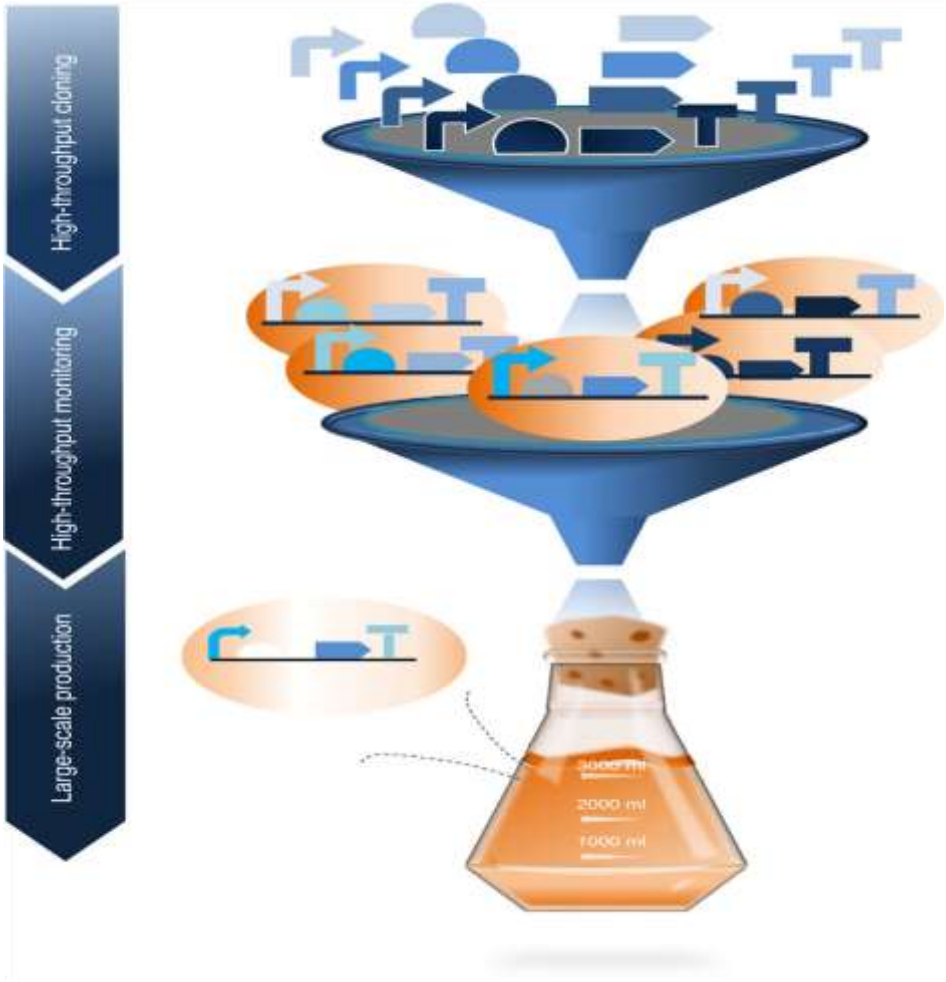

# INDUSTRIAL MICROBIOLOGY

***For***

**Applied Microbiology Diploma**




**Associated Prof./ Dr. Eman Abdel-Aty Alwaleed**

**2024.**

- **Vision and Mission of the faculty**

# Vision

The faculty of science seeks to achieve academic community and student dominated by science, realization, culture and challenge, where all aspects are in continuing dialogue, graduating alumni equipped with information that qualifies them to be productive and creative.

# Mission

The faculty aims at distinction on the local and regional level through:

- Cooperating with international and regional universities and institutions
- Doing targeted researches
- Providing distinct educational services to provide labor market with qualified alumni
- Providing community services and scientific consultations
- Ongoing training in education to meet the scientific progress

# SUBJECT INDEX

# 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 B2 ) production 3 scar bud

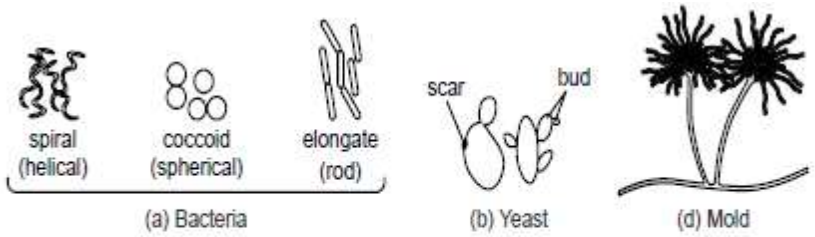



Fig. 1.1 Typical morphology of bacteria, yeast, and mold

## 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**

**Algae Biomass: Characteristics and Applications, Developments in Economic Aspects of Algae Biomass**

# Introduction

Biomass derived from algae is a valuable raw product for agriculture and the chemical industry. The chemical composition of the algae biomass obtained from the natural environment, culture under natural conditions, and culture in bioreactors determines its application to the energy، fuel, and cosmetic industries. The use of biomass, as well as the extracts derived from biomass, is discussed in regard to the economic aspect and life-cycle assessment. The economic aspects of obtaining biomass algae in the product life cycle are discussed for the bio-products industry.

- **Algae Biomass as Raw Product for Industry**

Because of the difficult environmental conditions of living، including periodic deficiencies in nutrients, the lack of access to light, space, limitations or temporal salinity, the organisms that live in water in order to survive have developed the ability to produce different chemical compounds through metabolic transformations. Among the water organisms of particular interest are algae. They are thalloid plants that show the ability to photosynthesize (Craigie et al. 2008؛ Craigie 2011). They are divided into two groups, depending on their biological structure: macroalgae and microalgae.

The biomass of these organisms has been used in many countries, those in Asia in particular, as a source of chemical compounds and nutrition for humans and animals, as well as a natural fertilizer in agriculture. These organisms are an excellent source of proteins, vitamin E, and beta-

carotene. The ones most often used are the green algae, or chlorophytes, whose thallus may contain chlorophyll (green pigment), xanthophyll (yellow), and carotene (orange pigment؛(the red algae (rhodophytes), which contain red phycoerythrin, blue-green phycocyanin, or green chlorophyll; and the brown algae (phaeophytes), whose thalli can be green, yellow, or brown (xanthophyll, fucoxanthin)

In the natural environment, algae are continuously exposed to stress related to access to light, temperature fluctuations, osmotic stress, and lack of water or water salinity.

In response, the plants synthesize a number of organic compounds whose task is to counteract the stress. The biologically active compounds synthesized in the algal cells in order to protect the plants against adverse conditions can be isolated from the biomass and be used as valuable raw products for industry. The organic compounds obtained in this way are both “bio” and “natural”.

Biomass has been used for many years as a food component for humans and animals, as well as a raw product for industry. Presently, about 15 million Mg products are obtained from algae annually. A small fraction of these products is used for obtaining extracts, the annual production of extracts is 25,000 Mg, and the production shows a tendency toward dynamic growth. In the understanding of pharmaceutical law (Pharmaceutical Law 2001), algae are classified as medical plants. Similarly, the Fodder Law (Minister of Agriculture 2005) defines fresh and processed algae as fodder substances of plant origin. The extracts from algae are classified differently. According to the EU laws (Community Register 2003), algae extracts are animal feed additives.

Consumers from Europe, in particular, those from Central and Eastern Europe, treat algae as a food component with reserve. In its unprocessed form, algae biomass is marketed as a diet supplement and is mainly made

up of microalgae– Spirulina. Algae extracts are easier to accept for consumers، so methods of algae processing are always eagerly sought.

The use of algae extracts is more easily approved, not only because of the psychological barrier but also because of the known composition and longer stability of the products، especially the certified ones, and the possibility of adding extracts – rich in valuable bioactive compounds – to different formulations. The algae extracts in which it was possible to isolate metabolites and secondary metabolites were found to contain antioxidants, bactericides, vitamins, mineral components, and polysaccharides (Alassali et al. 2016; Bedoux and Bourgougnon 2015). In the cosmetic industry, algae extracts are used as ingredients in creams, tonics, and shampoos، while dried and powdered algae are used for face masks and slimming baths.

**Algae biomass can be obtained for industry from:**

1- Natural water bodies (seas, lakes, rivers)
2- Algae cultivation under natural conditions in selected water reservoirs (ponds, algae farms)
3- Algae cultivation for industrial purposes under the controlled access of light, nutrients (N, P), and pH of the medium

Each of these methods provides raw products of different components, has a different impact on the natural environment, requires different costs, and shows different production capacities and different seasonal characters for the obtainment of biomass.

The composition of biomass strongly depends on the species of alga, which determines a given species' use.

According to Jung et al. (2013), global production of macroalgae has shown that macroalgae can be mass cultivated with currently available farming technology. Their various carbohydrate compositions imply that new microorganisms are needed to effectively saccharify macroalgae biomass. Up-to-date macroalgae conversion technologies for biochemicals and biofuels show that molecular bioengineering would contribute to the success of macroalgae-based biorefinery.

The industrial use of algae is the decisive factor in the rate of mass gain over time. Due to the high photosynthetic ability of macroalgae, they have the potential to generate and store sufficient carbon resources as needed for biorefinery.

- **Biomass Algae as a Raw Product for the Production of Biofuels**

All countries must take some precautions against the depletion of fossil fuels. The need to provide energy security, maintain economic growth, and restrict the effects of climate change has triggered an interest in biofuels. Production of sustainable bioenergy is of global interest. Algae offer great potential as a sustainable raw product. Much attention has been paid to the production of bioenergy from the algae biomass, taking into account the potential benefit for the natural environment when compared with the effects of conventional cultivations of bioenergy production and conventional fossil fuels. Algae are very promising, as they absorb considerable amounts of carbon from the atmosphere, as well as industrial gases, and can effectively use the nutrients from postindustrial processing waste andmunicipal waste. After the development of economically accept-able and environmentally justified technologies, algae cultivation could provide large amounts of biofuel

with a minimal effect on the environment (Singh et al. 2012; Ruiz et al. 2016).

In order to economically use algae biomass for biofuel production, first of all, a continuous supply of large amounts of dry biomass is needed. In the temperate climatic zone, the harvest of biomass from natural water bodies shows a seasonal character, with variations in algae species composition and different yields of the crop depending on the weather‹ species composition and diversity, and the considerable amount of pollution with toxic metals, pesticides, and toxins absorbed from the environment. Various species of filamentous alga prefer different conditions for a temporary increase in their vegetation season. Growth patterns for common filamentous algae indicate the occurrence of both an early spring species and, typically, a summer species in freshwater eco-systems in Poland (Pikosz et al. 2017). Thus, the harvesting of algae biomass for industrial purposes will be characterized by seasonality. Among the filamentous algae, which commonly occur in water ecosystems, Cladophora rivularis Hoek and C. fracta show higher aqueous water temperatures than C. glomerata (L.) Kütz‹. Tribonema vulgare Pascher, and Ulothrix variabilis Kütz‹ with an optimum of occurrence in water below 18 °C. Taking into account all macroalgae that form the densest patches, C. glomerata (L.) Kütz. occupies a distinct niche in Polish water ecosystems (Table 12.3). Moreover, C. glomerata L. Kütz., as the only species of homogenous macroalgae mass development, is able to achieve high biomass in freshwaters in a short time. This species forms its highest biomass concentration in two periods: spring (April–June) and autumn ( September–October), which confirms its strong response to colder water and also the possibility of harvesting biomass for several months each year. For other freshwater species of macroalga, optimum development is

limited to only a few weeks, making it difficult to accurately plan the collection of biomass. It seems that the impact of environmental factors on the size of freshwater macroalgae biomass is higher than that of the marine ecosystem, which adds an additional factor of high variability and seasonality of harvest. A lack of repeatability in the development of the same amount of bio- mass in natural aquatic ecosystems can negatively affect sub-sequent economic aspects. Table 12.3 Presence (light gray color) and growth intensity of macroalgae species determined by their ecological optimum (dark gray color) in freshwater ecosystems in Poland.

An important economic argument for development of algae biomass use is related to the necessity of improving recreational conditions of water reservoirs. Collection of algae biomass from beaches attractive to tourists has become a necessity, irrespective of the cost and manner of biomass utility, as it is vital for maintenance of the recreational value of the relevant regions. This way of collecting biomass seems to be the cheapest way to utilize waste that can become a raw product. The amount of biomass in water bodies and along their banks depends on the process of accumulation of organic matter at a rate higher than that of its assimilation; the organic matter can be provided in municipal and industrial wastewaters. Because of the weather conditions and character of the environment, it is difficult to predict the blooming and thus plan harvesting, transportation, and fast utilization. In addition, the algae collected from beaches are polluted with sand and litter and undergo fast decomposition. Presently, if algae biomass is collected, it is usually damp.

Another method for providing biomass supply for industry is the cultivation of algae in selected water bodies. Algae are characterized by a very fast pace of growth, and their cultivation permits very effective use

of space. From a unit area covered with algae, it is possible to get about 30 times more energy than from a unit of the same size in which bio-fuels of the first or second generation are being grown.

Biofuels of the first generation are obtained from corn, sugarcane, rapeseed, sugar beet, and others. Production of biofuels of the first generation is in competition with food production and is treated as a possible limitation for food production. Biofuels of the second generation are obtained from materials that are not used for food production, such as wood, straw, and other wastes from agricultural production (Carriquiry et al. 2011; Brennan and Owende 2010).

In 2013, biofuel obtained from algae was offered on the market for the first time. The Propel network of gas stations in San Francisco (USA) offered biodiesel with a 20% admixture of the component obtained from algae (Cardwell 2013).

Production of algae biodiesel on a mass scale must be evaluated, taking into account the size of resources, their avail- ability and stability of supply, the effect on the environment and economy, the yield of technology applied, and the life cycle of the product (Quinn and Davis 2015).

Microalgae are considered to be one of the most feasible options with the potential to serve as a major feedstock for biofuels and bioproduct production. However, the economic viability of commercial-scale production continues to be called into question by many researchers and investors.

Biomass from micro- and macroalgae contains significant amounts of water. Microalgae generally contain only  dry matter ( Chen et al. 2015). Therefore, there is a need to carry out parametric analyses so as to identify the influence of system configuration and process on their eco-

nomic viability. The results show that the most important cost-driving parameters are the pond, the harvesting, and the biomass drying process (Madugu and Collu 2016).

The biggest challenge for any biofuel technology is the drying out of microalgae. This process is the most energy consumptive (Bennion et al. 2015). Research on the economics of using algae for biofuel production is primarily based on experiences and calculations from other climates and adaptation to European conditions. Malic et al. have established that, for the climatic zone of Australia, bio-crude production is environmentally, economically, and socially sustainable.

To this end, an economic multi-region input-output model of Australia was completed through processing of the engineering data on algal bio-crude production. This model was used to undertake hybrid life-cycle assessment for direct measurement, as well as measuring the indirect impacts of producing bio-crude. Overall, the supply chain of bio-crude is more sustainable than that of conventional crude oil. The results indicate that producing 1 million Mg of bio-crude will generate almost 13,000 new jobs and 4 billion dollars' worth of economic stimulus. Furthermore, bio-crude production will offer carbon. Sequestration opportunities of the production process are net carbon-negative (Malic et al. 2015).

Slade and coworkers have shown three aspects of micro-algae production that will ultimately determine its future economic viability and environmental sustainability: the energy and carbon balance, environmental impacts, and production cost (Slade and Bauen 2013). We find that achieving a positive energy balance in the temperate climate of Europe will require technological advances and highly optimized production systems (Ghadiryanfar et al. 2016).

Biomass of microalgae is also considered as an alternative source of bioenergy. The techno-economic characteristics of macroalgae utilization

in European temperate zones was evaluated by Dave et al. (2013) in a selected anaerobic digester using the chemical process modeling software ECLIPSE. The assessment covered the mass and energy balance of the entire process, followed by an economic feasibility study, which included the total cost estimation, net present value calculation, and sensitivity analysis. The selected plant size corresponded to a community-based AD of 1.6 MW with a macroalgae feed rate of 8.64 Mg per day (dry basis).

The produced biogas was utilized in a combined heat and power plant generating 237 kW of electricity and 367 kW of heat. The break-even electricity-selling price in this study was estimated at around €120/MWh. On the grounds of different national and regional policies, this study did not account for any government incentives (Dave et al. 2013.(

Aitken presented a life-cycle assessment (LCA) that considered the energy return and environmental impacts of the cultivation and processing of macroalgae (seaweed) into bio- ethanol and biogas with a particular focus on specific species Gracilaria chilensis Bird, McLachlan and Oliveira and Macrocystis pyrifera (L.) Agardh) and cultivation methods bottom planting and long-line cultivation). The study was based mainly upon data obtained from a research conducted in Chile, but the results can be applied to other locations where similar cultivation is feasible (Aitken et al. 2014).

The cost-effectiveness of production of energy from macroalgae depends on the yield of macroalgae production and the cost of raw product acquisition and processing. The yield of macroalgae production varies from 150 to 600 Mg of fresh biomass per hectare per year and is much higher than the typical value for sugarcane, which varies from 70 to 170 Mg fresh biomass per hectare per year. This means that under the optimum conditions of cultivation, macroalgae could be a more effective source of energy than biomass from land plants. The most promising

species for biofuel production are the algae containing large amounts of oil.

From the point of view of societal development, the production of biofuels from algae is attractive, taking into account the following facts:

–Harvesting of algae biomass from water reservoirs does not compete with the production of farmland crops, as it does not affect agricultural land.

–Production of biomass from algae does not interfere with food production.

–Growing algae absorb large amounts of carbon dioxide from the atmosphere.

## 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 Boil for 15 min

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 polyethylenelined 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 m2 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/m2 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.

- **Algae Biomass as a Raw Product for the Cosmetic Industry**

Biomass of marine and freshwater algae is a good raw product for extraction of the bioorganic compounds used in the cosmetic industry (Pereira and Meireles 2010; Li et al. 2014؛Michalak and Chojnacka 2014, 2015; Goto et al. 2015؛Michalak and Chojnacka 2015; Messyasz et al. 2015a, b.(Green extraction technologies for high-value metabolites from algae .The current list of algae products is extensive.

Therapeutic supplements from microalgae comprise an important market involving compounds such as β-carotene and astaxanthin,polyunsaturated fatty acids (PUFA) such as DHA and EPA, and polysaccharides such as β-glucan. The dominant species of microalga used in commercial produc-

tion include Isochrysis, Chaetoceros, Chlorella, Arthrospira و Spirulina), and Dunaliella. The production of unsaturated algae DHA from bio- mass algae is an innovative approach to this problem. The optimum algae species and technology have been selected to ensure the production of unsaturated acid in amounts profit-able for the producers. DHA algal oil is produced via an algal fermentation process using Schizochytrium. Up to 50% of this species' dry cell weight can be made up of the fatty acids; approximately 30% of the total fatty acid content is DHA. It has been shown that S. limacinum can provide approximately 4 g of DHA for every liter of the medium، which is greater than the amounts obtained from the other species studied. Production of DHA oil from Schizochytrium is realized through the four-step technology (Oilgae 2016:(

–Fermentation (fermentation carried out under controlled conditions using carbon and nitrogen bulk nutrients, vita-mins, and trace mineral sources

–Intermediate product (algae cell concentrate and dried algae transferred to oil extraction process

–Oil extract (oil extract from dried algae by solvent (hexane) extraction. De-oiled biomass is separated by centrifugation or filtration. Solvent phase is crystallized and oil is extracted.

–Oil purification (Winterized oil is heated and pretreated with acid, neutralized, and centrifuged to get refined oil.

The search for biostimulants of plant growth (by extraction for ecological agriculture is another rapidly developing field.

The compounds obtained this way are expected to replace synthetic biostimulants and become natural products for agriculture consistent with the idea of "plant for plants".

Algae biomass contains small amounts of plant growth stimulants

The extraction of bioactive compounds from algae biomass requires the use of different extraction techniques.

A cost-effective analysis of these processes

# Chapter -3-

## 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<br>*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.

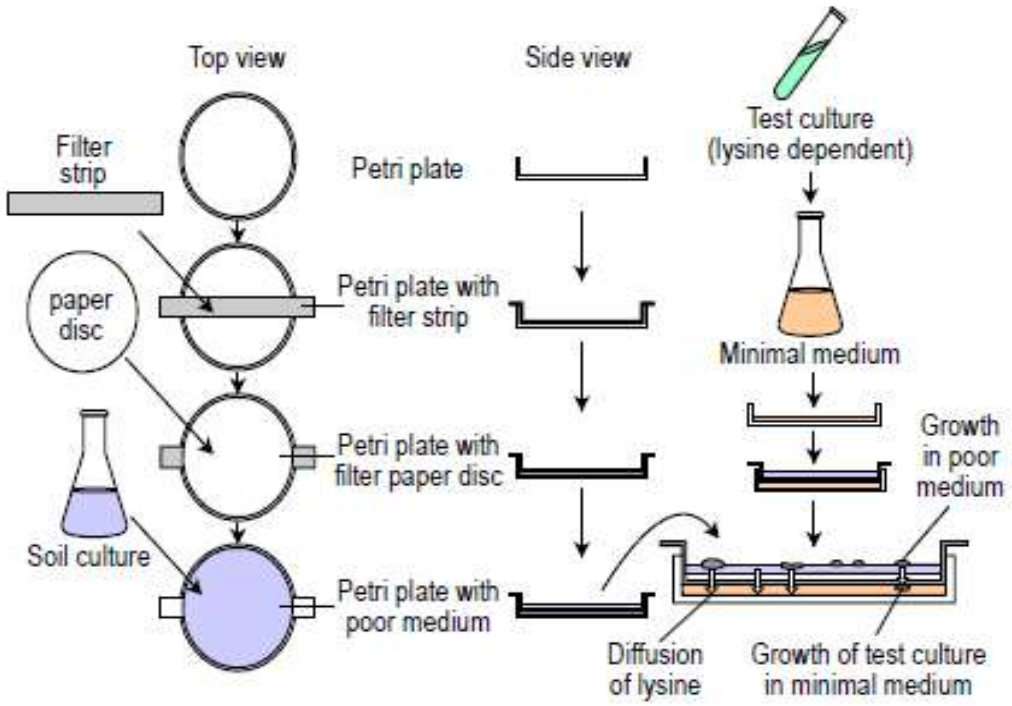



Fig. 2.1 Auxanographic screening of lysine producers

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.

7 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 sub-cultured 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

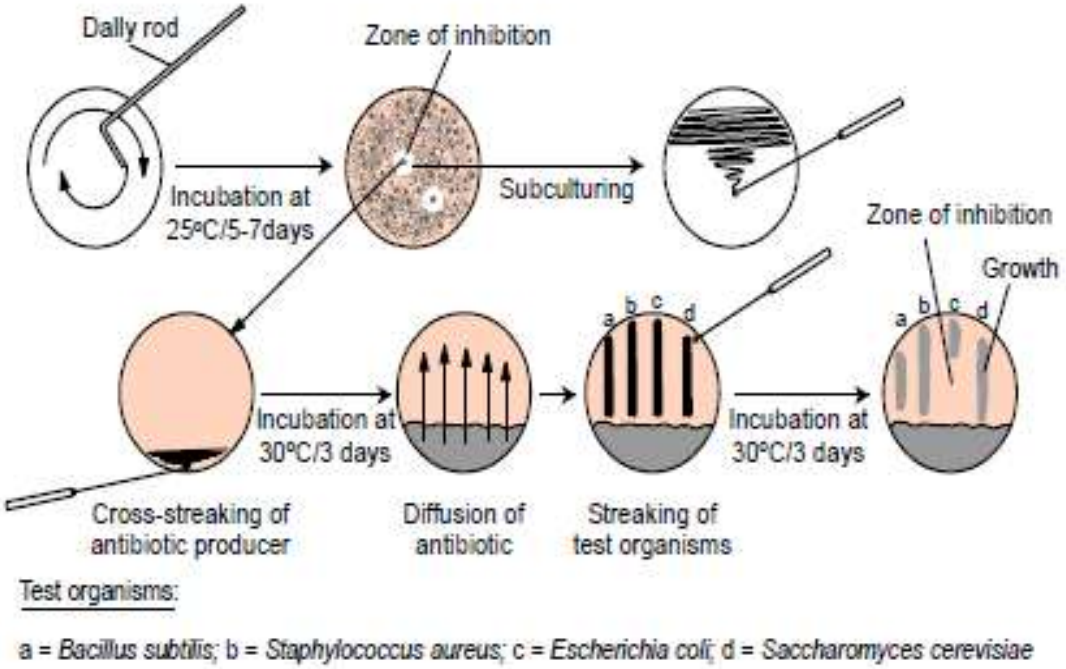



Fig. 2.2 Simplified protocol for the isolation of antibiotic producers from soil

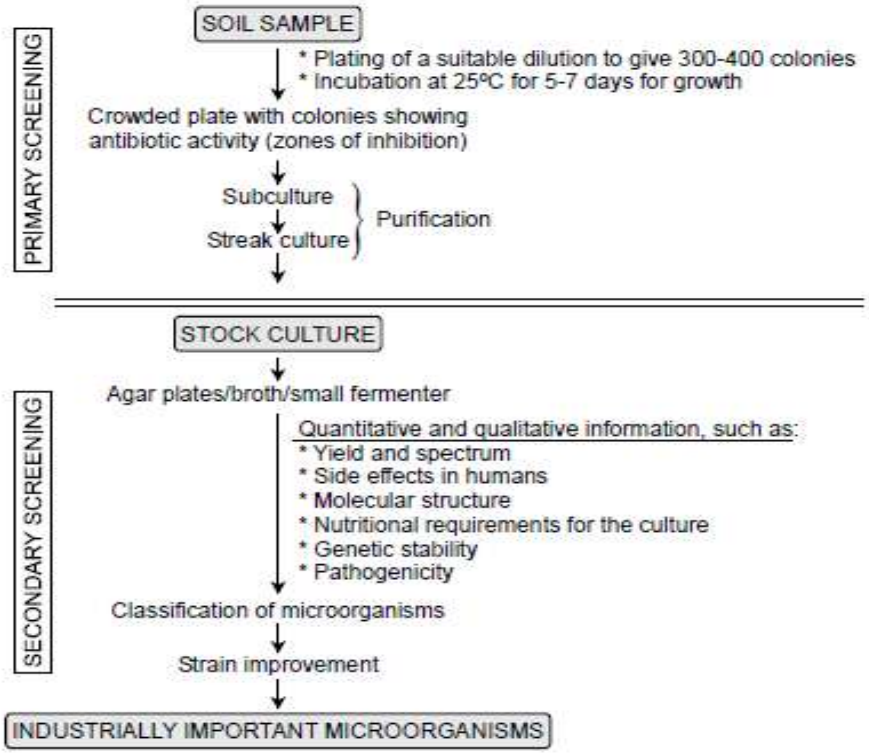



Fig. 2.3 Screening of antibiotic producer from soil

# CHAPTER 4

# GENERAL TECHNIQUES OF SELECTION OF MICROORGANISMS

## 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.

## 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.

## 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!

## 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

*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.

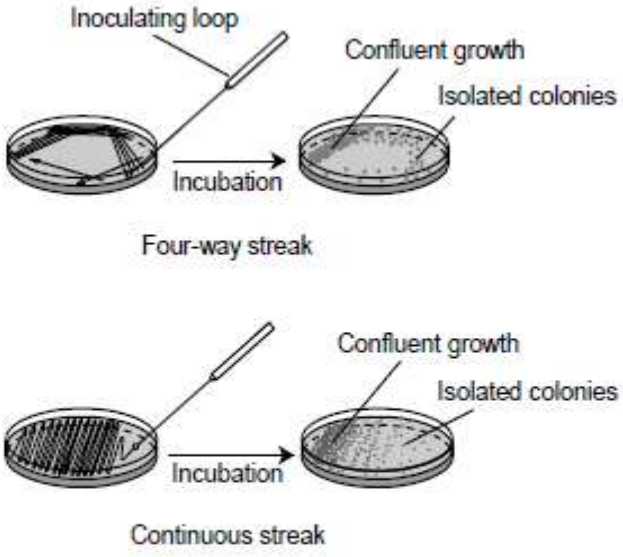



Fig. 3.1 Streak-plate isolation

### *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 sub-culturing 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.

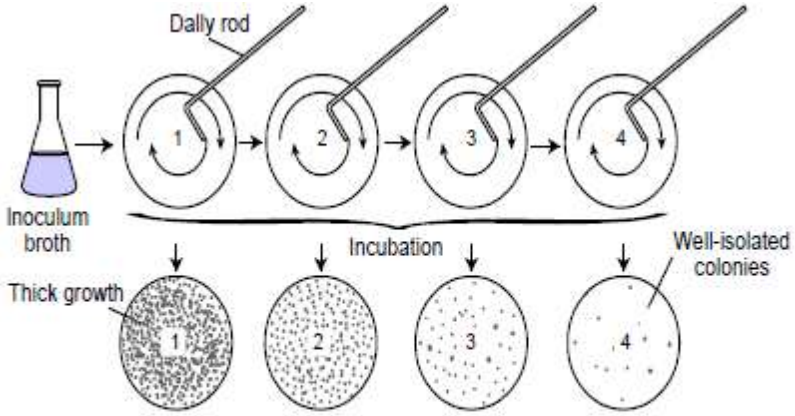



Fig. 3.2 Spread-plate isolation

### *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).

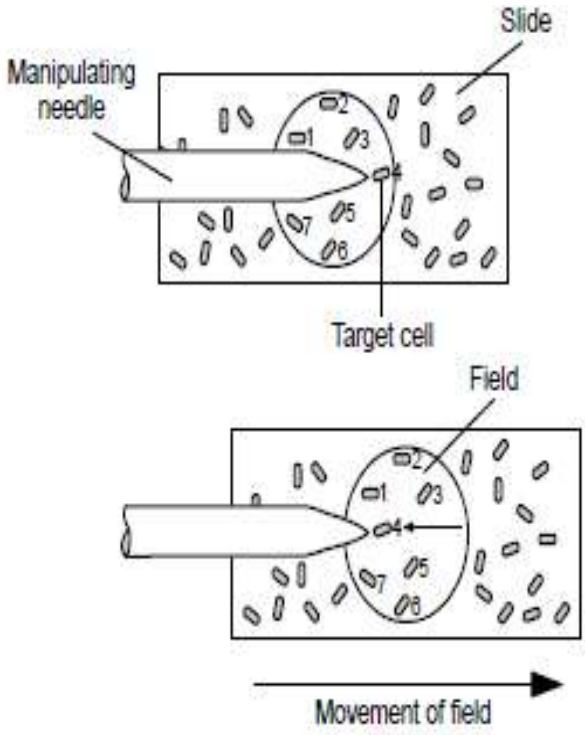



Fig. 3.3 Isolation by micromanipulator

## CHAPTER 5

## 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 off springs. 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 (HNO2)*

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.

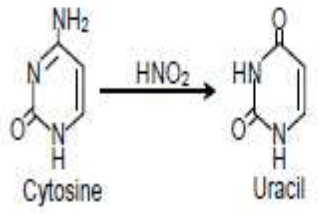



Fig. 4.1 Mutation by nitrous acid

 *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.

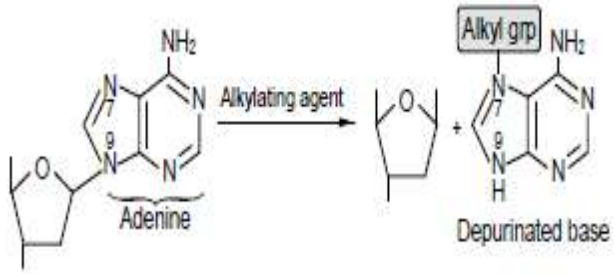



Fig. 4.2 Action of alkylating agent

 *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.

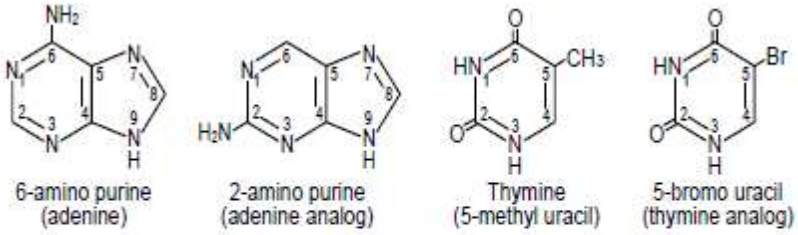
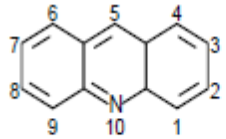



Fig. 4.3 Examples of base analogs

 *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(CH2 CH2Cl)2, where R is an alkyl, alkylamine, or alkyl chloride group.



Acridine orange

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* (nonionizing).

 ***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).

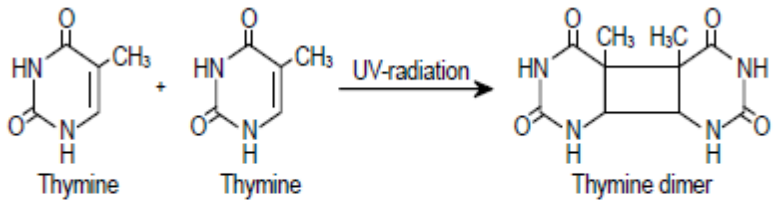



Fig. 4.5 Formation of thymine dimers

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).

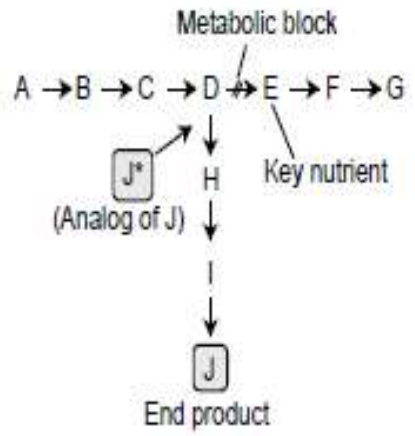



Fig. 4.6 Mechanism of auxotrophy

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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.

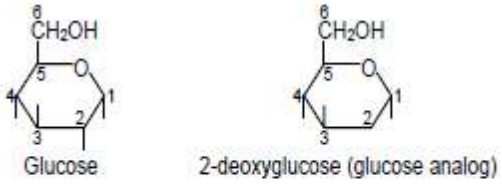



Fig. 4.7 Glucose and glucose analog

## 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(CH2CH2O)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 Ca2+ concentration (50 mMol/L); this approach is a combination of PEG and high pH with Ca2+ treatment, and is usually more effective than either treatment alone. PEG is negatively charged and binds to cations (e.g., Ca2+) 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).

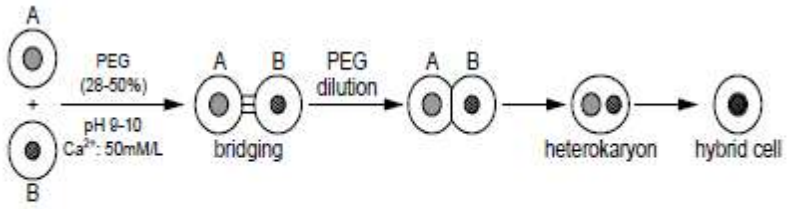



Fig. 4.8 Outline of PEG-induced protoplast fusion

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 6

# 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 Electronics 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, proteinenhanced 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 7

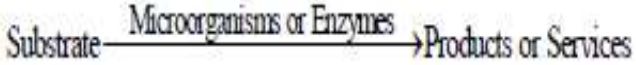
# CONCEPT OF FERMENTATION TECHNOLOGY

## 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:



## 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 ・m 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 sub-culturing, inoculum build up, fermentation, and sampling. A few examples of aseptic techniques are described in the following paragraphs.

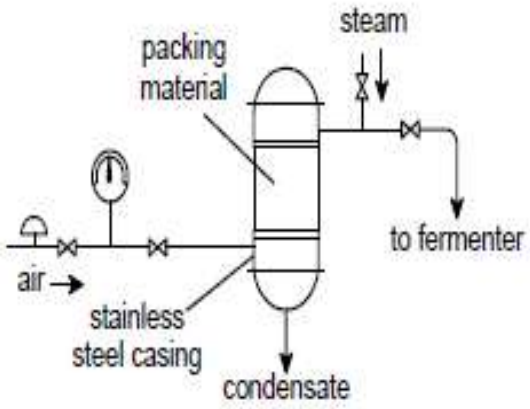



Fig. 9.1 Sterilization of air

*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.

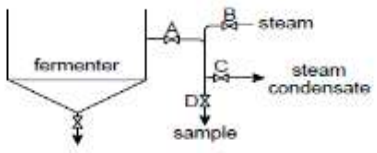



Fig. 9.2 Protocol for aseptic sampling

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.

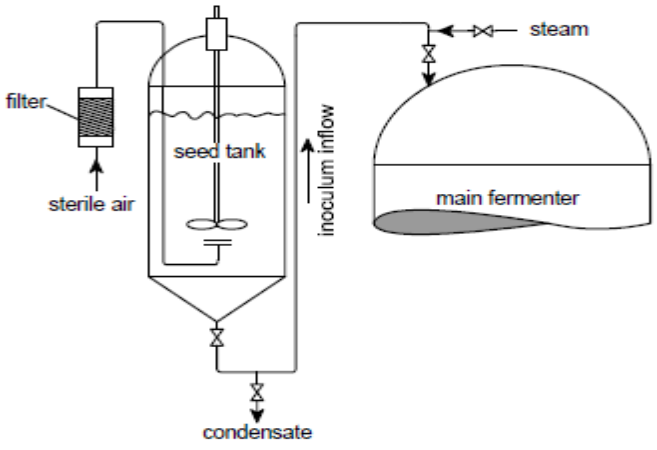



Fig. 9.3 Protocol for aseptic inoculation

### *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.)

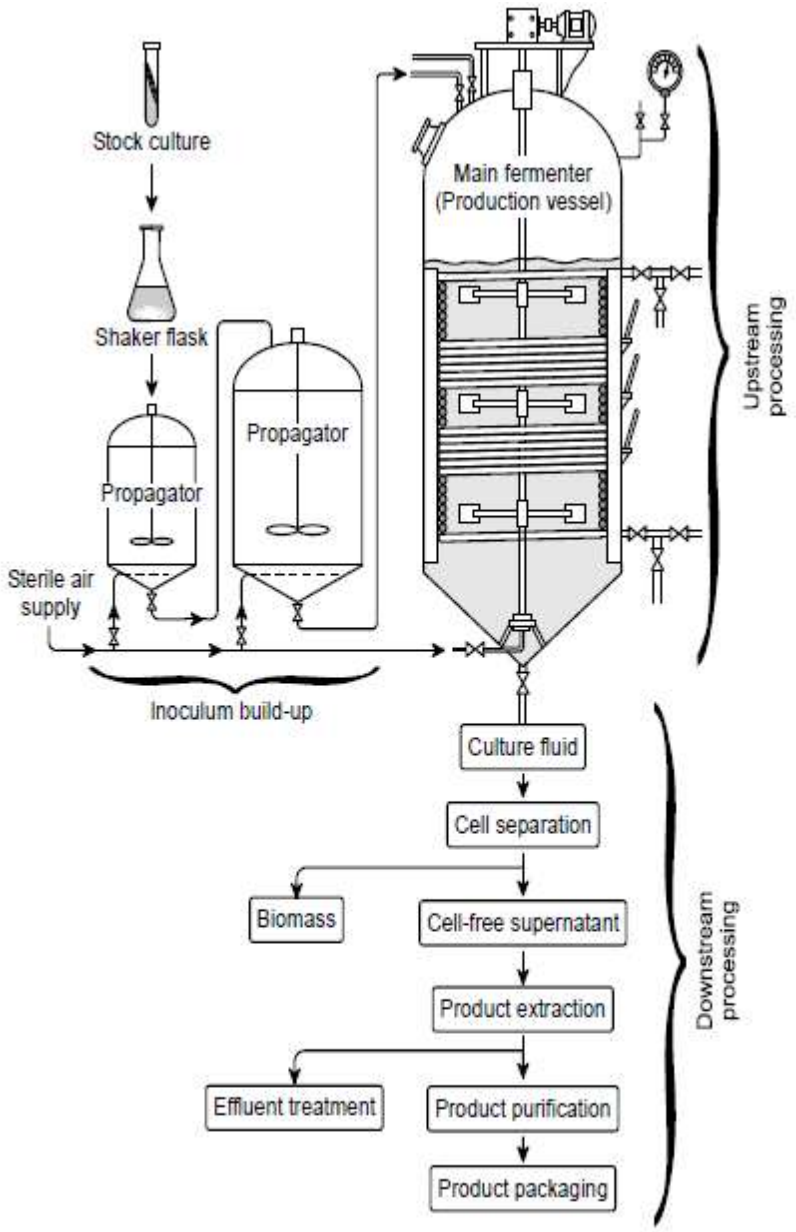



Fig. 9.4 The component parts of a fermentation process

## 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 (O2 supply and CO2 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 CO2 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.

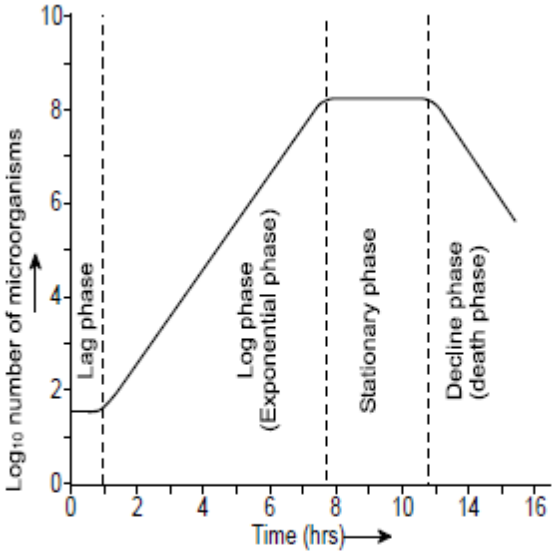



Fig. 9.5 The bacterial growth curve

### 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.

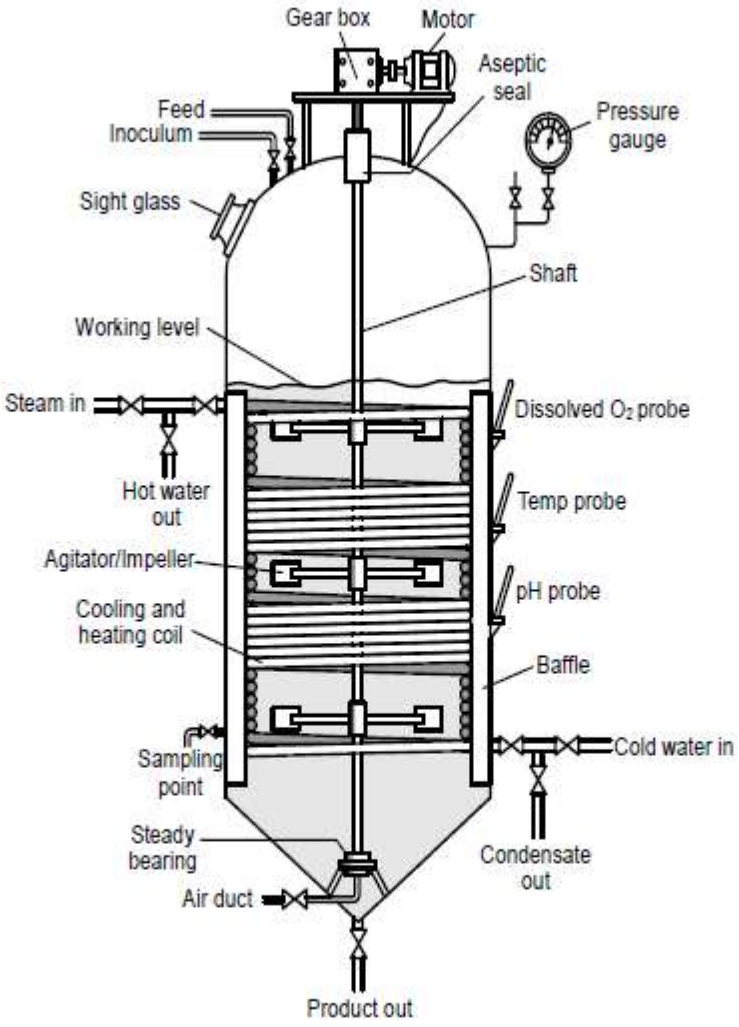



Fig. 9.6 A typical batch, stirred tank fermenter

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 dm3 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

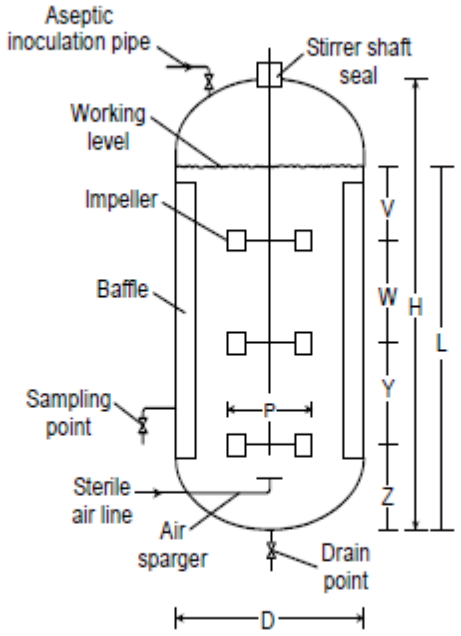



Fig. 9.7 Typical dimension for a stirred tank fermenter

## 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 Dt (tank diameter) above the base.

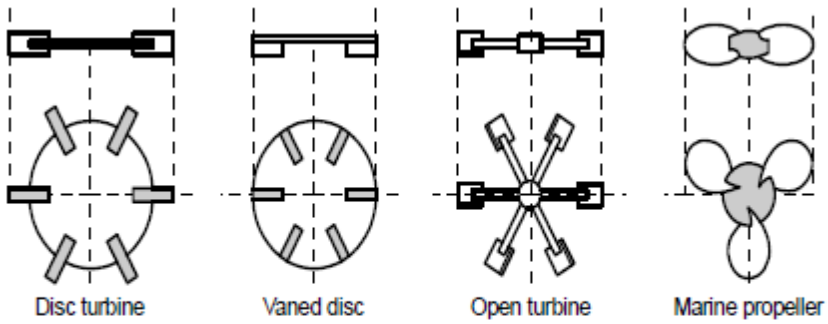



Fig. 9.8 Different types of impellers

### 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.

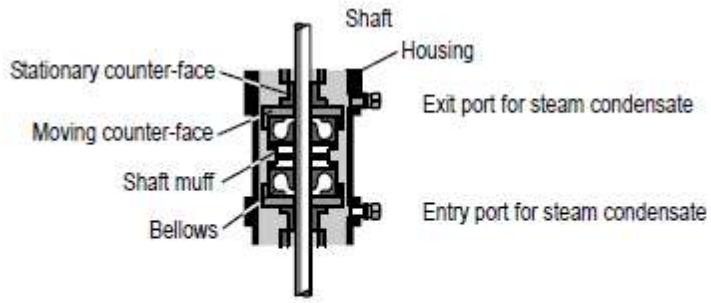



Fig. 9.9 Schematic drawing of mechanical seal

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 m3 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).

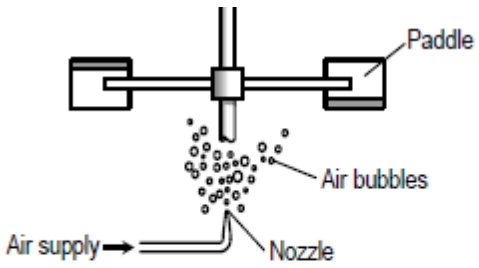



Fig. 9.10 Schematic drawing of nozzle

### 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 m2 may be taken as an average contact area for a 55000 dm3 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 O2 activity

5. pH

6. O2 and CO2 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.

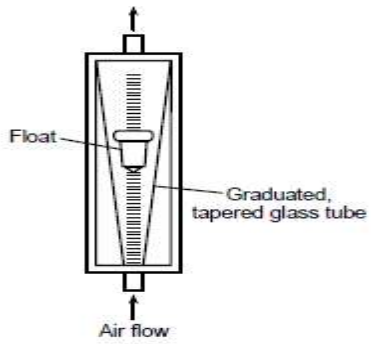



Fig. 9.11 Working principle of rotameter

**Agitation rate**

The rate of rotation is sensed by tachometers

**Dissolved O2**

Electrodes are used for the continuous measure of dissolved O2. Oxygen selectively diffuses through the membrane and produces signal. The current is directly proportional to the activity of O2 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 8

# 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 erequiring enzyme is given in Fig. 10.1.

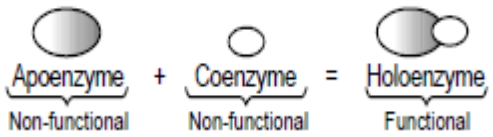



Fig. 10.1 Combination of apoenzyme and coenzyme to make holoenzyme

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 C6 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 1-104/s. The turnover number of 6・105 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.

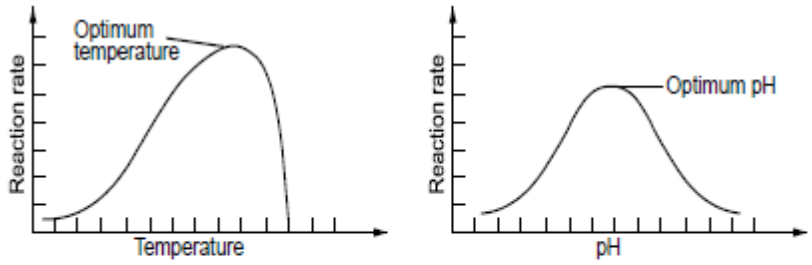



Fig. 10.2 The effects of temperature and pH on enzyme activity

### 10.4.2 EFFECT OF pH

The pH changes profoundly affect the ionic character of the amino- and carboxylicgroups 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.

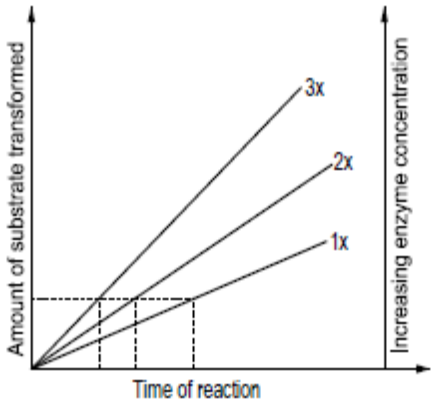



Fig. 10.3 Effect of enzyme concentration on reaction rate

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), $Km$ = Michaelis-Menten constant (expressed in moles/L), and $Vmax$ = 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.

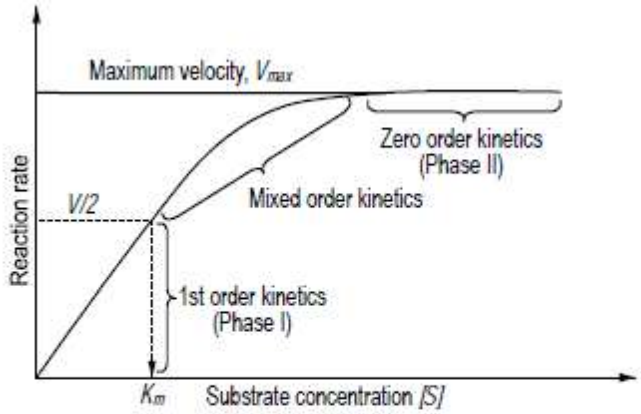
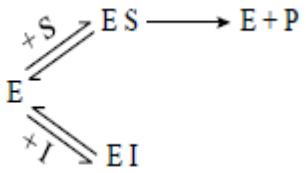



Fig. 10.4 Effect of substrate concentration on reaction rate

### *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, *Km*. Consider a typical enzyme-catalyzed reaction:

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

We can thus see from the initial equilibrium that *Km* 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).

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 *Vmax* and can even become zero on total inhibition. The *Km* value, however, is unaltered.

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

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

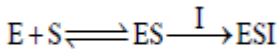
A highly dangerous nerve poison *diisopropyl fluorophosphates* works by inhibition of *acetyl cholinesterase*, the enzyme immediately associated with nerve function.

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.

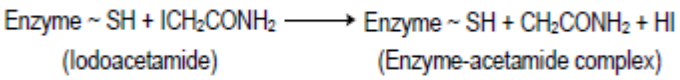
*10.4.5.3 Uncompetitive inhibition*

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 N reaction can be shown by:



Where, the notations carry usual meanings.




This type of inhibition is found in *multi substrate* reactions. Both *Km* and *Vmax* are

altered but the slope is the same as that of an enzymatic reaction free of inhibitors.

Experimentally, the value of *Km* 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.

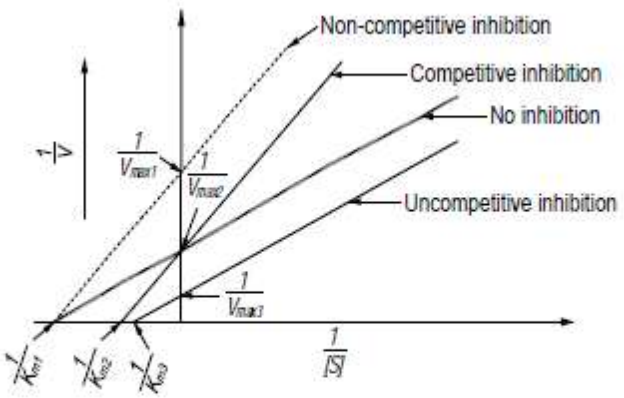



Fig. 10.5 Reciprocal plots of $v$ and [$S$] in the presence of different types of inhibitors

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 *Vmax*. Only the *Km* 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 (*Km'' < Km'*). 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*.

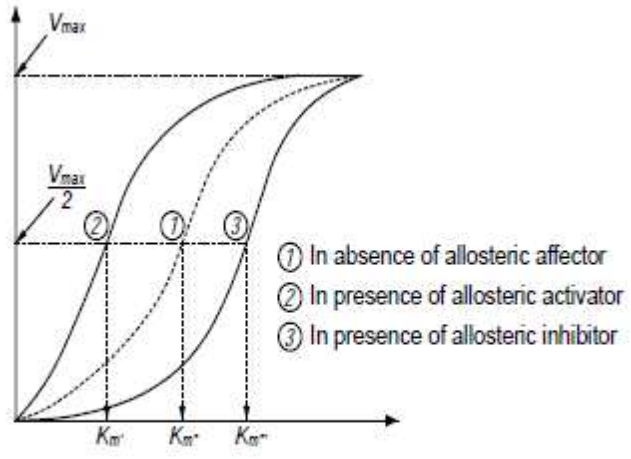



Fig. 10.6 Kinetics of reaction catalyzed by an allosteric enzyme

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.

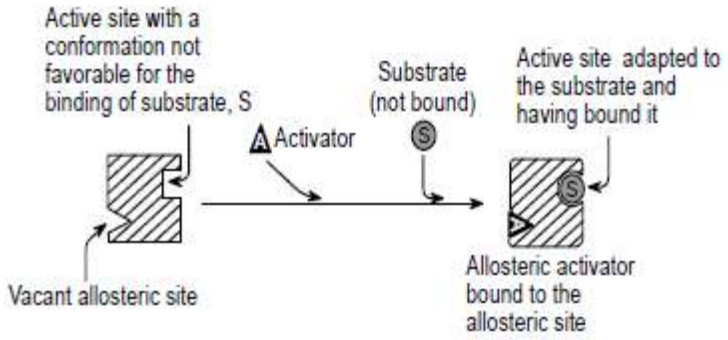



Fig. 10.7 Allosteric transition caused by allosteric activator

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 (*Km''' > Km'*, Fig. 10.7). The curve in this case is a true sigmoid. The *Vmax*, however, remains unchanged. Although most allosteric inhibitors are competitive by nature (*Km* changed, *Vmax* 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.

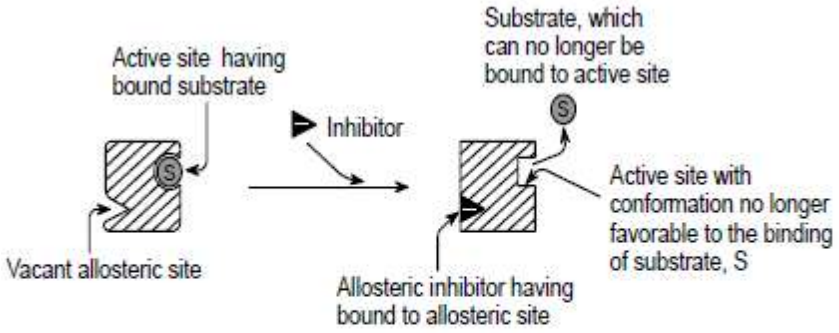



Fig. 10.8 Allosteric transition caused by allosteric inhibitor

## 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 NH4+ 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.

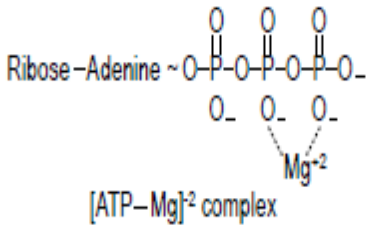



Fig. 10.9 The involvement of $Mg^{+2}$ in favoring substrate binding

## 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 $\Delta G$. Even the modest reduction in the value of $\Delta G$ leads to a very large increase in the reaction rates. For example, ΔG of an uncatalyzed breakdown of H2O2 into water and oxygen is  kJ/mole. The enzyme *catalase* requires only 30 kJ/mol for the same reaction. This lowering of energy is enough to yield 9 ・108-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., -NH2, -COOH, -CH2OH, 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.

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

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 $K1 >> K3$. 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,

$K1[E][S] + K4[E][P] = K2[ES] + K3[ES]$

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

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 $K4$ [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),

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

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

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

This shows that the unit of *Km* is the same as that of [S], i.e., moles/L. It can also be seen that *Km* represents substrate concentration, [S], at $v = ½$ *Vmax Km* 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, *Km* = [S], when $v = ½$

*Vmax*. Also, since $Km = (K2 + K3)/K1$, it is a ratio of complex dissociation to complex formation rate. Evidently, at *Km* values greater than unity the dissociation of ES complex dominates.

*Km* signifies many important meanings, some of which are:

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

2. High *Km* values indicate weak binding between E and S to form ES. Low *Km* values indicate strong binding of E and S. This is particularly true if $K2 >> K3$, which means that the product formation is negligible. Equilibrium exists between E+S and ES and *Km* will equal dissociation constant.

3. *Km* values can be used to identify whether a given enzymatic reaction is free from inhibitions, as also the type of inhibition.

4. *Km* values can be used to predict *Vmax* of a reaction at a given substrate concentration. Under practical condition, the observed velocity, *v*, becomes *Vmax* at $[S] \geq 100Km$. The reaction is then independent of [S] and exhibits *zero order* reaction. The basic assumption is that at $[S] >> Km$:

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

The reaction is then of 1st order.

The *Km* values of enzymes differ greatly. However, for most enzymes, the general range is between 10-1 and 10-6 mole/L. *Km* 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 *Km* Value

*Km* 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, *Vmax* 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:

The last equation is in the form of $y = mx + c$; $1/v$ and 1/[S] represent variables $y$ and $x$ respectively; $Km/Vmax$ and $1/Vmax$ 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 *Km* 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 $1/v = 0$, $1/[S] = -1/Km$, from which the value of *Km* can be calculated.

Similarly, for *Vmax*, we can use 1/[S] = 0 from the graph, in which case $Vmax = v$.

This is equivalent to saying that at infinitely high substrate concentration, $v = Vmax$, 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 CO2. 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.

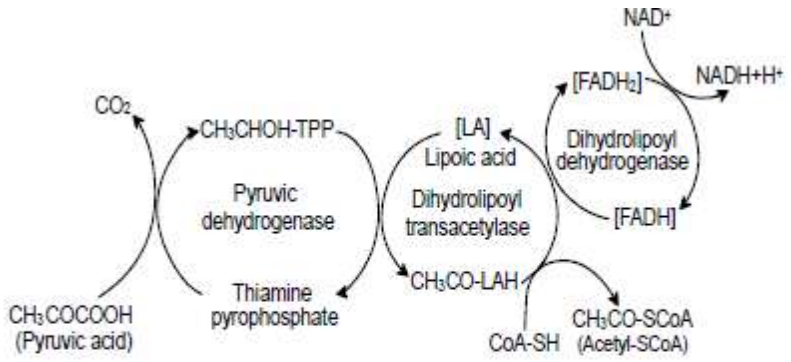



Fig. 10.11 Oxidation of pyruvate to acetyl-SCoA

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, $\mu$ = 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.

due to the mRNA carried due to the turnover synthesis over from the growth phase 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 them 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.

## 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 CO2 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.

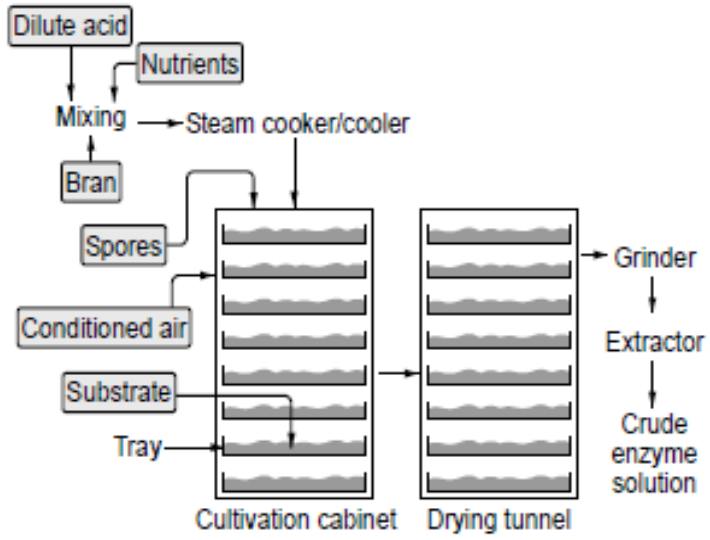



Fig. 10.12 Thin layer solid state fermentation

### *10.14.1.2 The deep bed process*

The deep bed process is a modification of the traditional solid-substrate (= solidstate) 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 m3. 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.

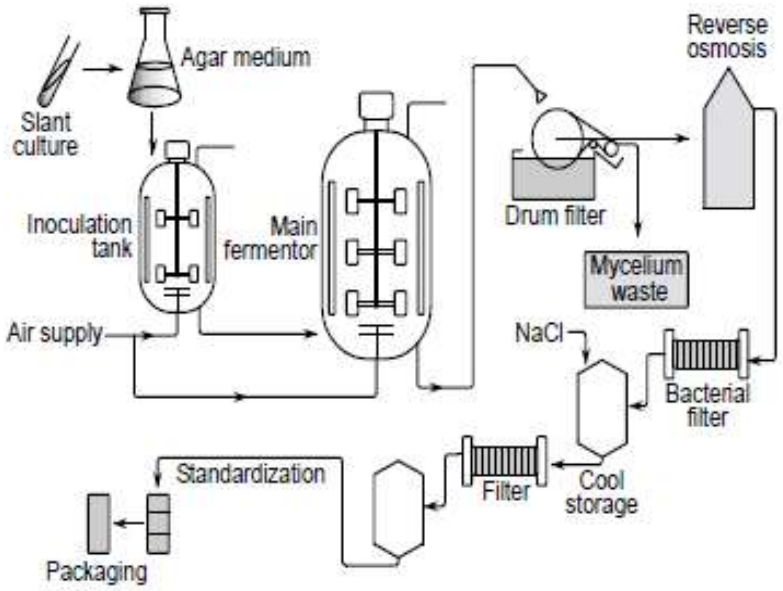



Fig. 10.13 Schematics of stages in the production of a liquid enzyme preparation

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.

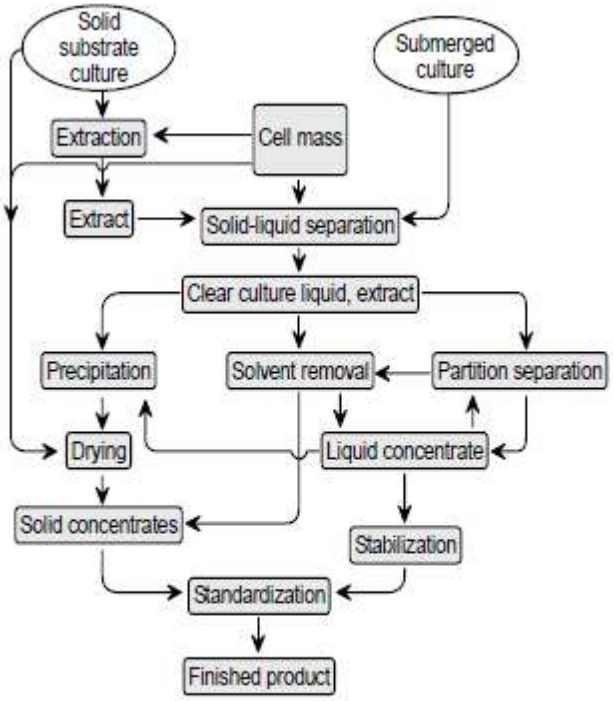



Fig. 10.14 Extraction and purification of an enzyme

### *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); $\tau$ = 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 ( ・) 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 ( ・25 = 33), ethanol ( ・25 = 24), and isopropanol ( ・25 = 18). Besides aliphatic alcohols, acetone ( ・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.*, *α-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.

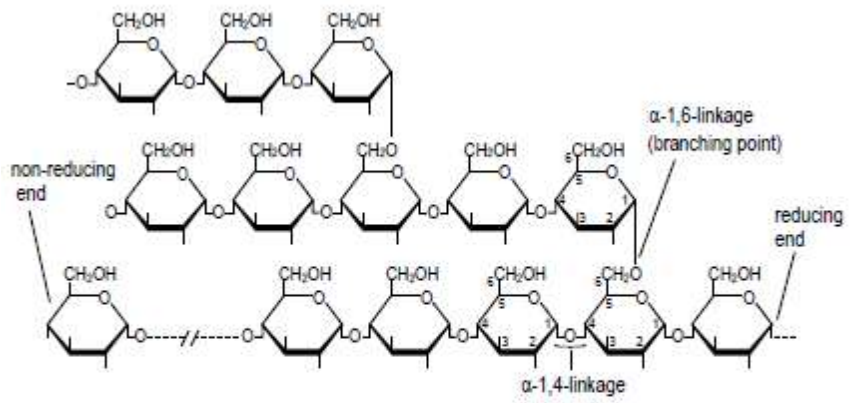



Fig. 10.15 Simplified structure of starch molecule

## 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 α-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, CaCO3 may be added.

*10.17.1.2 Bacterial α-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 109-1010 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 solidsubstrate 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 NH4+ 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 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 106 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 semipermeable 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.

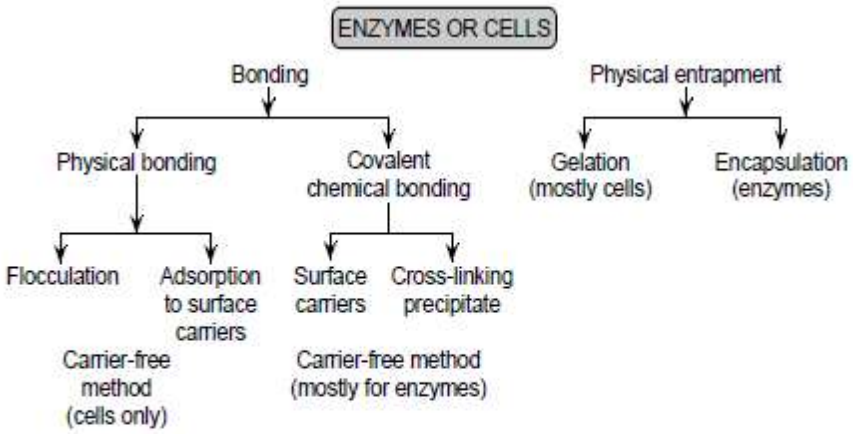



Fig. 10.16 Summary of cell and enzyme immobilization principles

### *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.

# CHAPTER 9

# MICROBIAL PRODUCTION OF ETHANOL

## 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.

$$\text{Ethylene} + H_2O \xrightarrow{H_3PO_4,\ 300^\circ C,\ 60\ \text{atm}} CH_3CH_2OH$$

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

## 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.

### *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.

### *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.

## INDUSTRIAL PRODUCTION

### 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.

## 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, (NH4)2SO4 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%) H2SO4.

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 cylindro conical 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).

**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) ・107 cells per ml of the main fermentation medium.

**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.

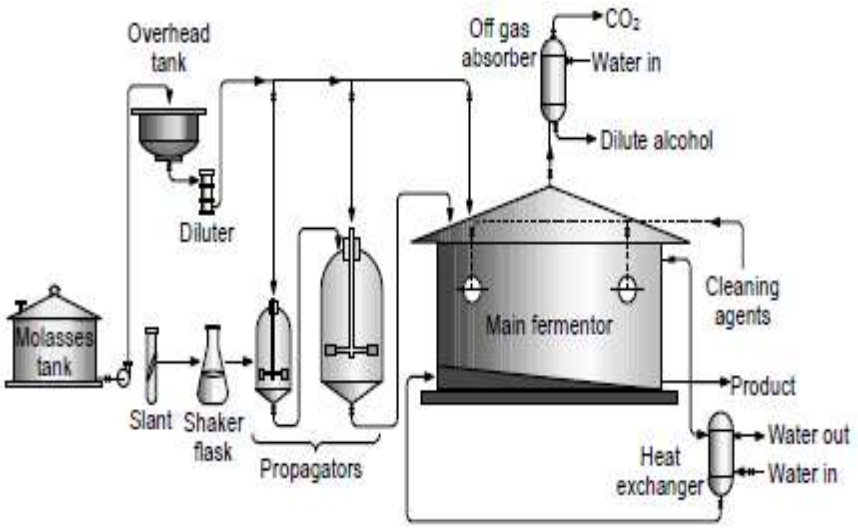



Fig. 13.1 Batch fermentation of ethanol

### 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.

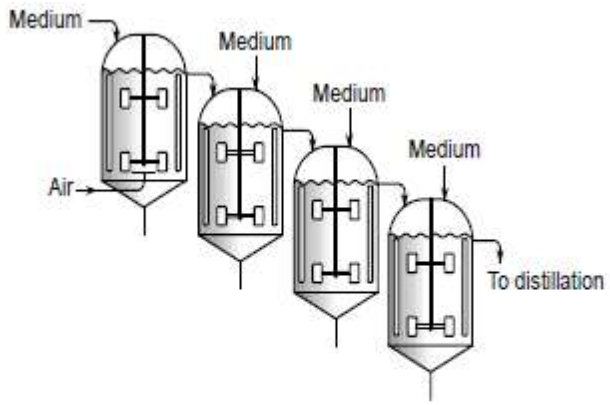



Fig. 13.2 Scheme for cascade process of ethanol fermentation

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 CO2. 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

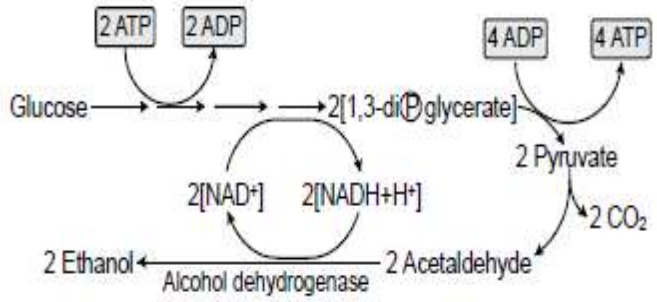



Fig. 13.3 Simplified pathway of ethanol biosynthesis

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.

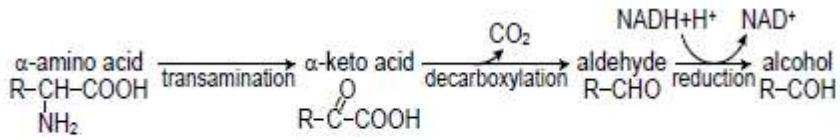



Fig. 13.4 Simplified pathway for higher alcohol biosynthesis

*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 *down comer*. Liquid falls through the down comer 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.*

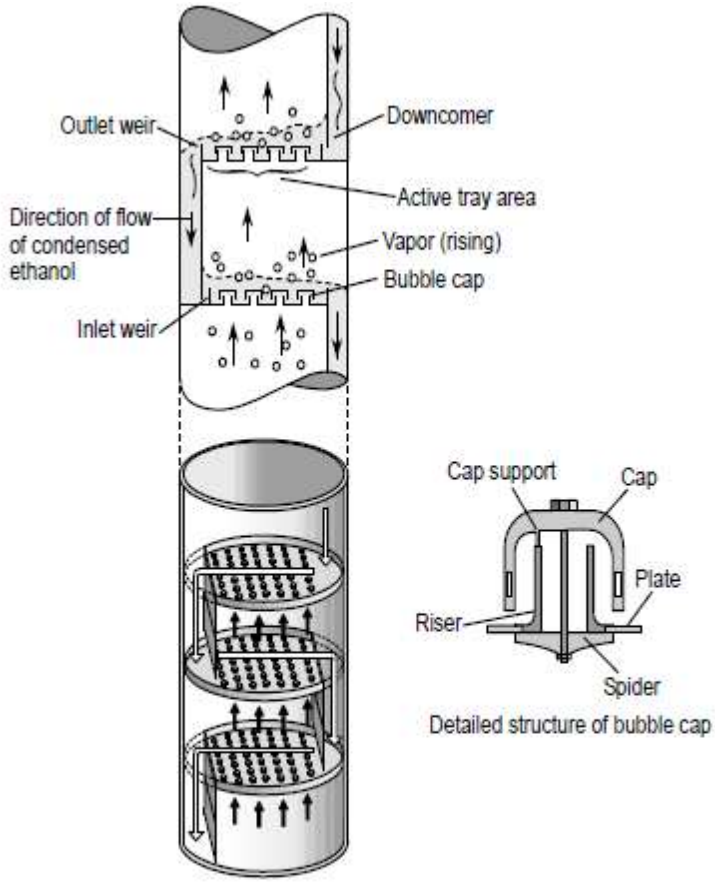



Fig. 13.5 Arrangement of plates in the column

*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.

*Fraction II*

This is the main fraction and contains 96% alcohol (rectified spirit). See

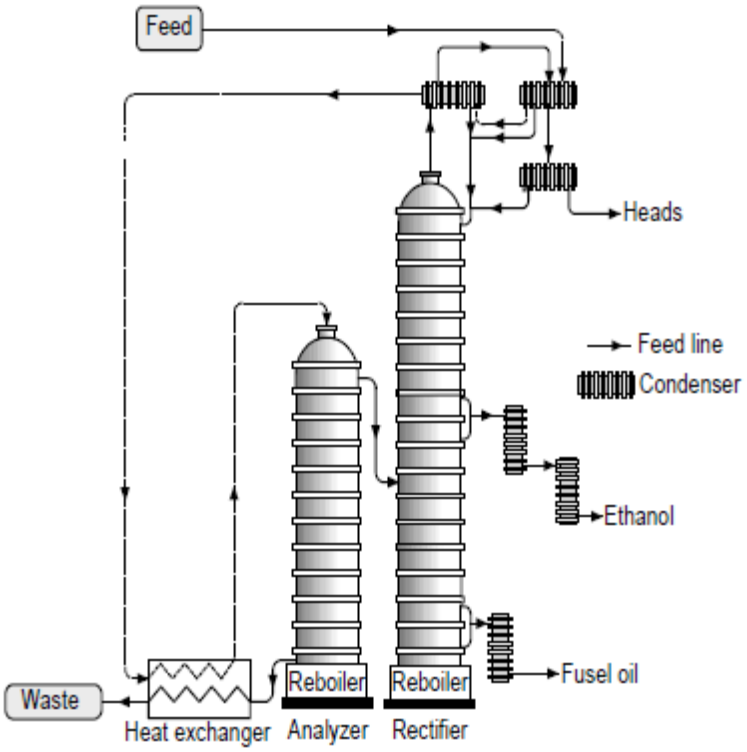



Fig. 13.6 Schematic diagram of ethanol distillation (two-column system)

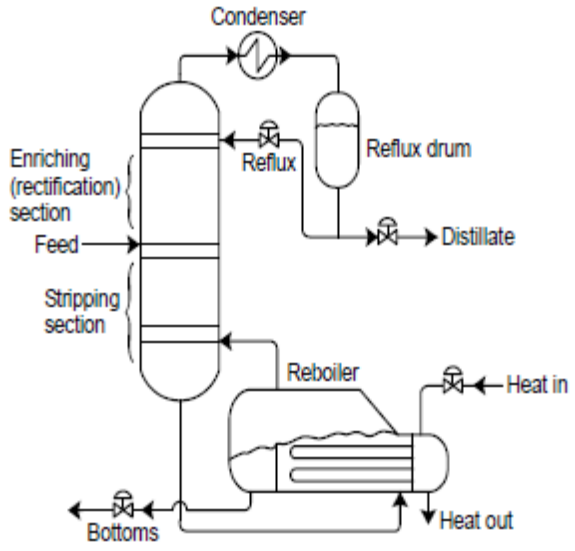



Fig. 13.7 Schematics of rectification and stripping sections

*Fraction III*

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 fuel 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, H2O, and CO2 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° over proof. Similarly, a spirit with 75° proof is 25° lower than proof spirit and hence can be written 25° under proof.

## 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.

# CHAPTER 28

# MICROBIAL PRODUCTION OF VITAMIN B12 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 B12. 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 B12

Vit B12 is a vitamin that is synthesized in nature exclusively by microorganisms. The Vit B12 needs of animals are covered by food intake or by absorption of Vit B12 produced by intestinal microorganisms. Humans obtain Vit B12 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 B12 per kg but isolation from these sources is expensive. Vit B12 was first obtained commercially as a by-product of streptomycin fermentation with yield of 1 mg/liter of broth. As demand of Vit B12 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 B12 production is based on media containing carbohydrate. Most Vit B12 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 B12) 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 B12 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 |
| $MgSO_4 7H_2O$ | 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*

| *Medium C* | Amount, g/liter (unless specified) |
|---|---|
| Sugar beet molasses | 100 |
| Yeast extract | 2 |
| $(NH_4)_2HPO_4$ | 5 |
| $MgSO_47H_2O$ | 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 |

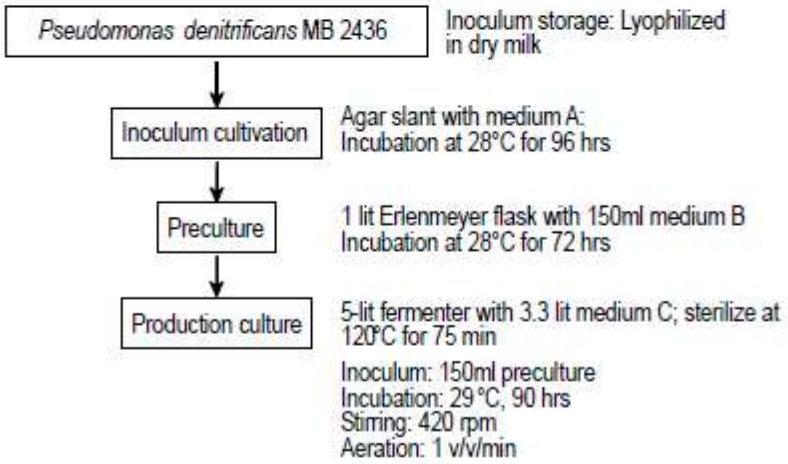



Fig. 28.1 Flow diagram of vitamin $B_{12}$ production

## 28.3 MICROBIAL PRODUCTION OF ・-CAROTENE

### 28.3.1 INTRODUCTION

Carotenoids are found in many animal and plant tissues but originate exclusively from plants or microbes. ・-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.

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 10

# BIOFERTILIZERS

## 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 ofmicroorganisms 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 NH3 or oxidation to $NO3^{-}$. 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:

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.

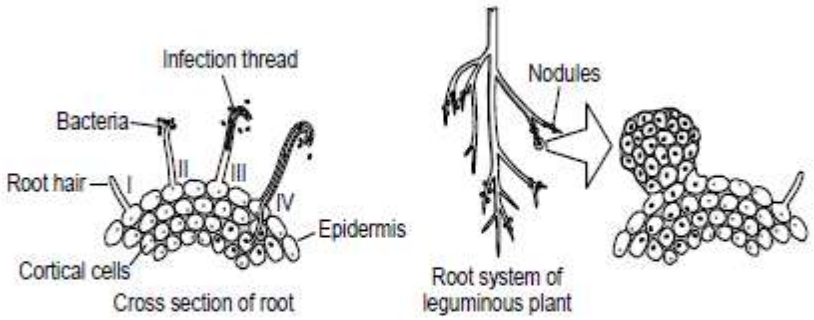



Fig 29.1 Different stages (I, II, II, and IV) of root nodule formation in legume plant

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 K2HPO4; 0.2 g MgSO4.7H2O; 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) × 109 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 105 and 106

5. Spread the seeds in shade for drying

6. Store the seeds at 4°C or use them in the farm

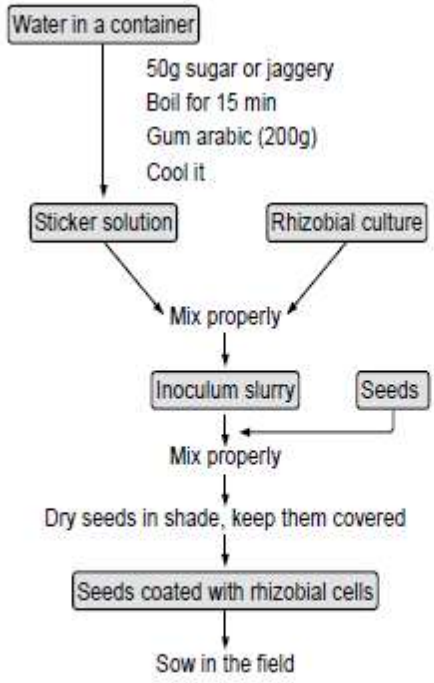



Fig 29.2 Procedure for seed inoculation with rhizobial culture

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