaction substrates for tryptophan synthesis are added and tryptophan which accumulates in the reaction mixture is optionally isolated.

In an example, the workers used recombinant *E. coli* MD33 (in which plasmid encoding for tryptophan was used, e.g., PIMS1015) as a tryptophanase producing

host. The engineered cells were first grown in a suitable broth at 30°C to maintain the plasmid in low copy number state. Once the desired cell density was reached, the temperature was raised to 37°C to induce an increase in the copy numbers of the plasmid, with a concomitant rise in the amount of tryptophanase produced. The tryptophanase levels reached 15-90% of total cell protein.

The cells were harvested and transferred to bioconversion vessel which contained cosubstrates consisting of ammonium acetate (870 m*M*), sodium pyruvate (620 m*M*), KH_2PO_4 (22 m*M*), pyridoxal-5'-phosphate (0.7 m*M*), and ethanol (870 m*M*). The pH and temperature were maintained at 8.5 and 30°C, respectively. Fermentation was carried out in a fed-batch mode by continuously feeding 5 *M* indole (suspended in ethanol) such that the concentration of indole in the fermenter was less than 10 m*M*. After a fermentation time of 75 min, the tryptphan titer was 24 g/liter, with a productivity of 19 g/liter/hr. More than 99% of the feed indole was found to be converted to tryptophan.

23.4.4.3 Production by enzymatic method

This method utilizes the enzyme *tryptophanase*, which catalyzes reversible synthesis of tryptophan. The enzyme is produced by bacteria such as *Achromobacter liquidium*, *Pseudomonas putida*, etc. Depending on the organism used, the major substrate can be indole or indole derivatives.

Interesting as the above two methods appear, they have not gained commercial importance because the costs of precursors such as indole and anthranilate are prohibitively high at the present time.

23.4.5 RECOVERY

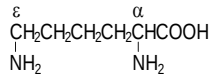
The culture broth is subjected to strongly acidic cation exchange resin. The adsorbed tryptophan is then eluted from the resin with 0.5 *N* aqueous ammonia, and crystallized to obtain crude crystals. The latter is dissolved in a small amount of hot, 50% aqueous ethanol, decolorized with activated carbon, and recrystallized to obtain pure tryptophan crystals.

A method patented by Kono and coworkers (1991) is claimed to be simple and economical. In this process, the crude tryptophan is dissolved in hot (~ 90°C) water-acetic acid solution (1+1) and heated for 2 hr. Activated carbon and filter aids may also be added in the mixture. The mixture is now cooled to 5-10°C to crystallize tryptophan (tryptophan crystallizes at concentrations exceeding 25 g/liter). The crystals are filtered, washed with cold water, and dried under reduced pressure. The purity and yield claimed for this process are 99.1% and 98.9%, respectively.

23.5 MICROBIAL PRODUCTION OF L-LYSINE

L-lysine or α, ε-*diaminocaproic* acid is indispensable to man. It is a limiting amino acid in cereal grains. Over 80% of the lysine commercially produced is used in animal feed, and the annual demand is rising at the rate of 10%.

The condensed formula of lysine is:



Commercially, lysine is available as *lysine monohydrochloride*. The amino acid can be produced by chemical as well as microbial method. The annual world production of lysine in 1995 was 70,000 MT. By 2002, the production volume reached about 600,000 MT.

23.5.1 BIOSYNTHESIS OF L-LYSINE

Lysine biosynthesis can occur by two different pathways, *viz*., (i) *Diaminopimelate pathway*, and (ii) *Aminoadipate pathway*. The former pathway is found in bacteria, certain lower fungi, algae, and higher plants while the latter is found in classes of lower fungi, higher fungi, and *Euglena* (flagellated protozoa).

In the diaminopimelate pathway, the carbon chain is synthesized from pyruvate and aspartate (and thus categorized as member of *aspartate family*). Other members of amino acids that share diaminopimelate pathway are threonine, isoleucine, and methionine. The important steps of diaminopimelate pathway are given in Fig. 23.5.

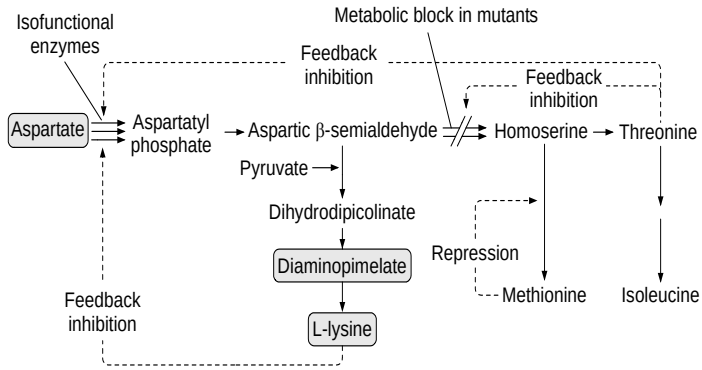


Fig. 23.5 Diaminopimelate pathway for lysine synthesis

23.5.2 PRODUCTION METHODS

Great developments have been made in lysine production technology since the first discovery of microbial production of lysine in the 1950s. Today, lysine can be produced by chemical-, microbial-, or enzymatic processes. The microbial process, which is still at the forefront, utilizes genetically improved microorganisms. The improvements are based on overproduction by the improved strains, development in fermentation protocols (media formulation, optimization of process variables, etc.), and refinement in purification techniques.

Microbial methods of production can be classified as:

1. *Production by homoserine auxotrophs*

2. *Production by multiply improved strains*
3. *Production by enzymatic method*

Although several methods of lysine production exist today, their details are still being closely guarded. Because of relevance, only a few methods will be described in the following sections.

23.5.2.1 Production by homoserine auxotrophs

The microorganisms used here are auxotrophic mutants of *C. glutamicum*. Double auxotrophs, which require in addition to homoserine at least one of amino acids, *viz*., thre, met or ile for growth have been found to be highly stabilized. It may be stated that the overproduction is due to the release of *aspartokinase* from concerted feedback inhibition by branch end products due to the metabolic block (Fig. 23.5). Fermentation is carried out in batch-, fed-batch-, or extended fed-batch mode. Patents are appearing for continuous fermentation also.

In batch fermentation, microorganism grows until one or more of essential nutrients is (are) exhausted or until fermentation conditions become unfavorable. In fed-batch- or extended fed-batch fermentations, one or more nutrients is (are) continuously or intermittently supplied to the culture medium, either from the beginning of fermentation or after the culture has reached a certain stage, or when nutrients are exhausted. The microorganism grows at a growth rate dictated by the rate or timing of nutrient feed. In general, a single nutrient (very often a carbon source such as glucose) is fed into the fermenter in order to overcome substrate inhibition and high osmotic pressure.

An interesting variant of extended batch or fed-batch fermentation is the repeated batch or fed-batch or fill-and-draw fermentation. In this method, a part of the fermentation broth is removed at a certain time of operation, while feeding continues. This extends the fermentation operation and leads to high product concentration.

The carbon sources for the production can be molasses, starch hydrolysates, and in some cases, acetic acid and ethanol. Cane molasses is the most important, though. The fermentation occurs at neutrality. NH_3 can be added to control the pH and meet nitrogen requirement. Biotin is very important for growth and production: it must be greater than 30 μg/liter. The requirement of biotin is variable and so is the explanation behind it. In biotin-dependent strains, the excretion results from the leaky cell wall. On the other hand, biotin is a coenzyme needed for the decarboxylation-conversion diaminopimelate to lysine.

The seed culture is prepared in stages (see Table 23.3). The final seed culture necessarily contains cane molasses for acclimatizing the organism with future environment. The production medium contains 20% glucose (from cane molasses) and 1.8% soybean meal hydrolysate. The amounts of growth factors (homoserine or threonine and methionine) are added in suboptimal levels. Since the most important intermediate is aspartic acid, its inclusion in the medium increases the yield of lysine.

Fermentation is carried out at about 28°C. Aeration is a crucial aspect of lysine fermentation. It is kept at greater than the actual requirement for respiratory growth. Oxygen deficiency may lead to lactic acid production at the cost of lysine, although not as significant as in the case of glutamate production. The duration of fermentation is about 3 days. The yield is about 40-50% based on sugar consumed.

Table 23.3 Composition of the seed culture medium for lysine production

Seed culture 1		*Seed culture 2*		*Main culture*	
Glucose	2%	Cane molasses	5%	Molasses	(20% glucose-equivalent)
Peptone	1%	$(NH_4)_2SO_4$	2%	Soybean meal hydrolysate	1.8%
Meat extract	0.5%	Cornsteep liquor	5%		
NaCl	0.25%	$CaCO_3$	1%		

23.5.2.2 Production by enzymatic method

This method is used by Toray Company, Japan. The method in principle utilizes two enzymes, *viz*., *racemase* and *hydrolase* to transform DL-α-*aminocaprolactam* (DL-ACL) to lysine. In the industrial process, DL-ACL is produced synthetically using chemicals such as NOCl, cyclohexane, NH_3, HCl, etc., in a series of reaction steps (Fig. 23.6). ACL is a compound industrially used in the prepraration of synthetic fibers, such as Nylon-6.

Racemization and hydrolysis are the final reactions for producing L-lysine. The reactions may be outlined as:

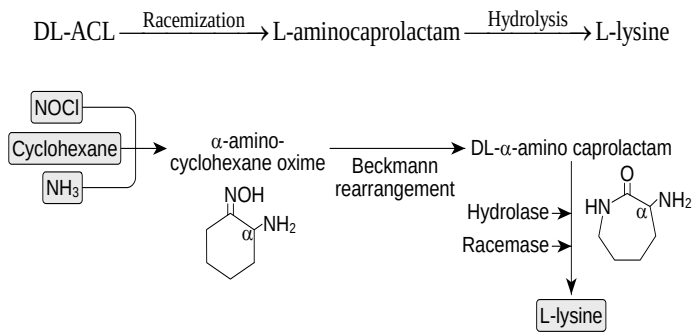


Fig. 23.6 Outline of enzymatic synthesis of L-lysine

Aminocaprolactam racemase (EC 5.1.1.15) is produced by *Achromobacter obae* using DL-ACL as an inducer. This enzyme specifically acts on neutral cyclic amides. *Cryptococcus laurentii*, another organism, produces hydrolase inductively in a medium containing L-ACL, glucose and other components. A similar optimum pH values for both the enzymes allows efficient conversion, which appears to be a single step.

Industrially, resting cells of the above two organisms are used for the production of enzymes. There is no detailed information about the use of enzyme but literatures

on its study are available. It has been reported that incubation of 100 ml of 10% DL-ACL (pH adjusted to 8 with HCl) with 0.1 g of acetone-dried cells of *C. laurentii* and *A. obae* nov. sp. at 40°C for 24 hrs resulted in 99.8% conversion of DL-ACL to L-lysine.

The amino acid produced by enzymatic means is relatively free from debris. It is therefore much easier to purify the amino acid. A very high-grade lysine can be obtained by carbon treatment and crystallization.

23.5.3 PURIFICATION OF LYSINE

Several methods are available for lysine purification, and still more are being developed. The extent of purification is dictated by the intended end use of the product. For animal feed supplement, the product can be produced in the form of liquid concentrate, powder, or grains. A general method for obtaining lysine of very high degree of purity (for food- and pharmaceutical use) is described in the following paragraphs.

The fermented broth (containing lysine) is treated with $Ca(OH)_2$ to bring the pH to 11. It is then heated to 100°C for 30 min and aerated at 1 vol/vol/min for 2 hrs. The resulting mixture is acidified to pH 5 with HCl or H_2SO_4 to precipitate the calcium (as $CaCl_2$ or $CaSO_4$). The precipitate and the cells are removed by filtration or centrifugation and the filtrate/supernatant is passed through cation exchange resin (IR-120, NH_4^+ type) in one or more stages. Then the ion exchange column is washed with distilled water. Next, lysine is eluted with 3% NH_4OH. The resulting product is concentrated by evaporation and the pH is again adjusted to 5 with HCl. The mixture is cooled to 20°C to obtain crystals of lysine-HCl (~ 98% purity). The crystals are recovered by centrifugation and then dried in fluidized bed to less than 1% moisture content.

CHAPTER 24

YEAST ENZYMES AND MINOR PRODUCTS

24.1 INTRODUCTION

Yeast enzyme production differs from other yeast processes, *viz*., bakers yeast production and SCP production in two ways:

1. Yeast enzymes are low volume-high value products
2. Fermentation process is largely secondary to downstream process

Yeast enzymes are of two types: (i) *intracellular*, and (ii) *extracellular*. Intracellular enzyme production poses certain problems: (i) enzymes are produced in very small amounts (due to feedback inhibition, repression, etc.), (ii) being inside the cell, they cannot be taken out unless a mechanism for disrupting the cells is used. This calls for added cost. Besides, there is the problem of separating nucleic acids that come along as contaminant. Unless in the case of very high value enzyme, production of intracellular enzyme is not cost-effective. This is the basic logic why emphasis has almost always been on the production of extracellular enzymes.

24.1 JUSTIFYING THE DESIGN OF SPECIALIZED EQUIPMENT

For intracellular enzyme production, construction of specialized equipment is not always justified. Since the fermentation is largely *secondary* to downstream processing, the former can be totally circumvented if a ready-source of biomass that contains the enzyme is available. In the case of extracellular enzymes, however, fermentation is a *must*, as it is during the course of fermentation that extracellular enzymes are secreted. Even in such cases fermentation economics must be thoroughly considered. The equipment constructed must be of multipurpose type to ensure flexibility in use. Thus, taking everything into account, it is essential that the equipment designed be small but flexible, for example, stirred tank with aeration.

Examples of some of the important yeast enzymes are: Invertase or sucrase(= β-D-fructosidase), lactase (= β-D-glactosidase), lipase, etc.

24.2 INVERTASE

The enzyme is also called *sucrase*. The scientific name is β-D-fructofuranoside fructohydrolase (EC 3.2.1.26) but can be called β-D-fructosidase in short. Both intra- and extracellular invertases are present in yeasts. Extracellular invertase is more abundant than the intracellular counterpart. Extracellular invertase is a glycoprotein (containing 50% mannose) with a molecular weight of 127,000. Intracellular invertase is non-glycosylated and has a molecular weight of 120,000.

The natural substrates of invertase are sucrose, raffinose, and stachyose. The enzyme works optimally at 60°C, has an optimum pH of 5.5, and acts selectively on the fructosidic linkage on the fructose side of the oxygen bridge. See Fig. 24.1.

Invertase was the first enzyme to be immobilized for use on an industrial scale. This was developed in the UK by Tate and Lyle during the early 1940s for syrup production.

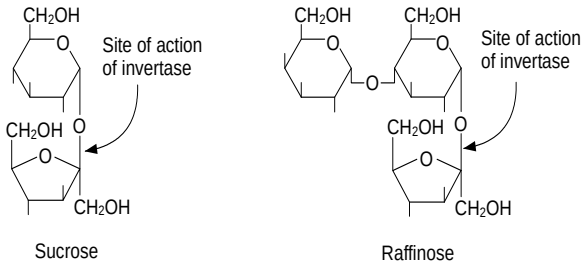


Fig. 24.1 Action of invertase on its substrate

24.2.1 PRODUCTION OF INVERTASE FROM BOTTOM YEAST

24.2.1.1 Fermentation

Today, invertase is produced from selected strains of *Saccharomyces cerevisiae*. The commercial production of invertase usually starts with an accumulation step. For this purpose, pressed bottom yeast is suspended in 20-fold amount of nutrient broth containing 4 parts $(NH_4)_2HPO_4$, 4 parts KH_2PO_4, 1 part $Mg(NO_3)_2$, and 1 part KNO_3. The mixture is aerated for 3-8 hrs while the pH and temperature are maintained at 4.5 and 28-30°C, respectively. During the same period, 3-20% sucrose solution is added continuously, a procedure that ensures reduced catabolite repression. At the end of the process the invertase activity of yeast increases by 15-fold.

For the most part, only a small amount of the invertase is produced intracellularly in the cytoplasm. The rest is extracellular, located within the cell wall or between the wall and the membrane. In fully repressed cells, all the enzymes are intracellular, thus indicating that the extracellular form is subject to more repression.

24.2.1.2 Recovery

First of all, yeast cells are concentrated by centrifugation. The release of invertase from yeast is achieved by destruction of the structures responsible for the retention of the enzyme. One method is autolysis with chloroform, toluene, or ethyl acetate at 30°C for not over 3 hrs. Alternatively, yeast cells can be disrupted in a homogenizer (Gaulin M3, at 550 bar) until about 75 g protein is obtained per kg of moist cell mass). The cells can be suspended in 0.1 *M* K_2HPO_4 buffer (pH 7.25) for the homogenization. Following extraction from yeast, comparatively high purification of

invertase is necessary for its application in foods because the enzyme preparation usually has an undesirable, irritating taste originating from yeast.

A method developed by Helmut and coworkers (1990) for the purification of invertase is described next. This method has been claimed to be more efficient than the conventional method.

The ruptured or lysed mixture (obtained as above) is cooled to 15°C, acidified with acetic acid or phosphoric acid (pH 4) and agitated for half an hour. Then the mixture is heated at 48-50°C for 9-10 min in a heat exchanger, the heat treatment being necessary to denature the proteins other than the invertase (which is relatively heat resistant). Next, the mixture is again cooled to 15°C, diluted in acetate buffer (pH 4) and the whole centrifuged to remove undesired proteins and cell debris. Finally, the supernatant is ultrafiltered to get permeate of invertase activity 3 U/ml.

Commercial invertase is available in liquid form that is stable for a year at low temperatures. The enzyme is generally stabilized with glycerol, which is added in amounts exceeding 55%.

24.2.2 BIOSYNTHESIS OF INVERTASE

In *Saccharomyces cerevisiae*, the ability to hydrolyze sucrose is conferred by any one of the six (or more) polymeric sucrose genes (denoted *SUC 1* to *SUC 6*), which reside at loci distributed throughout the genome. Strains unable to ferment sucrose can arise either by segregation during crossing over in strains containing *SUC* genes or by mutation of a known *SUC* gene. Segregated negatives are termed *SUC 0* to distinguish them from the mutational non-fermenters *suc 1, suc 2, suc 3, …..suc 6*. The presence of any one of the *SUC* genes in the genome leads to the production of any one of the two forms of invertases (intra- or extracellular, that is).

Invertase *m*RNA is continuously synthesized under repressive conditions and the level of this *m*RNA is regulated by the presence of glucose. The hexoses regulate them at the level of transcription. The expression of invertase *m*RNA present in the cell under repressive conditions is also regulated by glucose at the level of translation or secretion, or both. Consequently, under repressive conditions, invertase is destroyed before secretion occurs.

The two forms of invertases are encoded by two differently regulated *m*RNAs, which differ only at their 5' ends. One *m*RNA, which is glucose-repressible, encodes a signal-peptide containing precursor to the secreted invertase. However, another *m*RNA, which is constitutively synthesized, does not encode a complete signal-peptide sequence, and hence the translational product remains intracellular.

24.2.4 USES OF INVERTASE

- Analysis of sucrose in food products
- Manufacture of soft-centered chocolates, fondants, etc.
- Inversion of sucrose (to a limited extent)

24.3 LACTASE

Lactase is the trivial name for β-D-galactoside galactohydrolase (EC 3.2.1.23). It is an intracellular dimeric enzyme and is responsible for the hydrolysis of lactose into glucose and galactose subunits. The mechanism of hydrolysis is shown in Fig. 24.2. Maintenance of sulfhydryl status is required for enzymatic activity. The estimated molecular weight of lactase is 20300. It requires K^+ and divalent cations (Mn^{2+}, Mg^{2+}, etc.) for the activity.

Lactase is widely distributed in microorganisms, including the bacterium *E. coli*. However, relatively few species of yeasts are able to assimilate lactose. The species that have received interest to date are *Kluyveromyces fragilis* (syn: *Saccharomyces. fragilis*), *K. lactis*, *Candida pseudotropicalis* (imperfect form of *K. fragilis*), etc.

In general, the ability to assimilate lactose by *K. fragilis* is due to a lactose permease system (for the intact disaccharide) and a cytoplasmic β-galactosidase.

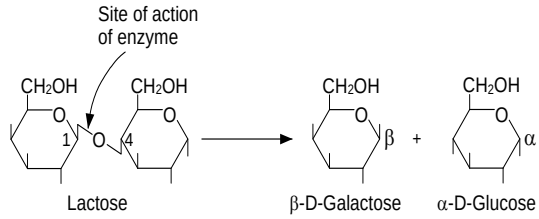


Fig. 24.2 Action of lactase on its substrate

Lactases from various microbial sources differ in properties such as pH optima. For example, pH optimum for bacterial lactase is around 7.0; that of fungal preparations near 5.0; and that from yeasts near 6.0. The lactase from *Corticium rolfsii* is distinguished by its unusual activity and stability at pH 1.8-2.0. Yeast lactases are activated by K^+ and NH_4^+ but inhibited by certain metals such as copper and iron.

24.3.1 PRODUCTION

A number of methods have been patented. In a method patented by Myrs (1956), the yeast may be grown on a whey medium. The whey derived from cheddar- or cream- cheese manufacture is treated to remove heat-coagulable protein by adjusting the pH to 4.5 and heating it at a temperature of 85-104°C, until coagulation is complete. It is then filtered off. Ammonia is added at the rate of 0.1%. The whey thus prepared is cooled to 30°C and inoculated with 10% by weight of actively growing yeast. The temperature is kept at 30°C, and air is supplied to the medium at the rate of 0.1 vol/vol/min during a period of about 24 hrs. The yeast cells propagated under this condition are separated from the spent medium by centrifuging and then washed with warm water. The cells are then quickly frozen at a temperature close to -1°C to inactivate the zymase. The resultant lactase-active, zymase-inactive product is dried under vacuum.

Where cell-free extracts are required, recovery can be affected by autolysis followed by salt- or solvent precipitation. A typical method of autolysis for the release of lactase from the cells entails pretreatment of cells with 80% ethanol for 1.5 hrs followed by autolysis at pH 6.6 and 28°C for 15 hrs. The yield is approximately 90% (Fenton, 1982). The diagrammatic representation of the production and recovery process is given in Fig. 24.3 (production: Myers; recovery: Fenton).

During production of lactase, maximum rate of lactase induction can be achieved by maintaining lactose concentration of above 2 m*M*/L in the medium. Glucose repression can be minimized by maintaining glucose level below 1 m*M*/L.

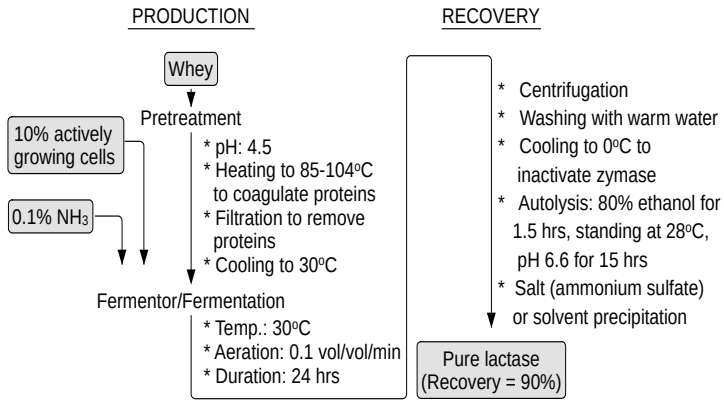


Fig. 24.3 Production and recovery of lactase

Another method of lactase production patented by Fenton (1982) will be described next. This method utilizes *Candida pseudotropicalis* UCD 55-31 and has been claimed to be superior to conventional methods.

An inoculum of *Candida pseudotropicalis* UCD 55-31 was prepared by cultivating the cells for 12 hrs at 28°C in 1 liter of nutritional medium of the following composition: whey 4%, $(NH_4)SO_4$ 0.5%, K_2HPO_4 0.5%, cornsteep liquor 0.1%, $MgSO_4.7H_2O$ 0.05%. The pH was adjusted to 4.5 with H_2SO_4 and sterilized at 110°C for 45 min.

Fermentation was carried out in fed-batch mode using an initial medium of following composition: KH_2PO_4 0.18%, $NH_4H_2PO_4$ 0.1%, $(NH_4)_2HPO_4$ 0.1%, $MgSO_4.7H_2O$ 5 mg/liter, $MnSO_4$ 5 mg/liter. The medium was sterilized by autoclaving at 121°C for 30 min. The inoculum was added at the rate of 10% (v/v).

A feed of following compostion was used: lactose 36.3%, yeast extract 0.6%, cornsteep liquor 0.3%, nicotinic acid 6 mg/liter. Sterilization of the medium was done at 110°C for 45 min. The medium was added continuously over the 60-hr fermentation at a feed rate of 0.006/hr. Dissolved oxygen was maintained at > 10 ppm with aeration (0.5-0.75 vol/vol/min) and agitation. Fermentation was carried out at 30°C and pH 4.5. After 60 hrs, the cell concentration reached 18 g/liter and lactase content 4200 units/g.

The cells were harvested by centrifugation. The cell paste was treated with ethanol (yeast biomass: ethanol = 1:3.5, m/v) for 90 min. Ethanol was later recovered by filtration. The cells were resuspended in 0.1 *M* potassium phosphate buffer (pH 6.6) so that the cell concentration in buffer was about 40 g/liter. Agitation was done at 30°C for 15 hrs.

The cells were next filtered and the lactase-enriched buffer collected. The enzyme solution had 65 units/ml of lactase activity. The yield was 45% based on the intracellular lactase content of the harvested cells.

Because lactase solution contained significant amounts of proteolytic enzymes, it was removed by heating the aqueous solution (containing 30-90% glycerol) to about 60°C at pH 6.4. The protease activity was reduced by 90%.

24.3.2 USES OF LACTASE

The most important use of lactase relate to hydrolysis of lactose in whey and milk. Hereditary intolerance to lactose precludes use of milk as a valuable protein source in large areas of Asia and Africa. In addition, lactose causes a number of problems in dairy and allied industries because of its poor solubility, resulting in crystallization in concentrated dairy products. Enzyme hydrolysis is helpful in overcoming these problems. Lactase is now utilized for accelerated ripening of cheese. The major applications may be summarized as in the Table 24.1.

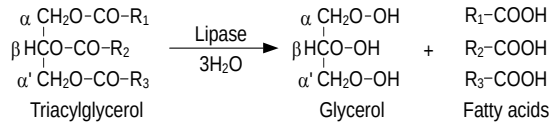
Table 24.1 Uses of lactase in food/feed industries

Raw materials	Product	Comments/advantages
Whey	Animal feed	Allows more whey to be incorporated in the animal food. Prevents lactose crystallization in whey concentrates
Whey	Lactose-hydrolyzed whey syrup	Used as food ingredient in bakery, confectionery, and ice cream products
Deproteinised whey	Lactose-hydrolyzed permeate syrup	Properties similar to glucose syrups of medium dextrose equivalent
Milk	Lactose-hydrolyzed milk	Improves digestibility in lactose intolerance. Increases sweetness. Prevents crystallization in milk concentrates

24.4 LIPASES

Lipases hydrolyze triglycerides to free fatty acids, partial glycerides, and glycerol. Their natural substrates are triglycerides of long-chain fatty acids which are insoluble in water. Lipases hydrolyze the ester bonds at the interface between aqueous phase (in which the enzyme is soluble) and the insoluble substrate phase. Their relative activity towards water-soluble fatty acyl esters is low. It is this ability to hydrolyze insoluble fatty acyl esters which distinguishes lipases from esterases.

The general name of the enzyme is lipase (EC 3.1.1.3). The action of the enzyme on triglyceride is shown in Fig. 24.4.



R refers to acyl group; α, β, and α' refer to position of attachment of fatty acids

Fig. 24.4 Action of lipase on its substrate

Commercial lipases are produced from *porcine* and *bovine* pancreas, *Candida rugosa* (yeast), *Rhizopus* / *Mucor* species (molds), and *Pseudomonas* species (bacteria).

24.4.1 LIPASES FROM MICROORGANISMS

Depending on the positional specificity, i.e., the ester linkages of the triglyceride they preferentially cleave, lipases can be divided in to two groups, *specific*- and *non-specific*. Non-specific lipases release fatty acids from all three positions of the triglyceride molecule. α, α'-specific lipases release fatty acids only from α and α' positions. The fatty acids present in the β-position will be spontaneously converted to monoglycerides in α or α' position, which will be broken down again by the same enzyme. Some of the important commercial lipases have been tabulated in Table 24.2.

Table 24.2 Properties of commercial lipases

Enzyme source	pH optimum	Temp. optimum, °C	Positional specificity
Pancreas	8.0	50	1,3 (i.e., α,α')
Candida cylindracea	7.5	50	Non-specific
Aspergillus niger	7.0	45	1,3
Mucor lipolyticus	7.5	50	1,3

24.4.1.1 Lipase from Candida antarctica

The lipase-producing yeasts of commercial interest are species of *Candida*, particularly *Candida rugosa* and *Candida antarctica*. Lipase from *Candida rugosa* is approved for food in Japan. Elsewhere, it is limited to use as processing aid: the lipase should be in an inactivated form in the food. A method described by Michio and coworkers (1987) for the production of lipase from *Candida antarctica* will be described next. This lipase is considered to be novel because it is non-specific, thermostable, and suitable for processing high-melting fats. They have reported the occurrence of two types of lipases, viz., lipase A (mol wt 43 kD, thermostable and isoelectric pH 8.0±0.2), and lipase B (mol wt 33 kD, more alkali resistant than lipase A, isoelectric pH 6.0±0.2).

Production

The slant culture of *Candida antarctica* strain DSM 3855 was cultured in a shaker flask at 26°C for 1 day in a sterilized, aqueous medium of following composition: peptone 0.6%, trypsin-digested casein 0.4%, yeast extract 0.3%, meat extract 0.15%, and dextrose 0.1%.

The main fermentation was carried out aerobically in an agitated, batch fermenter at 26°C for 119 hrs without pH control. The aqueous medium had following composition: pharmamedia® 4%, yeast extract 0.5%, sucrose 0.3%, soybean oil 3%, K_2HPO_4 5 mg/liter, and $MgSO_4.7H_2O$ 1mg/liter. The initial pH was kept at 6.2.

The lipase yield in the fermentated broth was found to be 157 LU/ml. The abbreviation LU refers to lipase unit (1 LU = amount of enzyme which liberates 1 μmole titrable butyric acid from tributyrate per min at 30°C and pH 7 with gum arabic as an emulsifier).

Purification

The cells were removed from the broth by centrifugation. The supernatant was collected and ultrafiltered. The permeate was mixed with 1 volume of 99% ethanol and stirred for 30 min at 4°C to precipitate the enzyme. The precipitate was recovered by centrifugation. The residual lipase in the supernatant was recovered by again precipitating with 2.5 volumes of cold, 99% ethanol and subsequent centrifugation. The precipitates were pooled (in the pellet form) and freeze-dried to obtain concentrate with 16,200 LU/g. Further purification was done by chromatographic method (hydrophobic interaction) and the resulting product vacuum-dried to obtain lipase concentrate with 92,000 LU/g.

2.4.2 USES OF LIPASE

Lipases have a number of food- and non-food uses. They can be used in the fruit juices, baked goods, and vegetable fermentation. Lipases find use in flavor development (by splitting low molecular weight fatty acids) in cheese, margarine, and butter. They are also used to improve emulsifying properties of ingredients (such as lecithin and egg yolk). In the fat and oil industry, lipases are used as hydrolytic agent and for interesterification. Lipases also have a number of non-food uses, such as in the production of pharmaceuticals and pesticides, waste management, and detergents. About 1000 MT of lipase is used in the detergent industry alone.

24.5 YEAST POLYGALACTURONASE

It is a soluble extracellular enzyme elaborated by *Saccharomyces fragilis* in complex as well as synthetic protein-free media. The enzyme appears to be constitutive. Yeast polygalacturonase (YPG) appears to be a single pectic enzyme produced by the yeast in nearly pure form. It has a specific activity of 0.179 polygalacturonase units per milligram of protein. Although it produces di- and galacturonic acid (monomer) by the hydrolysis of pectic acid, it is unable to attack digalacturonic acid and hence differs from mold polygalacturonase. Also, no esterase activity is associated with it.

A crude solution of the enzyme may be prepared by growing *Saccharomyces fragilis* strain 351 in synthetic yeast-nitrogen base (devised by Wickerham), containing 3% glucose. The yeast is cultivated in 2.5-liter flasks, holding 1 liter medium each, at 23-25°C for 4 days. The cells are separated from the medium by centrifuging. Toluene is added to the solution (as a preservative) containing the enzyme. The product is stored at 0°C. The enzyme may be concentrated to 13-fold by adsorbing it on pectic gel at a pH of 3.0 and eluting it with 1*N* acetate buffer at a pH of 5.

The reaction concerned in hydrolysis of pectic acid by yeast polygalacturonase follows 3 phases, *viz.*, (i) *Rapid linear phase*, (ii) *Slow linear phase*, and (iii) *Very slow phase*.

1. *Rapid linear phase*: Pectic acid → tetra- + tri- + di- + galacturonic acid
2. *Slow linear phase*: Tetragalacturonic acid → tri- + galacturonic acid
3. *Very slow phase*: Trigalacturonic acid → di- + galacturonic acid

The optimum pH for the rapid linear phase is 4.4. The slow linear phase starts after about 25% of the pectic acid is hydrolyzed. The optimum pH for this is 3.3 to 3.5. The third, or very slow, phase commences after about 50% of the pectic acid is hydrolyzed (also proceeds best at low pH). Yeast polygalacturonase is also called *endopolygalacturonase*, as the action on the pectic acid chain is random rather than regular. See Fig. 24.5 and 24.6 for an idea about pectic acid and pectin molecule. The term pectic acid is applied to pectic substances most commonly composed of polygalacturonic acids and essentially free from methyl ester groups. The salts of pectic acid are either normal or acid pectates. The compound is a polymer of D-galacturonic acids linked by α (1→4) glycosidic linkages.

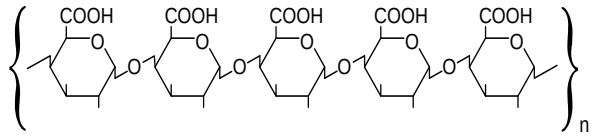


Fig. 24.5 Partial structure of polygalacturonic acid

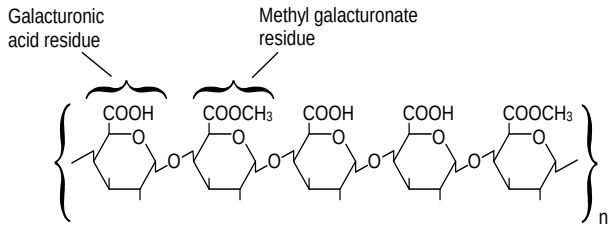


Fig. 24.6 Partial structure of pectin molecule

CHAPTER 25

MICROBIAL PROTEINS

25.1 INTRODUCTION

A major problem facing the world, in particular the developing nations, is the explosive rate of population growth. The number of humans in the world now totals 6.3 billion (as of 2006). It is increasing approximately by 94 millions annually and could well exceed 10 billions by 2050 if left uncontrolled. Conventional agriculture may well be unable to supply sufficient food - in particular, protein - to satisfy such demands. At least 25% of the world's population currently suffers from hunger and malnutrition; a disproportionate number of them live in the developing nations where wars, arid or changing climates, and infertile lands hamper productive agriculture.

To cope with the ever-expanding demand for proteins, several innovative alternatives and processes have been developed. The use of microbes as protein producers is one of them. Microbial proteins are more commonly called *single cell proteins* (SCP, a term coined at the Massachusets Intstitute of Technology around 1966), referring to the fact that most of the microorganisms used as protein producers grow as single cells or filamentous individuals rather than as complex multicellular organisms such as plants and animals. SCP is a generic name which refers to proteins, dry cells, or protein concentrates from microorganisms obtained by growing in large amounts in a variety of abundant and inexpensive culture media, and used as protein supplement for humans and animals. SCP must not be confused with *biomass* and *microbial biomass*. Mushroom, for example is simply a biomass.

The incidental consumption of microorganisms by man in and as food can be traced to prehistoric times but the realization or the microbe's contribution to total diet protein is only recent. The first conscious attempt to grow microbes for human food was made in Germany with the drying of brewer's yeast in 1910. Thereafter World War II followed, which prompted many European companies towards commercial production of SCP. Studies have multiplied after 1960 and today we have at our disposal a wide range of substrates, processes, and microorganisms to choose from. The three important concomitant events that contributed to progress in SCP production were:

1. Realization of acute shortage of protein in food and feed (especially after the war)
2. Realization of the fact that non-renewable resources could be microbiologically exploited
3. Realization of the fact that organic wastes could be used for protein production

There are several advantages of SCP over conventional crops used as protein source. Some of the more important advantages are:

- Rapid succession of generation of microorganisms
- High protein content (43-85% dry basis)
- Production is ecologically friendly
- Wide range of substrates can be used
- Production can be carried out throughout the year (i.e., does not depend on particular season as do the conventional crops)
- Consistent quality
- Little land requirement
- More easily modified (genetically)

25.2 SOME COMMERCIAL AND SEMI-COMMERCIAL PROCESSES

Several types of fermentation processes have been proposed for the production of SCP products. Notable among them are the British (ICI, BP, Quorn, Pruteen), American (Amoco), Japanese (Kanegafuchi), Finnish (Pekilo), Swedish (Symba), Canadian (Waterloo), Russian, and Cuban processes.

Except for the Waterloo process, the rest of the processes named above are of little attraction to developing or underdeveloped countries as these processes entail high-technology operation and use of rare- or expensive substrates (e.g., methanol, ethanol, etc.). Ironically, it is the third world where protein is needed most.

25.3 GENERAL CONSIDERATIONS

25.3.1 RAW MATERIALS

25.3.1.1 Carbon source

Two types of carbon sources can be used, *viz*., (i) *renewable*, and (ii) *non-renewable*. The choice of carbon source depends on availability of raw material and the type of microorganism.

Renewable resources include carbohydrates from agricultural and forestry products. Notable among them are bagasse, paper mill wastes, manure, whey, and fruit-processing waste. Although many bacteria can easily assimilate complex carbohydrates, there are some industrially important bacteria that cannot do the same: they need simple sugars. In such cases, the substrate must be supplied in pretreated form. Pretreatment of complex carbohydrates can be done by physical and/or chemical methods. A typical pretreatment used for bagasse from cane entails hydrolysis of the material with 10% NaOH solution for 1 hr at 180°C.

Non-renewable resources include petroleum hydrocarbon, natural gas, and chemicals derived from them (ethanol, methanol, chemical wastes). Methanol has received

special attention because of its high solubility in water, lack of explosion hazards, freedom from undesirable impurities, and ease of removal from the cell product.

25.3.1.2 Nitrogen source

Nitrogen sources include ammonia, ammonium salts, urea, etc. Chemical fertilizers can also be used when non-renewable carbon sources are used. Animal wastes also contain nitrogen in the form of urea, uric acid and other non-protein nitrogen. Meat processing wastes contain collagen and other protein nitrogen.

25.3.2 MICROORGANISMS FOR SCP

A large number of algae, yeasts, molds, and bacteria have been studied as SCP sources. Among the most promising genera and species are the following:

1. Algae: *Chlorella* sp. and *Scenesdesmus* sp., etc.
2. Yeasts: *Candida guilliermondii*, *C. utilis*, *C. lipolytica*, *C. tropicalis*, *Saccharomyces* sp., *Kluyveromyces fragilis*, *Debaryomyces kloeckeri*, etc.
3. Filamentous fungi: *Agaricus* sp., *Fusarium* sp., *Chaetomium cellulolyticum*, *Aspergillus* sp., etc.
4. Bacteria: *Aeromonas hydrophila*, *Alkaligenes eutrophus, Spirulina maxima*, *Methylomonas* sp., *Pseudomonas* sp., *Hypomicrobium* sp., *Acinetobacter* sp., *Flavobacterium* sp., and *Methylophilus* sp., etc.

25.4 BACTERIAL PROCESS

Non-photosynthetic bacteria are generally preferred. Notable among the bacteria used are given in Section 25.3.2 (above). In general, these bacteria are required to possess following properties:

- Ability to utilize cheap and readily available substrates for relevant fermentation
- Low requirement for nutrient supplement (e.g., growth factors)
- High specific growth rate, productivity, and yield on a given substrate
- pH and temperature tolerance
- Practicable aeration requirement and foam control
- Culture stability, including freedom from bacteriophages
- Non-pathogenicity to humans and animals
- Absence of endotoxin
- Lack of potential to mate with known pathogens (e.g., members of Enterobacteriaceae)
- Ease of separation from the growth medium by flocculation or agglomeration.

25.4.1 MEDIUM FORMULATION

The substrate should be nutritionally well balanced. The ratio of carbon to nitrogen (C:N) is maintained in the range 10:1 or less to favor high cell protein content and minimize the accumulation of lipids or cell storage substances (such as poly-β-hydroxybutyrate). Phosphorus requirement is met by adding feed-grade (rather than industrial grade) phosphoric acid to prevent contamination of the cell product with arsenic or fluoride. Other minerals are usually present in sufficient amounts in the dilution water. If needed, minerals should be supplied in the form of sulfates or hydroxides. Mineral salts in the chloride form cause corrosion of the equipment.

25.4.2 PROCESS CONDITIONS

The bacterial strain should preferably have a cardinal growth temperature between 37 and 55°C. This saves the cooling cost. The pH is maintained at 6-7.2 by the addition of ammonia (or ammonium salts) or phosphoric acid at regulated amounts. The fermentation can be either batch, fed-batch, or continuous.

For a batch process, the medium normally consists of renewable carbon sources. The concentration based on simple sugars such as glucose, sucrose, and lactose is usually in the range 1-10%.

In the case of hydrocarbon substrates, including methane, methanol, ethanol, *n*-alkane, etc., continuous processes are used for economic and process-control reasons. The substrate concentration is maintained at *close to zero* to prevent instability resulting from substrate inhibition. This is particularly important in methanol substrate because it exerts methanol toxicity.

Aeration, cooling, and foam control can be achieved by any of the conventional methods. With increasing cell yield, however, both oxygen demand and heat load decrease.

Reported cell yields for various processes based on carbohydrate or organic nitrogen substrate range from 0.25-0.61g dry matter/g substrate utilized. For hydrocarbons, the value may reach as high as 1.2 g dry matter/g substrate utilized.

The specific growth rates vary widely, depending on the choice of microorganism, substrate, temperature, feed rate, etc.

The often-cited example of bacterial protein product is that produced by Imperial Chemical Industries (ICI) and British Petroleum (BP). The ICI *Pruteen* process for feed-grade bacterial protein from methanol (using *Methylophilus methylotrophus*) has an operating temperature of 35-42°C. *Pressure Cycle* air-lift fermenter design is used for aeration. The design uses either air or air-and-agitator (combined) system for agitation. The specific growth rate is 0.5/h, cell density 30 g dry matter (dm)/L, and yield 0.5 g (dm)/g of substrate used. See Fig. 25.1 for an outline of the bacterial process.

25.4.3 PRODUCT RECOVERY

The product is recovered at a cell density of 30 g (dm)/L. Owing to the small size of the cells (1-2 μm) and low density (1.003 g/cm^3) conventional centrifuges cannot be used. Filtration is also unsuitable as the filters get clogged very soon. And, of course, one cannot use filter aids. Consequently, there has been considerable interest in recovery by agglomeration and flocculation of cells. The collected mass, after flocculation, can be centrifuged. The trade processes for recovering biomass are probably closely guarded secrets: ICI proprietary process carries out concentration without resorting to flocculation while Philips Petroleum Company relies on mixing the biomass first with much larger yeast cells. The mixed cell product is finally centrifuged for concentration.

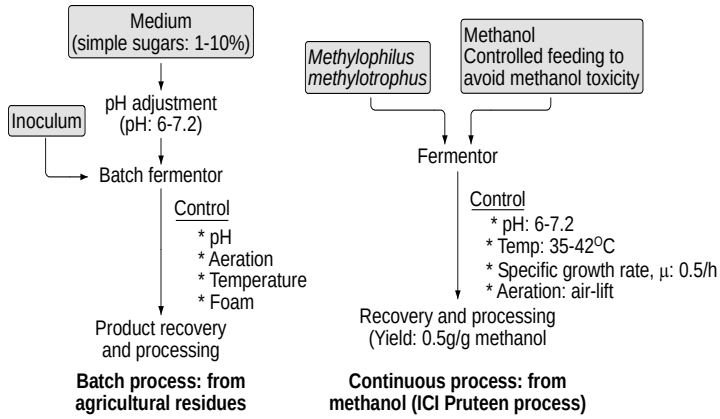


Fig. 25.1 Bacterial process for protein production

25.4.4 WASTE TREATMENT

It must be noted that the spent growth medium and cell wash waters from recovery processes have high BOD. Whenever possible, the spent medium should be purified, sterilized, and recycled. In some cases, low concentrations of inhibitory compounds may be very costly to remove from the spent growth medium. In any event, the residues from the cell separation must be treated before discharge.

25.4.5 PRODUCT QUALITY AND SAFETY

Bacterial proteins have three potential applications, *viz*., in (i) *animal feed*, (ii) *human food*, and (iii) *functional protein concentrates and isolates*. Nutritional and organoleptic characteristics are of paramount importance in the first two applications while functional effects in food products are important in the third application. Whatever the use, the product must be acceptable from sensory standpoint. Also, the product must be low in nucleic acid level and free from microbial toxins, pathogens, toxic heavy metals, and chemical residues.

25.4.6 NUTRITIONAL VALUE

The total nitrogen content in bacterial protein is as high as 13% or a protein content of 83% (using N×6.25). However, it must be appreciated that not all nitrogen is derived from protein. An actively growing bacterial cell can have nucleic acid content as high as 16% (on dry basis). Nucleic acids are detrimental to humans. Bacterial proteins in general tend to be deficient in methionine content from the standpoint of human and animal nutrition. From the data of some workers, performances of selected bacterial SCP products in animal feeding studies are:

- Protein Efficiency Ratio (PER) = 1.88
- Biological Value (BV) = 62-67
- Protein digestibility = 90%
- Feed conversion ratio (kg/kg weight gain) = 0.0569-0.757

(Note: for casein, PER = 2.5, BV = 75)

25.4.7 FUNCTIONAL QUALITY

Bacterial protein concentrates and isolates have been prepared for evaluation of functional effectiveness in food including:

- Water- and fat binding properties
- Emulsion and stability
- Gel formation and thickening
- Whippability

None of these functional proteins have been commercialized. Findings suggest that the effectives of these proteins fall short of those from soybean protein. Besides, the cost of obtaining safety data (for microbial protein products) to satisfy the regulatory agencies is normally very high.

25.4.8 REDUCTION OF NUCLEIC ACIDS

The problem encountered in the removal or reduction of nucleic acids has been the major limitation to the use of SCP products as human food. Some methods have been developed to address this problem but the common setback in them all is the high cost, which makes the process uneconomical. A typical process for reducing nucleic acids in bacterial proteins entails subjecting the cells to heat-shock at pH 5 at a temperature exceeding 60°C followed by raising pH between 6 and 10.

25.4.9 ECONOMIC CONSIDERATIONS

The capital cost of bacterial biomass production will depend upon the equipment requirement (for storing, processing and handling substrates, sterilization and cleaning operations, product separation, recovery and drying) and local land, site preparation, and construction costs. Bacterial SCP production costs are highly

dependent upon the cost of carbon and energy source, and it may range from 13-53%.

25.5 FUNGAL PROTEINS

Fungal proteins include proteins from filamentous fungi (molds), non-filamentous fungi (yeasts), and mushrooms. Fungal proteins are collectively called *mycoproteins*.

25.5.1 FILAMENTOUS FUNGI

Mycoproteins from molds can be produced by various methods, such as the Finnish Pekilo process, the Canadian Waterloo process, the Heurty process, etc. The advantages of using molds for the production of proteins are:

- Good at breaking down a wide range of complex substrates, e.g., cellulose, hemicellulose, pectin, etc.
- Can tolerate low pH values, which helps in resisting infection
- Few nutritional requirements for the culture
- Ease of recovery of the biomass by filtration
- Ease of handling and drying of biomass
- Structure conferred by mycelia can be used as a basis for food fabrication

There are disadvantages as well, for example:

- Comparatively poor growth rate
- Lower cardinal growth temperatures call for cooling requirements, and there are comparatively few thermotolerant strains to choose from
- Protein content generally is unfavorable compared to that from yeast and bacteria
- Fermentation broths are rheologically complex and difficult to aerate
- Production of a range of undesirable metabolites, e.g., oxalic acid mycotoxins, etc.
- Poor nutritional properties compared to proteins from yeasts and bacteria
- Genetically unstable

25.5.1.1 The Pekilo process

This commercial process has received clearance for animal feed in Finland. In this process, the substrate is a complex mixture of monosaccharides, acetic acid, and aldonic acids. The pH is maintained at 4-5, and the temperature of fermentation is around 38°C. The organism used is *Paecilomyces variotii*. Cell concentrations of approximately 13 kg/m^3 are normal at dilution rates of 0.14-0.3/h, giving a biomass productivity of 2.7-2.8 kg/m^{3}h. The carbon to nitrogen ratio can be 5:1 to 15:1. Recovery can be done by filtration or centrifugation. For drying, tray or belt dryers can be used. High temperatures are avoided as this markedly reduces the nutritional value. Normally, a temperature of 75°C can be used for 20-30 min to bring the moisture content below 10%.

25.5.1.2 The Waterloo process

Waterloo process is probably the most versatile of all the mold processes thus far used. It uses raw materials which occur universally in large quantities as waste biomass. The materials include agricultural wastes such as animal manures and crop residues (e.g., straw, corn stover, bagasse), and forestry residues (such as remnants and pulpmill sludge). The process concurrently alleviates environmental pollution frequently generated by these wastes.

Process description

The waterloo SCP process is based on the mass microbial cultivation of a new cellulolytic fungus *Chaetomium cellulolyticum*, in solid-substrate systems. The basic generic process uses a three-stage operation which involves: (i) thermal and chemical pretreatment of cellulosic material, (ii) aerobic fermentation of the pretreated material with nutrient supplements, and (iii) separation of the suspended solids (the product) from the fermented broth.

Cellulose materials provide the main carbon source for the fermentation. The main non-carbon nutrient supplements (N, P, K, etc.) are derived from synthetic chemical fertilizer blends or animal manure. If manure is used, it is pretreated by anaerobic fermentation to produce methane fuel gas as a byproduct. This fuel can be used to supply processing energy. See Fig. 25.2 for the outline of Waterloo process.

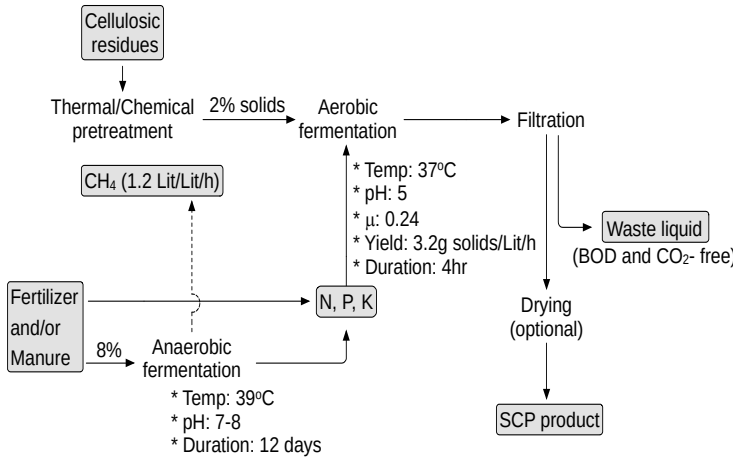


Fig. 25.2 Waterloo process for fungal protein production

The cellulosic material is pretreated with steam, hot water, or dilute alkali (depending on the feedstock type) to sterilize it and enhance its fermentability by swelling and/or partial delignification. A forage-grade carbohydrate co-product of unfermented cellulose may also be produced admixed with the main SCP product. This co-product is rendered digestible by the action of extracellular fungal cellulase, which is generated during the fermentation. Any residual lignin serves as direct diet roughage in the product. Because of the large mycelial growth forms, the SCP product can be recovered by simple filtration methods. The effluents of the process

are essentially free from CO_2 and BOD. The process is carried out in slurry or semi-solid system, depending on the feedstock type. The growth rate efficiency of *Chaetomium cellulolyticum* is 0.24/h, one of the highest known for cellulolytic fungi.

Product quality

The protein nutritional value of Waterloo SCP is comparable with the FAO reference standard (soymeal and a well-known fodder yeast). The average protein content of Waterloo SCP is 45% on dry basis, which is similar to that in commercial grade soymeal and fodder yeast. Although SCP products are often compared with soymeal, Waterloo SCP is more similar to meat in terms of protein quality and the spectrum of other nutrients such as fat and vitamins. It should be noted that Waterloo SCP is more attractive than the yeast for human use because of its lower content of nucleic acids and higher content of sulfur amino acids.

The product is gray in color and mushroomy is odor. It can be fabricated into granules, fibers, meat analogs and powder.

25.5.2 SCP FROM NON-FILAMENTOUS FUNGI

The organism in this category includes yeasts. Notable among them are *Saccharomyces cerevisiae*, *Saccharomyces fragilis*, *Candida utilis*, *Candida lipolytica*, etc.

25.5.2.1 General process

Some detail has already been given in production of feed yeast. To enhance protein accumulation, the carbon to nitrogen ratio is maintained at 7:1 to 10:1. The feed rate must also be carefully controlled so that the substrate is not utilized for microbial activities other than the accumulation of protein. Post fermentation treatment is done usually after the cell concentration reaches 2%. Recovery does not create problems because the cell size is large enough to be separated by centrifugation. Yeast cells can be recovered by decantation-centrifugation (including washing)-drying methods. After washing undesirable traces of medium, the biomass is further subjected to rotary vacuum filter (Fig. 17.10a and 17.10b). The cake contains 20-40% dry matter. This is again dried to 6-10% moisture content.

A method described by Marquez and coworkers (1989) for the production of SCP from yeast is described next.

25.5.2.2 Reduction of nucleic acid content

In yeasts, total nucleic acid content varies from 8-12% on dry basis. DNA accounts for about 1-2% of the total nucleic acid. Various methods are available for the reduction of nucleic acids in yeast cells. One method, patented by Robinson, entails alkali and/or heat treatment. Slurries of food-grade yeast cells, disintegrated cold at 8000 psig, are treated at pH 9.5 and 25-60°C for about 20 min after which they are recovered by centrifugation. The alkaline extract is adjusted to pH 6-8 and heated at temperatures of 110-120°C for 2-60 min. The treatment precipitates the protein, and

the RNA fraction is released into the supernatant. The heat treatment also sterilizes the protein fraction. The final RNA level comes down to about 2%.

The protein content in yeast SCP is 45-49% (dry basis). Fat, carbohydrate and ash contents are 4-7%, 26-36%, and 5-10%, respectively. Yeast SCP is rich in lysine but poor in sulfur amino acids.

25.6 SAFETY ASPECTS OF SCP

Microbial proteins have high nucleic acid contents, reaching up to 16% (dry basis). Nucleic acids present problem in man because they are only partially metabolized by humans. Humans do not have uric acid oxidase that converts uric acid to readily soluble (excretable) allantoin. Consequently, consumption by man of more than 2 g nucleic acid per day leads to development of kidney stone and gout. This amount is equivalent to 10 g of bakers yeast. Gout results from the precipitation of uric acid in joints. Uric acid in turn results from the incomplete metabolism of nucleic acids in man. Ruminants do not have this problem because they have uric acid oxidase needed for the metabolism of uric acid. Microbial proteins are therefore more suitable as feed than food.

CHAPTER 26

TRADITIONAL FERMENTED FOODS AND BEVERAGES

26.1 INTRODUCTION

Almost without exception, fermented foods were discovered before mankind had any knowledge of microorganisms other than as witness to the effects of their activity. It was simply an empirical observation that certain ways of storing food affected desirable changes in its characteristics. Much later, pure cultures were isolated and improved for specific applications in processing foods and beverages. Still later, purified enzymes and immobilized cells began to be used. More recently, microbial biomass production has been developed into an industrial activity to obtain protein-rich food/feed supplement.

Fermented foods and beverages have a significant role in all societies and result from the action of microorganisms or enzymes on a wide range of agricultural materials with associated desirable biochemical changes giving significant organoleptic improvement to the final product. As a result of the fermentation process the product is usually more nutritious, more digestible, has improved shelf-life, and is toxicologically and microbiologically safer.

Fermented foods and beverages are an accepted and essential part of the diet in almost all parts of the world. The preparation involves a wide diversity of raw materials as substrates, using technology from the most primitive to the most advanced, and achieving an astounding range of sensory and textural qualities in the final product. Some selected fermented foods and beverages are given in Table 26.1 and Table 26.2.

Originally the most important of these changes have been an improvement in the shelf-life and safety of a product. We now know that, in food fermentation, conditions of treatment and storage produce an environment in which certain organisms can flourish and these have a benign effect on food rather than spoiling it.

Fermented foods are of diverse nature: some resulting from lactic acid bacterial fermentation; some, alcoholic fermentation by yeasts; some, mold fermentation; some acetic acid bacterial fermentation; and many, by combination of these fermentations. Today a large majority of individuals preparing foods by these processes are practicing *rule of thumb* methods developed over a period of years by their forebears.

Fermentation and drying are two of the oldest methods of preparation and preservation of foods known to mankind. Even though the original physical and chemical characteristics of the foods may be altered during fermentation, their nutritive values are usually retained to a great extent. Many of the food preservation

practices antedate recorded history. Throughout the centuries fermentation has been (and still remains) one of the most important methods for preserving foods. Relatively few people, however, are aware that many food products consumed regularly are prepared and/or preserved by fermentation process.

Table 26.1 Some selected fermented alcoholic beverages

Beverage	Substrate	Microorganism(s)	Country
Wine	Grape juice	*Saccharomyces* sp.	Temperate: N and S hemispheres
Beer	Malt and adjuncts	*Saccharomyces* sp.	Industrialized countries
Sake	Rice	*Aspergillus oryzae* and *Saccharomyces sake*	Japan
Jand	Millet/rice	Wild molds, yeasts and bacteria from *murcha*	Nepal
Mead	Honey	*Saccharomyces* sp.	United Kingdom
Perry	Pear juice	*Saccharomyces* sp.	UK, France
Rum	Molasses	*Saccharomyces* sp.	World wide

Table 26.2 Some selected fermented foods

Product	Substrate	Microorganism(s)	Country
Dahi	Milk	Lactic acid bacteria	India, Nepal
Kinema	Soybean	Mixed flora (yeast/mold/bacteria)	Nepal
Natto	Soybean	*Bacillus natto*	Japan
Tempeh	Soybean	*Rhizopus oligosporus*	Indonesia
Sauerkraut	Cabbage	Lactic acid bacteria	Germany
Sinki and *gundruk*	Vegetables	Lactic acid bacteria	Nepal

The preparation and preservation of foods by fermentation processes are dependent upon the production by certain microorganisms of chemical substances that alter the flavor of the food and are generally inhibitive to the growth of undesirable microorganisms. The simplest example of such action is the inhibition of toxin-producing bacteria by lactic acid produced in many fermented foods.

Fermented foods are the result of the metabolic activity of a few species of microorganisms among the thousands of species of bacterium, yeast, and mold known to mankind today.

According to Steinkrauss (1997), fermented foods are food substrates that are invaded or overgrown by edible microorganisms whose enzymes (particularly amylases, proteases, lipases) hydrolyze the polysaccharides, proteins and lipids to

non-toxic products with flavors, aromas and textures pleasant and attractive to the human consumer. If the products of enzyme activities have unpleasant odors or undesirable, unattractive flavors or the products are toxic or disease-producing, the foods are described as spoiled.

Fermentation plays at least five roles in food processing:

1. Enrichment of the human dietary through development of a wide diversity of flavors, aromas and textures in food
2. Preservation of substantial amounts of food through the lactic-, alcoholic-, acetic-, alkaline-, and high salt fermentations
3. Enrichment of food substrates biologically with vitamins, protein, essential amino acids, and essential fatty acids
4. Detoxification during food fermentation processing
5. Decrease in cooking times and fuel requirements

26.2 CLASSIFICATION OF FERMENTED FOODS

Fermented foods can be classified in a number of ways. Steinkraus has classified fermented foods based on following categories:

1. *Fermentations producing textured vegetable protein meat substitutes in legumes/cereal mixtures*: Examples are Indonesian *tempeh* and *ontjom*
2. *High salt/savory meat-flavored/amino acid/peptide sauce and paste fermentations*: Examples are Chinese soy sauce and Japanese miso
3. *Lactic acid fermentations*: Examples are sauerkraut, cucumber pickles, yogurt, kefir, etc.
4. *Alcoholic fermentations*: Examples are wines, sake, palm wines, etc.
5. *Acetic acid/vinegar fermentations:* Examples are apple cider, tea fungus, coconut water vinegar of the Philippines, etc.
6. *Alkaline fermentations:* Examples are *kinema*, natto, Nigerian dawadawa, etc.
7. Leavened bread: Examples are yeast and sourdough breads
8. Flat unleavened breads

26.3 SAFETY OF FERMENTED FOODS

Fermented foods generally have a good safety record even in the developing world. Food fermentations that improve food safety are as follows:

1. Food fermentations involving lactic acid production
2. Food fermentations involving ethanol production
3. Food fermentations involving acetic acid production
4. Food fermentations involving highly alkaline conditions with liberation of free ammonia
5. Food fermentations carried out in the presence of high salt concentrations (above 13% w/w)

26.4 MISO

Miso is the Japanese name given to paste-like, salty food made from varying combinations and proportions of soybeans, barley or rye, and rice by a mixed fermentation with molds, yeasts, and bacteria. Although miso is a seasoning, it is also a traditional dietary staple used by the Japanese in preparation of soups for breakfast. Similar products are made and consumed in other parts of the Orient also, for example: *chiang* (China), *tauco* (Indonesia), *doenjang* (Korea), *tao chieo* (Thailand). Miso has a distinctive pleasant aroma resembling that of soy sauce. Some varieties, especially those that have greater proportion of soybean in the formula and have been fermented for a long time, have a very meat-like flavor.

There are several types of miso found in Japan, for example, *kome* miso or rice miso, *mugi* miso, *sendai* miso, *mame* miso, *shinshu* miso, *edo* miso, etc. The color of miso ranges from light white to reddish brown. White miso is preferred in western Japan, has a light color, a very sweet flavor, a low concentration of salt (5-6%), and a short fermentation period of about a week at 23-33°C. Edo miso, preferred around Tokyo, is reddish brown, has a low salt content, and requires two weeks of fermentation. In general, the higher the proportion of soybeans in the recipe, higher should be the salt content and longer the fermentation period. Overall, miso has 45-50% moisture, 4.5-13% salt, 4-11% fat, 4-38% reducing sugars, and 5-5.4 pH values.

26.4.1 COMMERCIAL PRODUCTION

Miso production is a two-stage fermentation process, *viz.*, aerobic, and anaerobic. The essential steps are: (i) preparation of rice/barley, (ii) preparation of koji, (iii) preparation of soybeans, (iv) mashing/mixing, and (v) anaerobic fermentation.

26.4.1.1 Raw materials

The basic raw materials for miso production are soybeans (yellow variety is preferred), polished rice, rye or barley, salt and alcohol.

26.4.1.2 Preparation of rice/barley

Barley/polished rice is washed and soaked in large fiberglass or epoxy-lined steel tanks holding 1000 kg or more of material. The duration is approx. 17 hrs. The soaked mass is transferred continuously on stainless steel mesh belts through two, long tunnel steamers. Steam is injected at 4.3 and 3.5 psig respectively in the first and second chamber. The partially steamed rice/barley may be rinsed with water as it passes from the first to the second chamber to remove free starch.

26.4.1.3 Koji preparation

The rice/barley is cooled in another conveyor belt and transferred to the koji fermenter. The rice/barley is inoculated with a pure strain of *Aspergillus oryzae* (*soyae*) spores selected for its ability to produce the required proteases, amylases, lipases, and other enzymes in proper proportions and quantities. The inoculation rate is 0.1% (w/w), or equivalently, 10^9 spores/g.

The koji may be fermented at 28-32°C for 3 days either in special rooms or mechanically agitated drums or rectangular tanks. This step implies the aerobic phase of miso production. Rotating drums are the easiest to explain here: they are about 1.8 m (diameter) × 3.6 m (length) with temperature, humidity, and air flow controls. The drums contain finger projections inside them for breaking up the developing koji as the drum is periodically rotated.

By the time fermentation terminates, the rice/barley is fully covered with white mycelia. It has a sweet, pleasant smell. Before sporulation occurs, further development of mold is terminated by either subjecting it to anaerobic brine fermentation, or cooled at low temperature, or mixed with 30% (w/w) of salt.

26.4.1.4 Preparation of soybean

Cleaned soybeans are hydrated to approximately double the weight by soaking them overnight in large tanks. If dehulled soybeans are to be used, dehulling can be done by either dry process (in burr mill) or wet process (in abrasive mechanical peeler). The beans are thoroughly cooked in large batch-type or continuous cookers (retorts/autoclaves) in steam or water at a temperature of 121°C for 30-40 min or higher temperature equivalents. The batch retort can hold 1000 kg or more of material. It is often mounted so that it can be rotated during the operation to hasten cooking and facilitate emptying. The beans are now emptied onto conveyor belts and rapidly cooled to prevent further darkening.

26.4.1.5 Mashing/mixing/preparation of green miso

The cooked beans are mashed and mixed with koji, required amount of salt added, and the mixture inoculated with either miso (from previous batch) or pure cultures of selected yeasts (e.g., *Saccharomyces rouxii*, *Torulopsis*) and bacteria (*Pediococcus halophilus*, *Streptococcus fecalis*). This mixture is called *green miso*. The bacteria produce the necessary acidity and yeasts produce alcohol, contributing to formation of esters, and aroma and flavor compounds. Machines similar to large sausage grinders can be used for mashing. Mixing continues in large mixing vats with heavy paddles. It is important to mix the ingredients well so that variation in salt concentration in the mash is less than 0.5%.

26.4.1.6 Anaerobic fermentation

The paste is then conveyed to temperature-controlled tanks or vats that can hold up to 12000 kg. Spigots allow the removal of liquefied *tamari* sauce as the fermentation progresses. The fermentation proceeds under anaerobic condition. The lower the salt content and the higher the proportion of rice/barley koji to soybeans used, the sweeter the resulting mixture. The more thoroughly the soybeans are cooked, the darker the color and the higher the salt content, the longer the fermentation, and also the more robust the meat-like flavor.

The duration of fermentation varies from 1 week (for white miso) to 2 years (for *mame* miso). At the end of the fermentation, the miso is blended, pasteurized in tube

heater, 2% alcohol added, and packed in unit sizes in plastic bags. See Fig. 26.1 for flow diagram of miso production.

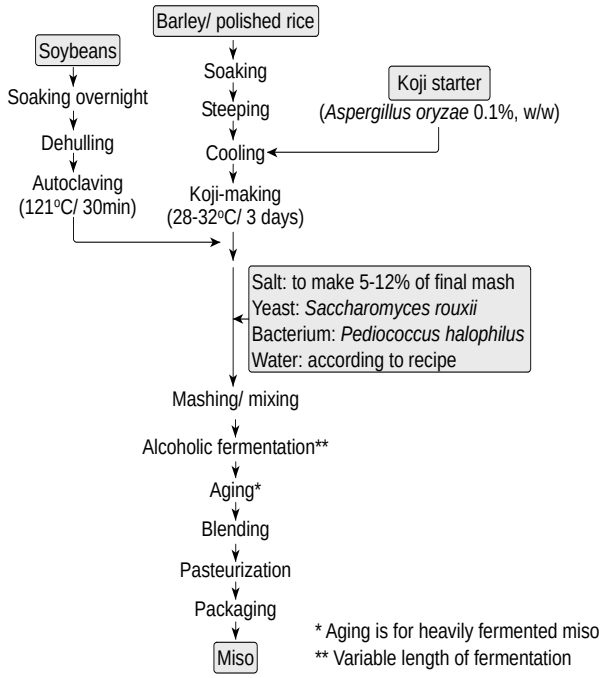


Fig. 26.1 Commercial production of miso from whole soybean

For heavily fermented miso, aging can be done by allowing the miso to stand at room temperature for about two weeks before pasteurization.

26.5 SOY SAUCE

Soy sauce is a light-brown to black liquid with meat-like salty flavor. It is prepared by hydrolyzing soybeans, with or without the addition of wheat or other starchy carbohydrate, in a strong brine (about 18% w/w) using enzymes produced by *Aspergillus oryzae* (*soyae*). A two-stage fermentation is used. An aerobic solid-state mold fermentation is followed by a mixed *Lactobacillus*-yeast submerged fermentation.

Soy sauce is the most widely consumed and the only oriental fermented product that has become well known in the cookery of western countries. The product is called *shoyu* in Japan, *chiang-yu* in China, *kekap* in Indonesia, *kanjang* in Korea, *toyo* in the Phillippines, and *see-iew* in Thailand.

Soy sauce can also be produced by chemical hydrolysis of proteins and starch. Although the hydrolysis is more complete the sensory quality is inferior to that produced by fermentation.

Shoyu is the Japanese name for soy sauce. *Koikuchi* means dark in color. The basic steps in commercial shoyu preparation entails: (i) Treatment of raw materials, (ii) Koji production, (iii) Mash production and aging, (iv) Brine fermentation, (v) Mash pressing, and (vi) Refining. See Fig. 26.2 for flow diagram of commercial soy sauce production.

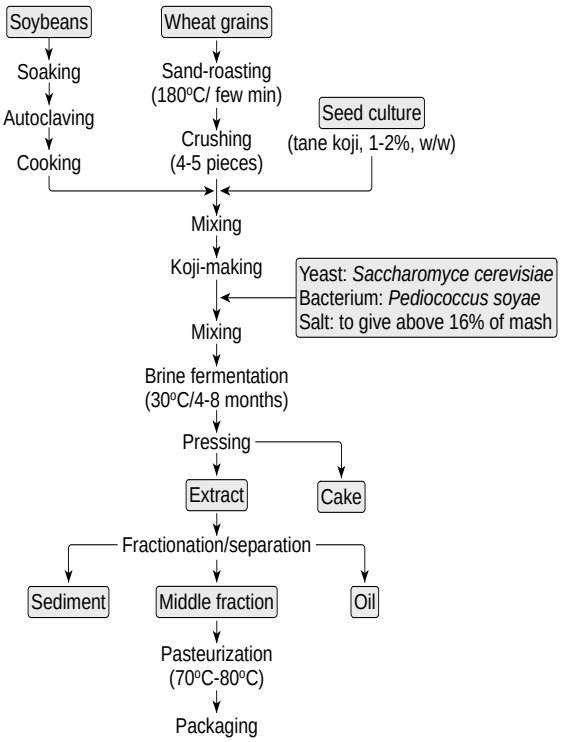


Fig. 26.2 Commercial production of soy sauce from whole soybean

26.5.1.1 Treatment of raw materials

Clean soybeans (whole/defatted grits) are soaked for 10-15 hrs in water, which is changed every few hours to prevent acidification by bacteria. The weight of the beans increases by about 2.1-2.15 times. The hydrated beans are cooked for 1 hr in steam at 10-14 psig in *NK rotary cooker* of capacity 1000 kg. The cooked beans are then cooled rapidly.

Whole-wheat kernels (soft type/low protein content) are roasted at 170-180°C in sand for several minutes (some workers mention roasting for less than a minute). A rotary cylinder about 0.7 m in diameter and 2 m in length (capacity: 500 kg/h), rotating at 25-30 rev/min is used. The sand is recycled and wheat grains crushed into 4-5 pieces in a roller mill. A slightly charred flavor is desirable.

26.5.1.2 Koji production

Typically, cooked soybeans are mixed with roasted wheat particles in the ratio 1:1. This mixture is inoculated with 1-2% (w/w) of seed koji, called *tane* koji, of *Aspergillus oryzae* pre-grown in polished rice. Some workers mention use of 0.1-0.2% koji. The mixture is transferred to porous stainless steel plates several meters in length and width and the material is maintained to a depth of 30-40 cm. Fermentation proceeds at 25-35°C. Careful control of temperature, aeration, and moisture allows complete white mycelial growth in 45 hrs, help prevent development of contaminants, and enhance development of proteolytic enzymes. Aeration is done by feeding humidified air through the bottom holes. The resulting product is called *shoyu koji* (or simply koji), which is a mixture of fungal hydrolytic enzymes and the substrate. As the fermentation continues, the *growth* turns yellow and dark green. The koji is now ready for brine fermentation.

26.5.1.3 Brine fermentation

The koji is mixed with 1.2-1.5 volumes of 23% salt solution. The mash, called *moromi*, is transferred to concrete, wooden, or resin-coated steel tanks of 10-20 m^3 capacity. It is important that the salt concentration be above 16%: this prevents putrefaction. Pure cultures of *Pediococcus soyae* and *Saccharomyces rouxii* are added to the moromi at the start and after one month of fermentation. The moromi is stirred occasionally in the early stages to distribute heat and mix up the mash properly. The fermentation normally continues for 4-8 months (1-3 years in traditional method). In some variations, the moromi is transferred twice to other vats during the fermentation period.

26.5.1.4 Pressing and filtration

The fermented moromi is filtered by pressing in hydraulic filter press through thick clothes, at 100 kg/cm^2 for 2-3 days. The cake, which typically contains 25% moisture, is used for animal feed.

26.5.1.5 Refining/pasteurizing

The extract thus obtained is separated into three fractions: (i) *sediments*, (ii) *supernatant middle layer*, and (iii) an *oily layer at the top*. The middle layer is further clarified by filtering through *keiselgel* to obtain raw shoyu. This portion is standardized with respect to salt, nitrogen level, etc., and pasteurized at 70-80°C in kettle or in heat exchanger. Pasteurization helps remove heat coagulable materials as well as preserve the product. The product is cooled, filtered again, and packed in 1-2-liter glass- or plastic bottles. Benzoic acid or *propyl-* or *butyl-p-hydroxybenzoate* is sometimes used as preservative. Shoyu can also be produced in the form of spray-dried powder. The oil and sediments find other uses. For example, the oil fraction can be used in paints as an antifreezing agent.

26.5.2 GENERAL PROPERTIES OF SHOYU

The chemical changes that occur in the production of shoyu and its flavor are complicated. More than 100 compounds have been reported as flavor components of soy sauce. The guaiacol compounds seem to have an important effect on overall

flavor of Japanese shoyu. Typical chemical composition of soy sauce from whole soybeans is given in Table 26.3.

Table 26.3 Some typical values of soy sauce from whole soybeans

Property/parameter	Value
Baume'	22.7
NaCl	18.5%
Total nitrogen	1.6%
Reducing sugar	1.9%
Alcohol	≈ 1%
pH	4.8

Shoyu and miso appear to be very similar both with respect to production, raw material, and sensory quality. Nevertheless, there are some fundamental differences between them, a brief mention of which appears in Table 26.4.

Table 26.4 Fundamental differences between miso and soy sauce

Description	Miso	Soy sauce
Raw material	Soybean, rice, barley, rye	Soybean, wheat
Koji material	Starchy items	Wheat and soybean
NaCl	5-12%	13-18%
Reducing sugars	10-38%	4-6%
Form of final product	Paste	Liquid
Yeast and bacteria for inoculation	About 10^5 each	About 10^{6-8} each
Duration of fermentation	Variable (1 week-2 years)	4-8 months

26.6 NATTO

Natto is a bacterial fermented oriental soybean product. It is popular in Japan (except northern Japan) and Korea. In Japan, natto is seasoned with soy sauce, salt or sometimes mustard, and served with rice.

Natto is characterized by persistent musty to ammoniacal flavor, and the presence of viscous sticky polymers. The sticky substance is due to glutamic acid polymer.

26.6.1 COMMERCIAL PRODUCTION

The soybeans are first soaked in water at 15°C for 16-20 hrs and then steamed under pressure (0.7 kg/cm^2) for 30-40 min. The cooked beans are inoculated with *Bacillus subtilis* (*natto*) and wrapped in paper-thin sheet of pinewood or packed in plastic packages weighing 80-120 g. The fermentation lasts for 15-20 hrs at 40-43°C in the package in which natto is sold. See Fig. 26.3 for an outline of commercial natto production.

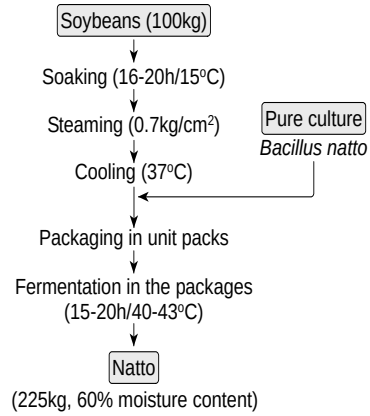


Fig. 26.3 Commercial production of natto

Natto has a short shelf-life, partly because of high moisture content (60%) and partly because it is usually prepared in small-scale plants with poor quality.

During fermentation, water-soluble nitrogen and ammonia nitrogen is greatly increased. There is no change in fat and fiber content but the carbohydrates totally disappear.

26.7 JAPANESE SAKE

Japanese sake is a clear, pale yellow, rice wine with an alcohol content of 15-16% (or higher), a characteristic aroma, little acid, and slight sweetness.

26.7.1 INDUSTRIAL PRODUCTION

The industrial production of sake involves various steps. The essential steps are described in the following paragraphs:

26.7.2 SELECTION/PREPARATION OF RICE

Rice of short-grained variety is considered best for sake production. The rice should be finely polished to remove proteins, lipids and minerals. It is then washed and steeped in water (until 25-30% water uptake), drained (4-8 hrs) and steamed (30-60 min). Steaming sterilizes and gelatinizes starch and the total water uptake amounts to 35-40%. The rice is cooled to 35°C for koji manufacture and 10°C for the preparation of *moromi*.

26.7.3 KOJI PREPARATION

The rice prepared as above is inoculated with spores from *tane* koji (at the rate of 60-100 g/100 kg prepared rice). Tane koji is prepared by culturing *Aspergillus oryzae* on soaked, steamed, polished rice for 5-6 days or until abundant sporulation. The inoculated rice is heaped on the floor of a room with controlled humidity and held at 26-28°C. The internal temperature of the heap rises to 31-32°C. After 10-12 hrs, the

mold spores germinate. The rice is mixed and after 20-24 hrs the developing koji is placed in 15-45 kg capacity wooden boxes. Mixing is done every 6-8 hrs to avoid overheating. After 40 hrs, the temperature of the developing koji reaches 40-42°C, and the mycelia will cover the grains. The mycelia contain sufficient hydrolytic enzymes so that the koji can be used for saccharification of starch in the main mash.

26.7.4 PREPARATION OF KOONTOKA-MOTO

Koontoka-moto is a hot-mash, rapid-saccharification procedure used for the preparation of yeast starter (moto). After a 6-hr starch hydrolytic step at 56-60°C with koji amylases, the mash is cooled, acidified and filtered. The filtrate is used to grow pure sake culture.

More recently, aerobically propagated compressed sake yeast has become available commercially and can be inoculated directly as 7% (w/w) of the total rice used in a moromi mash. Acidification of the mash is carried out with lactic acid. This method eliminates the necessity of preparing moto.

26.7.5 MAIN FERMENTATION

For the main fermentation, mash (moromi), unsterilized koji, steamed rice, and water are fermented in 6-20-kL tanks, each containing 1500-10,000 kg of rice. The yeast population in moromi is built up in a stepwise manner over a period of 3 days. Moto mash is combined with equal quantities of rice and water, reducing the yeast count by two-thirds. After 2 days at 12°C, the yeast population rises to 10^8/g and mash is diluted again by about one-half. The rice-koji-water mixture is added at 9-10°C to suppress the growth of contamination microorganisms. The following day, a third addition is made at 7-8°C, again reducing the yeast by one-half. In this way, the yeast population of 2.5×10^8 cells/g is reached after about 1 week of fermentation. Such stepwise fermentation permits careful temperature control, important in balancing saccharification and fermentation rates. With such control, ethanol concentration approaches 20% *abv* in 20-25 days.

The moromi tends to form a rather viscous foam that may occupy one-third of fermenter volume.

The mash is pressed, the liquor settled for 5-10 days, filtered, blended, and settled again for 30-40 days. Everything needs to be carried out at low temperature. The wine is pasteurized at 55-65°C and aged at 13-18°C with or without activated carbon. Blending, dilution with water, filtration, and bottling marks the final steps of sake preparation. 1000 kg of polished rice yields 3000 liters of sake (20% *abv*) and 200-250 kg of residue (sake-*kasu*).

26.8 *KINEMA*

Kinema is a fermented soybean food product indigenous to Nepal. It is mostly prepared and consumed (and sometimes sold) by Limbus of eastern Nepal, especially in the hills. The preparation is limited to household level. Methods followed for its preparation are often subject to variation. Some of the more

important factors contributing to variation are: locality, convenience, availability of raw materials, and processing steps.

26.8.1 TRADITIONAL PROCESS OF *KINEMA* MAKING

Kinema preparation is a relatively simple process. The traditional process entails cooking of soybeans (white or brown), cooling to room temperature, mixing with a small amount of vegetable ash, wrapping with banana leaves or rice straw, and leaving it for 2-3 days in a warm place for fermentation. The beans are usually mashed in wooden pestle or macerated with hands so as to split them apart. A well-fermented *kinema* has a slimy appearance (stringy when touched), tends to form cake, and has a persistent *nutty* to *musty* flavor. Unless dried, the preparation has a very short shelf-life (2-3 days) at room temperature.

Kinema preparation does not require any addition of microbial culture: it is a spontaneous fermentation. The microbial flora present in the banana leaves or rice straw (sometimes other leaves are also used) act as an inoculum. Wood ash, which is so often considered an essential ingredient in *kinema* making, may not be indispensable for the fermentation itself but can be considered desirable in that it may furnish certain minerals to the organisms. The condition also becomes alkaline, which favors fermentation. The use of ash can also be related to the development of characteristic taste in *kinema*. See Fig. 26.4 for an outline of traditional *kinema* making.

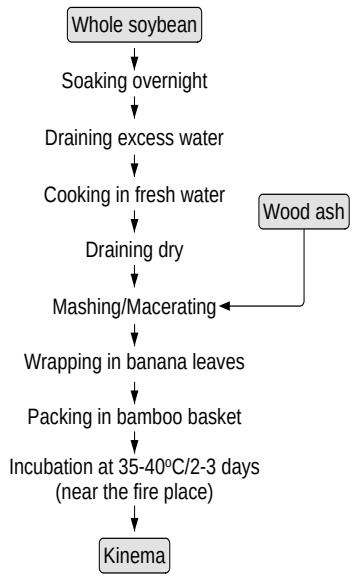


Fig. 26.4 Traditional preparation of *kinema*

With respect to properties, *kinema* falls in a position somewhere between the two familiar oriental fermented soybean products, namely, *tempeh kedele* and *natto*. Tempeh is a traditional mold-fermented food native to Indonesia (see later). The most popular type of tempeh is produced from soybeans and is known as tempeh

kedele. Natto is bacterium-fermented soybean food product of Japan (discussed earlier).

Because of the nature of fermentation, the quality of *kinema* obviously never remains consistent. Since rice straw as well as banana leaf is used as the source of inoculum the quality of the final product can only be as good as the quality (and relative proportions) of microorganism present in the source. The final product has the characteristics of natto as well as tempeh but since the fermentation produces a more ammoniacal odor, the characteristics of tempeh are usually masked. Overall, *kinema* more resembles natto than tempeh. The sticky substance present in *kinema* has been identified as exopolypeptides of D-isomeric glutamic acid having γ-glutamyl peptide bonds. The organisms, according to T.B. Karki (1994), are: *Bacillus subtilis, Enterococcus faecium, Candida parapsilosis*, and *Geotrichum candidum*

Natto, tempeh kedele, and *kinema* have many things in common, for instance:

1. Soybean is the basic raw material for preparation
2. They are traditional fermented foods of the orient
3. Traditional methods of preparation depend on spontaneous fermentation

Of the differences, the more important ones are as in Table 26.5:

Table 26.5 Fundamental differences between natto, *kinema*, and tempeh

Parameter	Natto	Tempeh kedele	*Kinema*
Organism	Bacteria	Mold	Mixed flora
Flavor/odor	Musty to ammoniacal	Nutty	Nutty to musty
Use	Condiment, in soups, etc.	Staple food, usually deep fried	Condiments, in soups, curry, and as *chutney* substitute
Incubation temperature	40-43°C	35°C	30-40°C

26.8.2 NUTRITIONAL SIGNIFICANCE

Soybeans are fermented not primarily for preservation. In fact, the fermented product has a very short shelf-life. Flavor development is the principal reason of such fermentations. Nevertheless, among other things, fermentation of soybeans also leads to following advantages:

1. Leaches out during cooking flatulent principles like stachyose and raffinose
2. Decreases/destroys antinutritional factors like trypsin inhibitors, lectins and phytic acid
3. Increases soluble nitrogenous substances

26.8.3 PALATABILITY

Palatability of fermented soybean products is largely a matter of food habit. What can be mouth-watering to one can be offensive, or even revolting to another. For a beginner, natto and *kinema* can be revolting but for a habitual consumer the same flavor can be highly appetizing. Tempeh kedele has a much milder and nutty flavor and is being slowly accepted in the West also.

26.9 TEMPEH

Tempeh is an oriental mold-fermented food indigenous to Indonesia. The most popular type of tempeh is produced from soybeans (preferably white) and is known as *tempeh kedele*.

26.9.1 TEMPEH ORGANISM

The fermentation is invariably a mixed culture of molds, yeasts, and bacteria but the most important component appears to be *Rhizopus oligosporus*, although other *Rhizopus* species and *Mucor* are also often isolated. One of the better producers of tempeh has been identified by Hasseltine *et al* (1963) as *Rhizopus oligosporus* Saito NRRL2710.

26.9.2 METABOLIC CHARACTERISTICS OF THE ORGANISM

Rhizopus oligosporus Saito NRRL2710 has a low amylase activity but high protease and lipase activity. Fatty acids in soybeans are the principal source of carbon and energy. Stachyose and raffinose are not utilized but common sugars such as glucose, fructose, etc., support excellent growth. By virtue of high protease activity, the organism can hydrolyze and utilize soybean proteins for nitrogen source. Proline, glycine, aspartic acid, and leucine are excellent sources of nitrogen but tryptophan supports no growth at all. It can utilize ammonium salts but not sodium nitrate.

Bacterial contamination is generally not encountered. Because the organism produces an antibacterial agent, and also because it has the unique characteristic of fast growth rate, there is little chance for bacteria to gain ground before the tempeh fermentation is complete.

26.9.3 COMMERCIAL TEMPEH PRODUCTION PROCESS

Commercial tempeh preparation starts with dehulled, full-fat soybeans or soybean grits. Grits yield better quality tempeh. The soaking time is usually 30 min to an hour but some investigators consider this step superfluous. Commercial fermentations use pure culture in regulated amounts. Banana leaves may be optional (see Fig. 26.5).

26.9.3.1 Processing loss

The dehulling, soaking, washing, cooking, and fermenting steps employed in the preparation of tempeh all contribute to loss of soybean constituents. The total loss of solids ranges from 24 to 48%, depending on the variety of soybeans and the

process used. The more significant losses are in dehulling and cooking. Loss can be minimized by using less water during cooking but the fermentation as well as the quality of tempeh will not be sound. In such cases, the tempeh shows less mold development and much sporulation (and therefore discoloration). The flavor and odor are also unpleasant and poor. The factor responsible for this is the presence of heat-stable and water-soluble mold inhibitor in soybeans. This factor also inhibits the formation of proteolytic enzymes by *Rhizopus oligosporus*. Therefore, soaking and cooking of soybeans in excess water (which is discarded later) are essential to tempeh making.

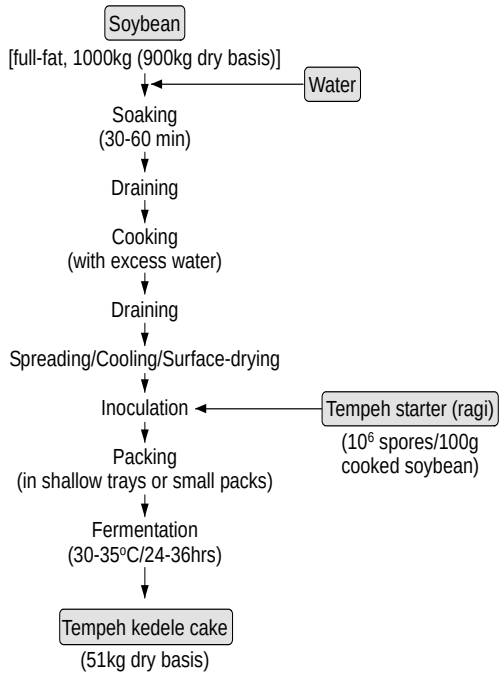


Fig. 25.5 Commercial preparation of tempeh kedele

26.9.3.2 Mold management

Traditionally, small pieces of tempeh from previous fermentation serve as inoculum. The fungus is then propagated mainly by means of fast-growing mycelia. The disadvantage of this method is that it can lead to contamination by undesirable microorganisms. Moreover, the inability of mycelia to survive adverse temperatures and dehydration makes mycelia unsuitable for long-term preservation. For long-term storage, either lyophilized cultures or other suitable modifications are prepared. Spores may be produced in rice, soybeans, or wheat substrate. Soybean as a substrate results in unpleasant flavor and odor while wheat bran results in poor sporulation. Fermentation in rice of 40% moisture level for 4-5 days at 32°C produces good growth and spores. The whole can be made into slurry by blending with sterilized water and then freeze-dried. The spores show comparable viability even after storage for 6 months in plastic packages at 4°C.

26.9.3.3 Inoculum size

The amount of inoculum required to make satisfactory tempeh is significant because fermentation time becomes too critical if the amount of inoculum is too large. On the other hand, too small an amount of inoculum provides chance for contaminating bacteria to grow. A level of 10^6 *Rhizopus oligosporus* spores per 100 g of cooked soybeans seems appropriate.

26.9.4SHELF-LIFE OF TEMPEH

Fresh tempeh has a shelf-life of only one to two days as sporulation of mold discolors the product and a rich ammoniacal odor develops as proteolysis proceeds. The release of ammonia makes the product noxious. Its shelf-life, however, can be prolonged by various methods. In Indonesia, it is cut into slices and sun-dried. An alternative method could be to first blanch the sliced tempeh to inactivate the mold and enzymes and then freeze it.

26.9.5 PALATABILITY

Fresh tempeh has a pleasant nutty flavor and odor. It is free from *beany* flavor and odor of raw soybeans and is therefore highly palatable. It is the only Oriental fermented product that has been extensively investigated in the West. Many countries in the West have now begun to consume tempeh.

26.9.6 NUTRITIONAL VALUE

Tempeh has a superior nutritional value over unfermented soybeans. Undesirable soy components such as flatulence factors and trypsin inhibitors, etc., are removed and/or destroyed during cooking and draining. Lipids become more resistant to autoxidation. Niacin, riboflavin, Vit. B_6, and pantothenic acid increase after fermentation. The food also contains beneficial antibacterial components. At any rate, it is a potential source of low-cost protein that can have significant role in solving protein malnutrition.

26.9.7 CONSUMPTION PATTERN

Tempeh, in its various forms and types, is consumed in Java, Indonesia, and some other Oriental and western countries. In Indonesia, the annual production of tempeh is over 80,000 MT, which accounts for about 14% of the total soybeans produced there. Indonesians consider tempeh to be a nourishing and easily digestible food. They use it as a main dish. In Java, the per capita daily consumption of fresh tempeh is in the order of 20-120 g.

The food is consumed in a variety of ways. The more common ways are:

1. Slicing, dipping in salt, and deep frying in coconut oil
2. Including pieces of tempeh in soups

26.10 TEMPEH BONGKREK

The preparation of this type of tempeh is principally similar to that of tempeh kedele. It is popular in Java. The raw material used here is the coconut press cake. Tempeh bongkrek has been associated with occasional outbreaks of food poisoning due to the bacterium *Pseudomonas cocovenenans* growing in the product and elaborating the toxins *bongkrekic acid* and *toxoflavin*. Since 1951, at least 1000 people are known to have died as a result of this intoxication. Consequently, the Indonesian Government prohibited the production of tempeh bongkrek in 1988.

26.11 ONTJOM

Ontjom is also mold-fermented product native to Indonesia. It is prepared from peanut press-cake. The organism used is either Rhizopus *oligosporus* of *Neurospora intermedia*. The preparation has a fruity/mincemeat character.

26.12 FERMENTED VEGETABLES

Most horticulture products can be preserved by lactic acid fermentation. In the West the most important commercially are cabbage, cucumbers, and olives. Fermented vegetables, commonly cabbage, in Korea is known as *kimchi*. The two most common lactic acid-fermented vegetable products of Nepal are *gundruk* and *sinki*.

26.12.1 SAUERKRAUT

Sauerkraut production is thought to have been brought to Europe from China by the Tartars. Initially, the shredded cabbage was deliberately made sour by adding sour wine and vinegar. This produced a literally "sour cabbage". But as we know and define now, *sauerkraut is a clean, sound product of characteristic flavor, obtained by full fermentation, chiefly lactic, of properly prepared and shredded cabbage in the presence of not less than 2%, nor more than 3% salt*. It contains, upon completion of fermentation, not less than 1.5% of acid, expressed as lactic acid.

26.12.1.1 The technology

Like a number of other traditional vegetable fermentations, the commercial process of sauerkraut production is technologically simple, but involves some interesting and complex chemistry and microbiology.

Usually, where commercial sauerkraut production is practiced, special cabbage cultivars are grown. These improved cultivars are well-adapted to mechanical harvesting and at the same time inherently contain less water. The outer leaves are removed mechanically and the cabbages decored before cutting into shreds of about 1 mm (0.8-1.0 mm). The finely cut long shreds, called "slaw", are then conveyed by belts or carts to the vats or tanks for salting and fermentation.

The level of salting is critical to obtaining a satisfactory product. It must be within the range 2-3% w/w and is normally about 2.25%. Uniform distribution of salt throughout the mass of cabbage is very essential. Too little salt (less than 2%) causes

product-softening to an unacceptable level. Too much salt (over 3%) interferes with the correct microbial sequence, delays fermentation, and depending on the amount of oversalting, may produce a product with a sharp, bitter taste, causing darkening of the color, or favor growth of pink yeasts. The salt serves a number of purposes, namely:

- It extracts moisture from the shredded cabbage by osmosis to form the brine in which the fermentation will take place
- It helps inhibit some of the natural microflora of the cabbage, such as pseudomonads (which would otherwise cause spoilage) and helps select the lactic acid bacteria
- It helps maintain the crisp texture of the cabbage by withdrawing water and inhibiting endogenous proteolytic enzymes which cause the product to soften
- Finally, salt contributes to flavor of the product.

Commercially, sauerkraut fermentation is often carried out in concrete vessels with synthetic polymer lining to protect the vessel from attack by the acid brine. After salting and dumping in the vat, the whole is sealed by covering with plastic sheeting. The sheet is then filled with brine on the top to press the sheeting on the cabbage, thereby expelling the entrapped air. See Fig. 26.6 for an idea of the packing process.

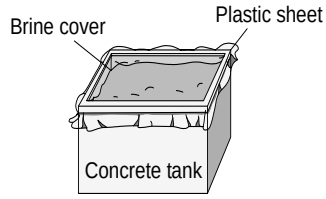


Fig. 26.6 Packing of slaw for fermentation

26.12.1.2 Fermentation condition

Temperature is a controlling factor in the sequence of desirable bacteria in the sauerkraut fermentation. At the optimum of 18.3°C or lower, the quality of sauerkraut is generally superior in color, flavor, and ascorbic acid content because the heterofermentative bacteria exert a greater effect. Under such a condition, the fermentation is complete in 1-2 months. At a temperature of 32°C, the fermentation time is as short as 8-10 days, but at the cost of quality. Higher temperatures lead to essentially homofermentative mode and the kraut is reminiscent of acidified cabbage. Acidity, coupled with saturation of the mass of the product with CO_2, is sufficient to provide conditions necessary for the preservation of sauerkraut.

26.12.1.3 Microbiology of sauerkraut fermentation

Although commercial starter-cultures for sauerkraut fermentation are available, they are not widely used: rather, spontaneous fermentation is generally employed. The fermentation is initiated by *Leuconostoc mesenteroides*, which is among the less acid-and-

salt tolerant lactic acid bacteria (LAB) but grows the fastest during these early stages. As a heterofermenter, it produces CO_2, which replaces the entrapped air and helps establish anaerobic conditions within the product. This event prevents the oxidation of Vitamin C and loss of color. Since fructose is present as an alternative electron acceptor, the bacterium also produces appreciable amounts of acetic (ethanoic) acid acety-ScoA (the major contributor to sauerkraut flavor). As the pH drops due to acid production, *Leuconostoc* is inhibited and replaced, first by heterofermentative lactobacilli (such as *Lactobacillus brevis*), and then by homofermentative *Lactobacillus plantarum*. Acid accumulation continues in the form of lactic acid although the pH stabilizes somewhere around 3.6 (the pK_a of lactic acid). At the end of fermentation (which can last from 4-8 weeks) the total acidity of the product is 1.7-2.3%, expressed as lactic acid, with the ratio of volatile to non-volatile around 1:4.

This succession of microorganisms produces interesting changes in the kraut during fermentation. At the commencement, lactic acid bacteria comprise only about 1% of the total microflora, but many of the non-lactics fail to grow. Two days later, lactic acid bacteria account for more than 90% of the total microflora. During this time they produce sufficient acid to decrease the pH to below 4, further inhibiting the competing microflora. Underlying this overall dominance by lactic acid bacteria is a natural succession of different species which contribute to the characteristic flavor of sauerkraut. Fig. 26.7 shows some changes observed during sauerkraut fermentation.

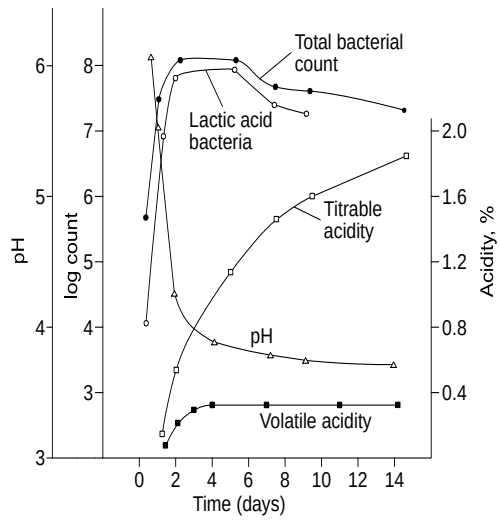


Fig. 26.7 Chemical and microbial changes during sauerkraut fermentation

26.12.1.4 Defects in sauerkraut

Defects in sauerkraut arise mainly as a result of yeast and mold growth. They can produce off-flavor/odor, loss of acidity, a slimy softened product as a result of pectolytic activity, or a pink coloration due to the growth of *Rhodotorula* (yeast). In the early stages of fermentation, *Leuconostoc mesenteroides* fermenting sucrose will preferably utilize fructose, polymerizing the glucose moieties to produce dextran

slime. However, this is transient and the slime is later degraded and utilized by other lactic acid bacteria.

26.12.2 *GUNDRUK* AND *SINKI*

Gundruk is a non-salted, lactic fermented vegetable product indigenous to Nepal. It is made from vegetable leaves (e.g., mustard, radish, *rayo*). The fermentation is a spontaneous one, caused by epiphytic LAB that inhabit the vegetable leaf (the raw material).

Gundruk is believed to have existed in Nepalese culture since time immemorial. In the rural areas (where *gundruk* is generally produced), *gundruk* is primarily used for flavor reasons and as an alternative to green vegetables in the lean season. It is valued for its uniquely appetizing flavor. In Nepal, about 2% of the *gundruk* production has been industrialized. It is sold in the market in the dried form.

The quality of *gundruk* mainly depends on the balanced production of lactic acid and acetic acid (ratio of about 10:7). *Gundruk* fermentation is primarily initiated by heterolactic lactobacilli such as *Lactobacillus cellobiosus* and *Leuconostoc mesenteroides*. The fermentation is subsequently completed by the more acid producing homolactic bacterium *Lactobacillus plantarum*.

26.12.2.1 Technology

The methods of *gundruk* preparation tend to differ slightly, depending on the geographical region, raw material, and the people preparing it. An oversimplified method of *gundruk* preparation (suitable in small batches) is shown in Fig. 26.8.

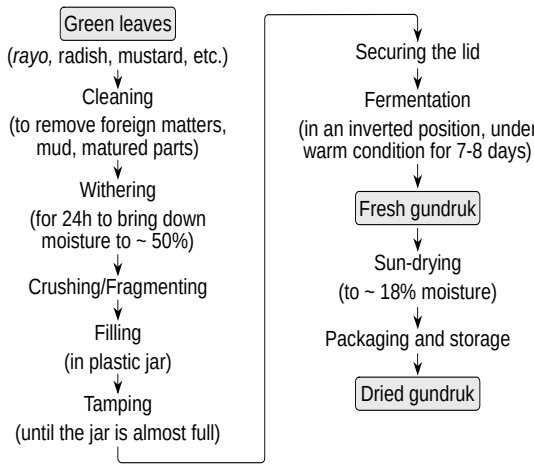


Fig. 26.8 Simplified process of *gundruk* preparation

A generalized method of *gundruk* production is rather straightforward: it entails wilting of vegetable leaves in the sun for a few days, crushing, tamping in containers (usually made out of bamboo stem, called *dhungro*), and fermented in a warm place

for a week to several days. The first sign of fermentation is the appearance of froth (that oozes out of the container) and this is generally followed by leaching out of the brown-colored juice. In general, it takes 10-15 days for the completion of fermentation. The final operation in *gundruk* preparation consists of drying (to moisture content around 10%) in sun and making ready (for example, packaging) for sale, storage or distribution.

Gundruk is used as pickle or soup, alone or in some suitable combination with other vegetables, and served generally in the main course.

Sinki is another similar lactic-fermented product in which radish is used as the raw material. *Sinki* has a light- and more acceptable appearance. The production process is more or less similar to that of *gundruk*. In the glut season, when large quantities of surplus radish need to be fermented, the fermentation is carried out in pits (~ 1 m^3 space) dug in the ground. The pit is first warmed up by burning dried leaves and twigs and then lined (internally) with banana leaves. Radish pieces (which have been previously wilted and crushed) are then tamped in. An outer covering of banana leaves and an additional layer of other covering materials (e.g., straw) are given to provide a facultative environment for the fermentation. The whole is left undisturbed for 10-15 days for fermentation. Drying, storage, and consumption of *sinki* are similar to that of *gundruk*.

26.13 *JAND*, *NIGAR*, AND *RAKSI*

Jand is an alcoholic beverage (undistilled) indigenous to Nepal. It is prepared by solid-substrate fermentation of starchy cereals like corn, rice, wheat, and millet. Millet is the material of choice because it is claimed to produce superior quality of *jand*. *Murcha*, a starter culture, is used as the inoculum in traditional fermentation. Murcha contains saccharifying molds, lactic acid bacteria, and fermenting yeasts. *Jand* is therefore the result of concerted action of these microorganisms on the cooked cereal. Fig. 26.9 shows a simplified biochemistry of *jand* fermentation.

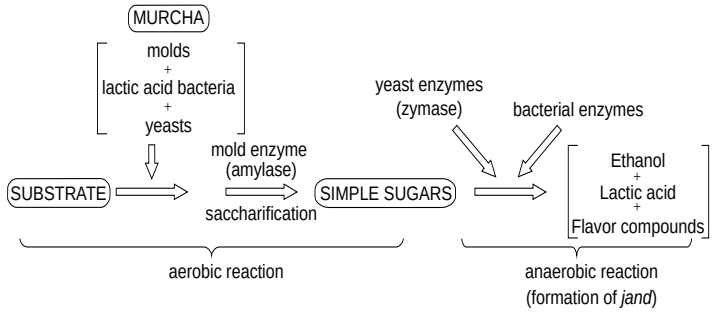


Fig. 26.9 Simplified biochemistry of *jand* fermentation

The basic steps followed in the traditional *jand* making are: cooking of cereal, cooling to room temperature, mixing with murcha powder, leaving it for a day or two for biomass build-up (of essential organisms, viz., yeasts and molds), and fermenting in tightly plugged containers. Originally, close-necked earthen pots were used for

fermentation but now plastic containers have largely replaced them. The duration of fermentation may range from a week to several months, during which 4-15% alcohol by volume (*abv*) may develop.

Due to continued saccharification and ethanol production, the mash gradually turns limpid. At some point, a nearly clear supernatant is observed in the fermentation vessel. This liquid is called *nigar* and is much prized by habitual *jand* drinkers. *Nigar* can be categorized as a *cereal wine* (similar to Japanese *sake*) while *jand* (which contains live yeasts and suspended particles) has been classified by various workers as a category of *cereal beer*. See Fig. 26.10 for an outline of the traditional method of *jand* preparation.

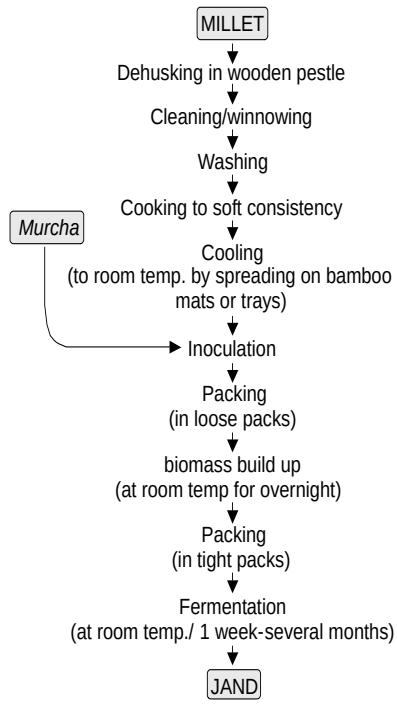


Fig. 26.10 Traditional preparation of *jand*

During serving, the mash is taken out, mixed with requisite amount of water, squeezed, strained, and the cloudy extract drunk. Another variation of consuming *jand* is as *tongba* (or *tumba*). In this variation, *jand* is loosely stuffed into a cylindrical jug, lukewarm water poured in, a small bamboo tube (with a pair of eyeholes at one end) inserted through the mash, and the extract sucked in (Fig. 26.11). This variation is a more standard form of taking/offering *jand* in traditional ceremonies and ritual rites.

The traditional method of *jand* preparation has many shortcomings. The quality of murcha is never consistent and so is the quality of *jand*. Optimum fermentation conditions are difficult to maintain. Besides, sanitary conditions are not adequately maintained in the tribal method. As of now, some studies are available that indicate that use of pure cultures (isolated from *murcha*) can be an attractive alternative.

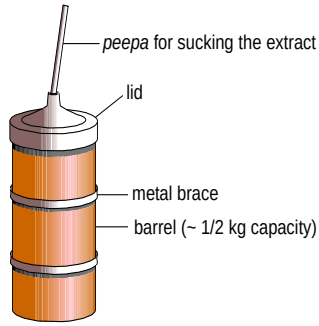


Fig. 26.11 *Tongba* (a variation of serving *jand*)

When *jand* is pot-distilled, it becomes *raksi*, which is an unaged traditional spirit of varying alcohol contents (15-50% abv). The product likens whiskey and is very popular among the ethnic groups of Nepal. The pot distillation assembly used in the locality for *raksi* production is shown in Fig. 26.12.

The traditional *raksi* making apparatus is rather inefficient. An improved design the author has proposed for a 150-liter mash (the traditional still uses 15-20 liters of mash per batch) is shown in Fig. 26.13. However, this design has not been tested so far.

The scheme (Fig 28.11) utilizes low pressure steam to heat the fermented mash in a steam-jacketed kettle. The boiler has two chambers, only one of which can be used for steam generation at a time. The condensate from the still jacket is led through a non-return valve into the boiler (compartment from where steam is not being led out). To regulate the flow of steam, fire can be shifted alternately (manually) so that only one of the compartments is heated (for steam) while the other compartment receives hot water. The entire design does not use any pumps and is fully gravity-based.

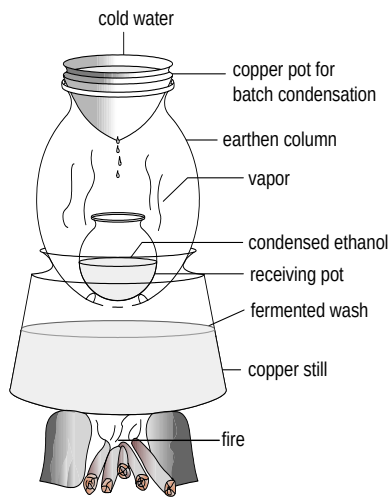


Fig. 26.12 *Raksi* making apparatus

The rectification column is made up of a cylinder that is packed with broken glass chips. The entire set is made of steel that is readily available locally. The equipment can be completely fabricated in a local workshop that has facilities for rolling the iron/steel sheet and welding. Valves can be purchased from retail stores.

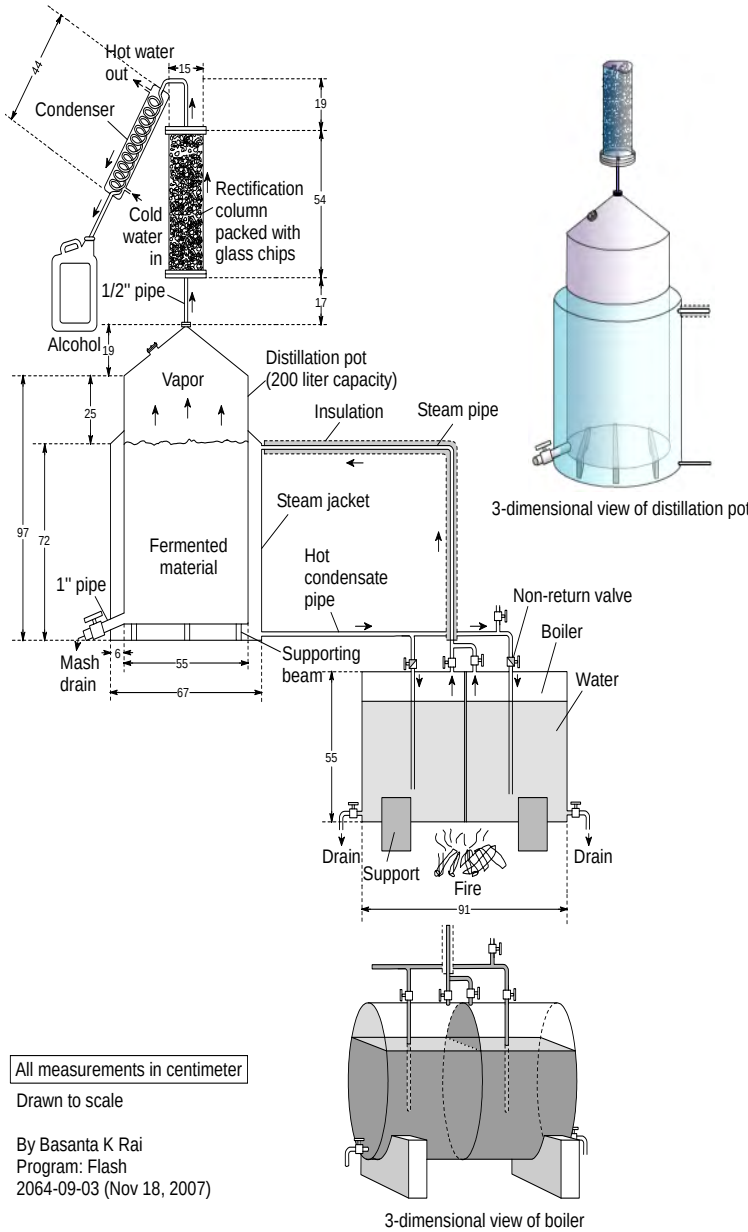


Fig. 26.13 Proposed improved distillation set for *raksi* making

CHAPTER 27

MICROBIOLOGICAL ANALYSIS OF NUTRIENTS

27.1 INTRODUCTION

Because of the similarity in nutritive requirements of microorganisms and experimental animals, it is possible to use microorganisms to determine quantitatively many of the substances that are known to be essential constituents of all living cells.

Microbiological assay involves the use of microorganisms as reagents for quantitative determination of certain chemical compounds, particularly vitamins, amino acids, growth factors, and antibiotics. Microbiological assay are highly specific and unusually sensitive. For example, as little as 0.1 nanogram of biotin/ml can be detected using the organism *Lactobacillus casei.*

The basic principle upon which microbiological assay depends is that in the presence of limiting amounts of certain compounds, the amount of microbial growth is a function of the amount of these compounds.

The response measured depends on the effect of the substance on the metabolism of the organism. The responses are of two main types, *viz.*, (i) growth response and (ii) metabolic response.

27.2 GROWTH RESPONSE

The response is *positive* (promotion of growth) in assay of nutrients and *negative* (inhibition) in assay of antibiotics. The growth response can be measured by methods such as *numerical counts, optical density, weight of cells, area* (of the growth of cells on the surface of the medium,) etc. Additionally, the growth response may be a *definite end point* or an *all-or-none* response.

27.3 METABOLIC RESPONSE

In the case of metabolic response, whether positive or negative, metabolic products or changes in some function may be measured. Among the measurable metabolic responses are acid production, CO_2 production, O_2 uptake, reduction of nitrates, hemolysis of red blood cells, antiluminiscent activity, and inhibition of spore germination. Not all of these responses are easy to measure, though.

The microorganisms used for assay include bacteria, yeasts, fungi, and protozoa. The use of bacteria generally poses fewer problems than the use of other groups of microorganisms. They have been used to assay proteins, amino acids, carbohydrates, and vitamins, and to evaluate antiseptics, disinfectants, and chemotherapeutic agents. Lactic acid bacteria (including the genera *Lactobacillus*, *Streptococcus*, and *Leuconostoc*)

equal or surpass all other groups of microorganisms in the complexity of their nutritional requirements (and hence their usefulness).

There are some requirements for an organism to be useful in microbiological assay. The ideal test organism should:

1. Be sensitive to the substance being assayed
2. Be easily cultivated
3. Have metabolic function or response that is readily measurable
4. Not be susceptible during assay to variation in either its sensitivity or phase.

As an example of microbiological assay of nutrients, the use of *Lactobacillus arabinosus* for the assay of *niacin* can be mentioned. This bacterium requires niacin for growth. When it is inoculated into a medium containing all the necessary nutrients except niacin, growth will not occur. If niacin is added to this medium the organism will grow and the growth obtained, within limits, will increase as the amount of niacin is increased. It is therefore possible to prepare a standard curve relating growth to the amount of the vitamin. If a substance of unknown niacin content is added to the medium and the test is carried out in the usual manner, the amount of growth measured can be compared with the standard curve (of niacin), and from this the amount of niacin in the unknown sample can be extrapolated (see Fig. 27.1 for an idea). Some examples of the microorganisms used for the assay are given in Table 27.1. The amount of niacin can be obtained either from the graph (slope) or by linear regression by first drawing a trendline (Excel program can be used) to yield an equation of the form: $y = mx + c$, where: y = absorbance, m = slope, x = concentration of nutrient, and c = intercept.

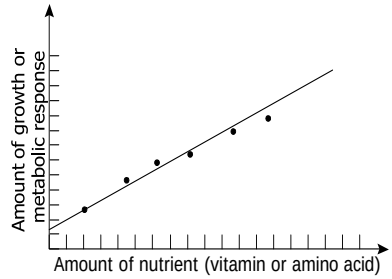


Fig. 27.1 The standard curve for microbiological assay

Table 27.1 Examples of microorganisms used in microbiological assay

Organism	Compound assayed
Streptococcus fecalis (bacteria)	Several amino acids
Tetrahymena gelei (protozoan)	Folic acid
Neurospora crassa (mold)	Biotin
Saccharomyces carlsbergensis (yeast)	Pantothenic acid
Ochromonas malhamensis (alga)	Vitamin B_{12}

27.4 METHODS OF MICROBIOLOGICAL ASSAY

There are four main methods of microbiological assay by which the potency of samples and standard solutions can be compared. They are: (i) diffusion, (ii) turbidity or dilution, (iii) gravimetric, and (iv) metabolic response methods.

27.4.1 DIFFUSION METHOD

The assayed substance is allowed to diffuse through solid media (in which the culture has been inoculated), and the zone of growth (or inhibition) of the test organism formed around the application point (or area) of the substance is observed. The size of the zone is a function of the concentration of the assayed substance. The function can be expressed as a *linear* relationship between the size of the zone and the logarithm of concentration of the substance. By measuring the distance the substance diffuses and comparing it with that of a known standard preparation, the potency of the sample can be assayed.

Diffusion may be of two types: (i) *linear*, which occurs when the substance is placed in contact with a column of seeded agar in a capillary or test tube, and (ii) *radial*, which occurs around a suitable reservoir containing the substance on a seeded agar plate.

The horizontal (radial) diffusion can be carried out by two common methods: (i) *cylindrical method*, and (ii) *cup-plate method*. In the former method, cylinders are embedded to a fixed height in the solidified agar (see Fig. 27.2). In the cup-plate method, a *depression* is made in the solidified seeded agar by removing a slug with a cork-borer. The test material is placed in the cylinder (or depression, as the case may be) and the diameter of the zone of growth or inhibition due to diffusion of the test substance is compared with standard concentration of the assayed substance.

The *drop-plate* and *paper disc* method is a variation of the horizontal diffusion method. In the drop-plate method, the test substance is placed directly on the medium whereas in the paper-disc method the substance comes in contact with the medium indirectly through paper disc (see Fig. 27.2).

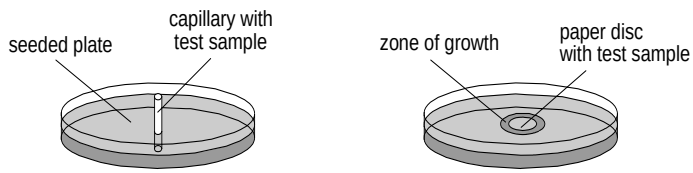


Fig. 27.2 Linear and radial diffusion assay

27.4.2 TURBIDIMETRIC AND DILUTION METHODS

The distinction between *dilution-* and *turbidimetric* methods is that the former gives an *all-or-none* end point in broth or agar, whereas the latter measures graded growth or metabolic response.

The serial dilution method for the assay of antibiotics is important for food analysts. In this method, several dilutions of the test-substance in small tubes is inoculated with a test organism, incubated, and the lowest concentration of the substance that causes apparently complete inhibition of growth of organism is taken as the minimum inhibitory concentration.

In turbidimetric assay, graded concentrations of the test substance are added to a series of test tubes or flasks containing a liquid nutrient medium. The medium is inoculated with the test organism and incubated for a suitable time. The response of the test organism is measured in a photometer, the scale reading of which may be converted by calibration curve prepared with graded amounts of pure substance to determine the potency of the assayed sample.

27.4.3 GRAVIMETRIC METHOD

The response of the test organism to graded concentrations of the analyzed substance is determined after a suitable incubation time by measurement of the amount of growth in terms of dry cell weight. Under the conditions of assay, this weight is proportional to the concentration of the limiting factor. The majority of the gravimetric methods use *Neurospora crassa* as the test organism.

27.4.4 METABOLIC RESPONSE

The response of the test organism to various concentrations of the assayed substance is evaluated, after a suitable incubation period, as a change in specific measurable metabolic parameters. Several parameters can be measured; however, acid production is the only one used widely. The acid production is determined by titration.

Although the basic principle of microbiological assay appears straightforward, in practice the protocols can be quite involved. The complexity involved in sample preparation can be taken as a good example. In this particular case, the sample must not contain any components that may interfere with the response of the test organism. Since the sample can come from a wide variety of sources, it becomes a practical limitation to provide any generalized, foolproof method for the preparation of the sample whereby it can be used for all assays. Another example of practical complexity is in the measurement of cell response in terms of weight. In this case, the cells must be meticulously washed free from the medium and the true dry matter content determined.

27.5 EXAMPLE: ASSAY OF FOLIC ACID

Test organism: Streptococcus fecalis ATCC 8043

Inoculum: Prepare a *stab culture* using medium of following composition: Peptone 05g, dextrose 1.0 g, anhydrous sodium acetate 0.6 g, kH_2PO_4 0.2 g, agar 2.5 g, distilled water 100 ml. Maintain the pH at 6.8. The rest of the process is as follows:

- Inoculate from stab-culture a tube of broth of the same composition as above but without agar.

- Incubate overnight at 37°C
- Transfer a suitable aliquot from this to 50 ml of sterile assay medium and incubate at 37°C for 5 hrs.
- Use assay medium of following composition for the assay of folic acid:

Components	Amount
Vitamin-free *caesamino* acid	5.0 g
Sodium citrate	26 g
Dextrose	10 g
K_2HPO_4	3.2 g
L-cystine	0.38 g
Distilled water	1 liter

- Dissolve L-cystine in dilute HCl and other ingredients in distilled water. Adjust pH to 6.8, add stock solutions[♣], and make to desired volume.
- *Standard and dilutions*: dilute standard folic acid and sample suitably to finally contain 1 mμg per ml.

♣ Stock solutions contain special compounds such as amino acids, vitamins, nitrogenous bases, etc., needed for the growth of the test organism. Usually, several stock solutions are used for a given assay media. For folic acid assay, 5 different stock solutions are needed.

ASSAY PROCEDURE

1. Use 25 mm × 200 mm *lipless* tubes with loose-fitting aluminum caps for the assay
2. Prepare a series of tubes containing different aliquots between 0.5 ml and 5 ml of the standard and sample dilutions
3. Raise the volume to 5 ml with distilled water
4. Add 5 ml of a *double-strength* assay broth (the one given above is a single-strength broth)
5. Autoclave the medium at 15 psig/15 min and cool
6. Inoculate and incubate the tubes at 37°C overnight
7. Arrest growth of microorganisms by adding formalin
8. Measure turbidities (or percent transmittances) using suitable photoelectric colorimeter
9. Draw a standard curve of % transmittance against the concentration of vitamin on an arithmetic graph paper
10. Find the equivalent values of the standard for the responses of the sample by interpolation and compute folic acid activity in the sample

CHAPTER 28

MICROBIAL PRODUCTION OF VITAMIN B_{12} AND ß-CAROTENE

28.1 INTRODUCTION

Microorganism can be used for the production of vitamins like thiamin, riboflavin, and cyanocobalamin. Some other vitamins like Vit C can also be produced microbiologically by *Acetobactor suboxidans*. So far, commercial fermentation has been economical only for the production of riboflavin and Vit B_{12}. Microbial production of β-carotene is less cost effective than chemical synthesis but due to the rising cost of raw materials, fermentation process may be more economic.

28.2 MICROBIAL PRODUCTION OF VITAMIN B_{12}

Vit B_{12} is a vitamin that is synthesized in nature exclusively by microorganisms. The Vit B_{12} needs of animals are covered by food intake or by absorption of Vit B_{12} produced by intestinal microorganisms. Humans obtain Vit B_{12} only from food since the vitamin synthesized by microorganisms in the large intestinal tract cannot be assimilated. Activated sludge from sewage treatment contains 4-10 mg Vit B_{12} per kg but isolation from these sources is expensive. Vit B_{12} was first obtained commercially as a by-product of streptomycin fermentation with yield of 1 mg/liter of broth. As demand of Vit B_{12} increased, fermentation processes were developed with high-yielding strains. Commercial production is currently carried out entirely by fermentation. The current annual production (World) is over 15000 kg. Vitamin B_{12} production is based on media containing carbohydrate. Most Vit B_{12} fermentation processes use glucose as carbon source. Several producing strains are known, some of which are:

Microorganism	Production, mg/liter
Streptomyces olivaceus	3.3
Micromonospora sp	11.5
Propionibacterium freudenreichii	19.5
Propionibacterium shermanii	23.0
Pseudomonas denitrificans	60.0

Propionibacterium and *Pseudomonas* are the commercially used genera.

28.2.1 PROCESS BASED ON *PROPIONIBACTERIUM FREUDENREICHII*

Propionibacterium freudenreichii as well as other mutant strains are used in a two-stage process with added cobalt (10-100 mg/L). In the preliminary anaerobic phase (2-4 days), 5′ deoxyadenosyl cobinamide is mainly produced. In the 2nd aerobic phase (3-4

days), the biosynthesis of 5,6-dimethyl benzimidazole takes place so that 5′ deoxyadenosyl cobalamine (known as coenzyme B_{12}) can be produced.

As an alternative to this two-stage batch process, both stages can also be operated continuously in two tanks. During the recovery process, the cobalamins (which are almost completely bound to cell) are brought into solution by heat treatment (10-30 min at 80-120°C, pH~ 6.5-.5). They are then converted chemically into more stable cyanocobalamine. The raw product with about 80% purity is used as feed additive. Additional purification is done (95-98% purity) for medicinal use.

28.2.2 PROCESS BASED ON *PSEUDOMONAS DENITRIFICANS*

Pseudomonas denitrificans has been found to be the most productive species among Vit B_{12} producing microorganisms. In this one-stage process, the vitamin is produced during the entire fermentation. Cobalt and 5,6-dimethyl benzimidazole must be added as supplements. Sugar beet molasses is used as low cost carbon source, which also contains betaine (which is assumed to cause activation of biosynthesis or an increase in membrane permeability).

The media composition for different stages of production is given below. The production flow-diagram is given in Fig. 28.1.

Medium A	Amount, g/liter (unless specified)
Sugar beet molasses	60
Yeast extract	1
N-Z amine (enzymatic casein hydrolysate)	1
$(NH_4)_2HPO_4$	2
$MgSO_47H_2O$	1
$MnSO_4.H_2O$	0.2
$ZnSO_4.7H_2O$	0.02
$Na_2MoO_4.2H_2O$	0.005
Agar	25
Tap water	To make 1 liter
pH	7.4

Medium B

Same as Medium A but without agar

Medium C	Amount, g/liter (unless specified)
Sugar beet molasses	100
Yeast extract	2
$(NH_4)_2HPO_4$	5
$MgSO_4 7H_2O$	3
$MnSO_4.H_2O$	0.2
$Co(NO_3)_2.6H_2O$	0.188
5,6-dimethyl benzimidazole	0.025
$ZnSO_4.7H_2O$	0.02
$Na_2MoO_4.2H_2O$	0.005
Agar	25
Tap water	To make 1 liter
pH	7.4

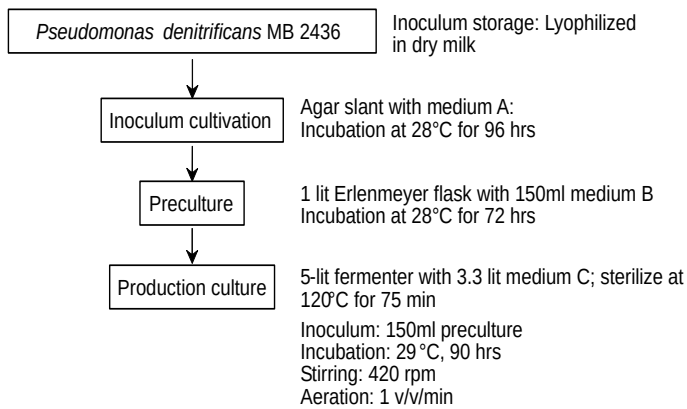


Fig. 28.1 Flow diagram of vitamin B_{12} production

28.3 MICROBIAL PRODUCTION OF β-CAROTENE

28.3.1 INTRODUCTION

Carotenoids are found in many animal and plant tissues but originate exclusively from plants or microbes. β-carotene (provitamin A) is converted into vitamin A in the intestinal mucous membrane and is stored in the liver as the palmitate ester. There is a good demand for β-carotene as provitamin A and as food coloring agent. Other carotenoids such as lycopene or xanthophylls do not have provitamin A activity but are used as food coloring agents. Carotenoids are synthesized by chemical means or by microorganisms but the fermentation process is not economical. Production processes for several carotenoids is given in Table 28.1.

Table 28.1 Production processes for several carotenoids

Carotenoid	*Organism*	*Medium*	*Time (days)*	*Yield (mg/liter)*
β-carotene	*Blakeslea trispora*	CSL, distiller's solubles	~ 8days	3000
Lycopene	*Streptomyces chrestomyceticus*	Starch, soymeal	6	500
Zeaxanthin	*Flavobacterium* sp	Glucose, CSL		335

28.3.2 PRODUCTION PROCESS USING *BLAKESLEA TRISPORA* STRAINS

The production flow diagram using *Blakeslea trispora* strains NRRL 2456(+) and NRRL 2457 (-) is given in Fig. 28.2 and the media compositions for the same are given in Table 28.2.

Isoniazid and kerosene are sterilized separately. After 48 hrs, 1 g/liter of β-ionone and 5 ml kerosene/liter are added. Glucose feeding (total addition of 42 g/liter) is done until the end of fermentation.

The observation that production occurs during the process of zygospore formation in this organisms has had an impact on process development. When cultures of both sexual forms (+) and (-) strains are mixed, a significant increase in carotene production in the (-) strain is achieved. The production is also increased by trisporic acid. Another activator of β-carotene synthesis is isoniazid, particularly in combination with β-ionone. Alone, β-ionone is toxic to the production organism, but in the presence of plant oils, it promotes carotene production. The addition of purified kerosene to the medium doubles the yield.

Table 28.2 Medium composition for β-carotene production

Medium A	*Amount, g/lit*	*Medium B*	*Amount, g/lit*
Cornsteep liquor	70	Distillers solubles	70
Corn starch	50	Corn starch	60
KH_2PO_4	0.5	Soybean meal	30
$MnSO_4.H_2O$	0.1	Cottonseed oil	30
Thiamin-HCl	0.01	Antioxidant	0.35
Tap water	To make 1 liter	$MnSO_4.H_2O$	0.2
		Thiamin-HCl	0.5
		Isoniazid	0.6
		Kerosene	20ml
		Tap water	To make 1 liter
		pH	6.3

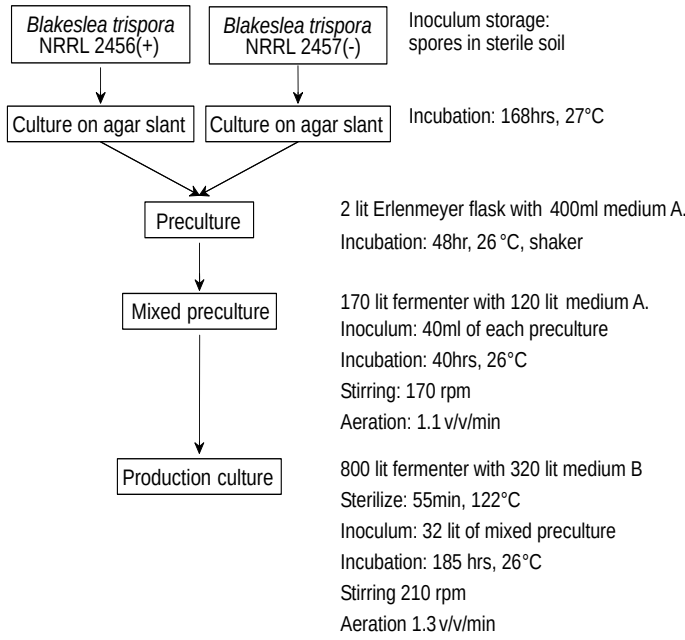


Fig. 28.2 Flow diagram of vitamin β-carotene production

Because of the low stability of β-carotene within the cells, the addition of an antioxidant is necessary during the fermentation process. The carotenoid-rich mycelium can be used directly as a feed additive. To obtain pure β-carotene, the mycelium is removed, dehydrated (with methanol), extracted with methylene chloride (75-92% yield) and the crude product is further purified.

CHAPTER 29

BIOFERTILIZERS

29.1 INTRODUCTION

The term *biofertilizer* denotes the 'nutrient inputs of biological origin for plant growth'. Here biological origin should be referred to as microbiological process synthesizing complex compounds and their further release into outer medium, to the close vicinity of plant roots which are again taken up by plants. Therefore, the appropriate term for biofertilizer should be 'microbial inoculant'. In the recent years, use of microbial inoculants as a source of biofertilizers has become a hope for most countries, as far as environmental- and economical viewpoints are concerned. Development and use of biofertilizers is mainly concerned with the exploitation of a group of *nitrogen fixing* organisms called *diazotrophs* for harvesting atmospheric nitrogen for plant crops.

Nitrogen compounds account for 40-50% of the dry matter of protoplasm of plant cells. Nitrogen is therefore required in large quantities by growing plants and is indeed the key to soil fertility. Plant crops obtain nitrogen from fertilizers and atmospheric nitrogen. Atmospheric nitrogen is in fact the cheapest and ubiquitous source of nitrogen for the plant kingdom.

The big reservoir of atmospheric nitrogen, however, is not directly available to the crop plants: plants simply cannot use the atmospheric dinitrogen (molecular nitrogen). An important intermediary involved here is the heterogeneous group of microorganisms collectively called *diazotrophs*. This group of organisms, limited in type, is able to change the dinitrogen into forms readily assimilable by crop plants, either by reduction to NH_3 or oxidation to NO_3^-. This microbial process of producing the inorganic forms of nitrogen from molecular nitrogen is known as *nitrogen fixation* or *diazotrophy*. Nitrogen fixation is of great economic importance in agriculture. The soil supports the plant growth indefinitely when it is replenished with nitrogen taken away (by crop plant year after year) and this task is carried out by diazotrophs.

The various microorganisms that have realized or potential applications as biofertilizer are:

1. Bacteria: *Rhizobium* sp, *Azospirillum*, *Azotobacter*
2. Fungi: *Mycorrhiza*
3. Blue-green algae (cyanobacteria): *Anabena*, *Nostoc*
4. Fern: *Azolla* (containing a symbiont *Anabena azollae*)

The diazotrophs exhibit two modes of nitrogen fixation, viz., (i) non-symbiotic and (ii) symbiotic. Those microorganisms that pass independent life and fix atmospheric

nitrogen are known as *free-living diazotrophs*, notable among which are species of *Azotobacter*, *Bacillus*, *Clostridium*, and *Anabena*. By analogy, those microorganisms which establish symbiotic relationships with plants for fixing nitrogen are called *symbiotic diazotrophs*. The plants and the symbiotic diazotroph exhibit mutualism whereby the plant exchanges carbohydrates (energy source) with the diazotroph for the nitrogen the latter fixes.

The modes of nitrogen fixation, however, are not confined to any particular group of microorganisms. In fact, the same microorganism may exhibit both the modes of diazotrophy. A remarkable characteristic that all diazotrophs share is the presence in them of an enzyme complex called nitrogenase which helps in the conversion of atmospheric nitrogen into ammonia. The overall reaction scheme is:

$$N_2 + 6e^- + 12\ ATP + H_2O \xrightarrow{\text{Nitrogenase complex}} 2NH_4^+ + 12\ ADP + 12\ P_i + 4\ H^+$$

At present, diazotrophs cultured in commercial scale for the biological nitrogen fixation are mostly based on rhizobial-, cyanobacterial-, and mycorrhizal cultures.

The symbiotic relationship between legumes and rhizobia is the most talked-about topic as regards symbiotic nitrogen fixation. The relation has been found to be extremely specific (commonly described by what is called *host specificity*). Stated differently, *Rhizobium* species or strains effective for one group of legume plants are less effective or ineffective for another group. Even within the species, certain strains are more effective than other with the given host plant. For the purpose of inoculation, and commercial preparation of the bacteria, legumes are therefore classified into seven major categories as given in Table 29.1.

Table 29.1 Species of *Rhizobium* and cross inoculation groups of hosts

Legume category/group	*Rhizobium* species
Soybeans	*Rhizobium japonicum*
Peas and vetch	*Rhizobium leguminosarum*
Beans	*Rhizobium phaseoli*
Lupines	*Rhizobium lupini*
Cowpeas	*Rhizobium* sp
Alfalfa	*Rhizobium meliloti*
Clover	*Rhizobium trifoli*

Before rhizobia can fix nitrogen, they must establish themselves in the cells of the root tissue of the host plant. Infection of the root hair system by rhizobia is closely associated with the formation of 'infection thread' that develops into certain root hairs. The bacteria invade the host plant cells via this infection thread, causing enlargement and an increased rate of cell division. This event leads to the formation of abnormal growth (nodule formation) in the root system. Within the nodules the bacteria convert free nitrogen to nitrates, which the host plant utilizes for its development. See Fig. 29.1 for an idea.

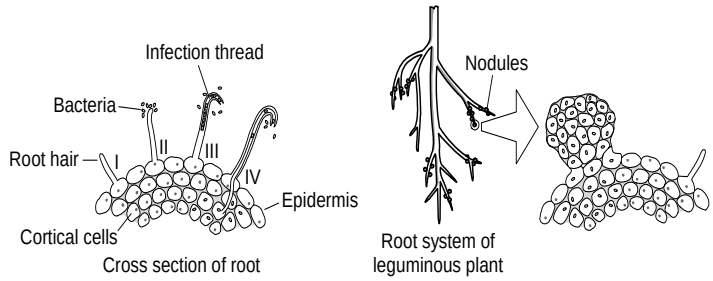


Fig 29.1 Different stages (I, II, II, and IV) of root nodule formation in legume plant

Once the inoculant having high nitrogen-fixing ability is introduced into the field, it promptly enters into ecological competition with indigenous strains already present in the soil. Sometimes, the introduced strain may not actually populate the roots but may simply be overwhelmed by the indigenous strains. This event renders the microbial inoculant ineffective.

29.2 PRODUCTION OF RHIZOBIUM CULTURE

Bacteria to be inoculated in soil as biofertilizer need to be multiplied on artificial media to harvest on a large scale so that they can be supplied to farmers.

Strains of *Rhizobium* are grown in Yeast Extract Mannitol (YEM) broth, the composition of which is: 1 g Yeast extract; 10 g Mannitol; 0.5 g K_2HPO_4; 0.2 g $MgSO_4.7H_2O$; 0.1 g NaCl; 1000 ml Distilled water. The pH is maintained at 6.5-7.0.

The principal steps for mass cultivation are:

1. Sterilize the growth medium and inoculate with broth of mother culture prepared in advance
2. Incubate for 3-4 days at 30-32°C
3. Test the culture for its purity and transfer to a large fermentor equipped with temperature control and aeration device. Allow aerobic fermentation for 4-9 days. There should be profuse growth of bacteria
4. Check the quality of the broth
5. Blend the broth with sterile carrier, e.g., peat, lignite, farmyard manure and charcoal powder. The carrier should contain (1-4) × 10^9 rhizobial cells/g
6. Pack the culture in polyethylene bags and store at 4°C or supply to the farmer

The increase in yield of legumes by using rhizobial culture ranges from 2.4% (*Vigna munga*) to 16.4% (arahar: *Cajanus cajan*).

29.2.1 APPLICATION OF RHIZOBIAL CULTURE AT THE FARM LEVEL

There are variations in the method of application of rhizobial culture at the farm level. One very successful method entails seed inoculation with aqueous suspension

of carrier culture during sowing (Fig. 29.2). The method of preparing seed inoculant is as follows:

1. Prepare 10% sugar or jaggery solution by boiling in water and then cool
2. Add Gum Arabic (10%) to help rhizobial cells stick to the seed
3. Add the carrier-based rhizobial culture to the solution and mix well. For one hectare, 400 g of charcoal-based culture would be sufficient
4. Add seeds in the slurry and again mix well. The number of rhizobial cells per seed should be between 10^5 and 10^6
5. Spread the seeds in shade for drying
6. Store the seeds at 4°C or use them in the farm

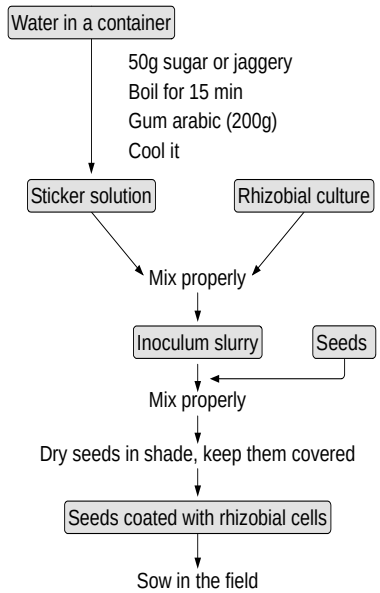


Fig 29.2 Procedure for seed inoculation with rhizobial culture

29.3 PRODUCTION OF BLUE-GREEN ALGAE

In water logging conditions, cyanobacteria multiply, fix atmospheric nitrogen, and release it into surroundings in the form of amino acids, proteins and other growth promoting substances. The process of application of blue-green algal culture in field as biofertilizer is also called *algalization*. Algalization has been reported to increase yield in paddy by around 1200 kg/ha.

The biofertilizer is beneficial for paddy. The mass cultivation of cyanobacterial fertilizers is done in various ways, viz.,

1. Cemented tank method
2. Shallow metal trough method
3. Polyethylene lined pit method
4. Field method

The polyethylene-lined pit method is most suitable for small and marginal farmers. In this method, small pits are prepared in the field and lined with thick polyethylene sheets. Mass cultivation of cyanobacteria is done by using any of the four methods under the following steps:

1. Prepare the cemented tanks, shallow trays of iron sheets, or polyethylene-lined pits in an open area. Width of tanks or pits should not be more than 1.5 m. This will facilitate the proper handling of culture
2. Transfer 2-3 kg soil (collected from open place for 1 m^2 area of tank) and add 100 g of super-phosphate. Water the pit to about 10 cm height. Mix lime to adjust to pH 7.0. Add 2 ml of insecticide, e.g., malathion to protect the culture from mosquitoes. Mix well and allow soil particles to settle down
3. When the water becomes clear, sprinkle 100 g of starter inoculum on the surface of water
4. When temperature remains between 35 and 40°C during the summer, optimum growth of cyanobacteria is achieved. Always maintain the water level to about 10 cm during this period
5. After drying, the algal mat will get separated from the soil and form flakes. During summer, about 1 kg pure algal mat/m^2 area is produced. These are collected, powdered, kept in sealed polyethylene bags, and supplied to the farmers

The algal flakes can be used as starter inoculum if the same process is repeated.

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Essentials of Industrial Microbiology is basically a compilation of a series of lectures on INDUSTRIAL MICROBIOLOGY and MICROBIAL BIOCHEMISTRY I have delivered over the years to B. Tech (Food Technology) and B. Sc. (Microbiology) respectively at Central Campus of Technology, Dharan, Nepal.

The chapters included herein more than cover the current syllabus of Industrial Microbiology for B. Tech (III year). Within the scope and limitation of the syllabus, I have tried to put together information as meticulously as possible. Some descriptions have become outdated, genetic engineering in particular. However, the basic concept is still useful. The book contains a large number of cross-referenced diagrams, tables and index to assist the students/readers.

Thanks are due to those authors whose books I have freely consulted. As an acknowledgement, I have appended a short bibliography, which I hope will be helpful to the students in finding out additional reading materials.

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