SATHYABAMA
INSTITUTE OF SCIENCE AND TECHNOLOGY
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SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

UNIT – I – INDUSTRIAL MICROBIOLOGY – SMB2204

SATHYABAMA INSITUTE OF SCIENCE AND TECHNOLOGY SCHOOL OF BIO AND CHEMICAL ENGINEERING

SMB2204	INDUSTRIAL MICROBIOLOGY	L	T	P	Credits	Total Marks
		3	1	0	4	100

COURSE OBJECTIVES

- The objective of this course to enable students to correlate metabolic aspects of industrially relevant microorganisms with the corresponding biotechnological products, and learn the basics of simple strategies for strain improvements encompassing both classical and metabolic engineering methods, design medium and downstream processing.

UNIT 1 HISTORY **12 Hrs.**

Brief history and developments in industrial microbiology Types of fermentation processes - Solid-state and liquid-state (stationary and submerged) fermentations; batch, fed-batch (Eg. Baker's yeast) and continuous fermentations

UNIT 2 BIO-REACTOR **12 Hrs.**

Components of a typical bio-reactor, Types of bioreactors-Laboratory, pilot- scale and production fermenters, constantly stirred tank and air-lift fermenters, Measurement and control of fermentation parameters - pH, temperature, dissolved oxygen, foaming and aeration

UNIT 3 UPSTREAM PROCESSING **12 Hrs.**

Sources of industrially important microbes and methods for their isolation, preservation and maintenance of industrial strains, strain improvement, Crude and synthetic media; molasses, corn steep liquor, sulphite waste liquor, whey, yeast extract and protein hydrolysates

UNIT 4 DOWNSTREAM PROCESSING **12 Hrs.**

Cell disruption, filtration, centrifugation, solvent extraction, precipitation, lyophilization and spray drying. Citric acid, ethanol, penicillin, glutamic acid, Vitamin B12 Enzymes (amylase, protease, lipase)
Wine, beer

UNIT 5 IMMOBILIZATION **12 Hrs.**

Methods of immobilization, advantages and applications of immobilization, large scale applications of immobilized enzymes (glucose isomerase and penicillin acylase)

Max Hours.60

TEXT / REFERENCE BOOKS

1. Patel A.H. (1996). Industrial Microbiology. 1st edition, Macmillan India Limited
2. Okafor N. (2007). Modern Industrial Microbiology and Biotechnology. 1st edition. Bios Scientific Publishers Limited. USA
3. Waites M.J., Morgan N.L., Rockey J.S. and Higton G. (2001). Industrial Microbiology: An Introduction. 1st edition. Wiley – Blackwell
4. Glaze A.N. and Nikaido H. (1995). Microbial Biotechnology: Fundamentals of Applied Microbiology. 1st edition. W.H. Freeman and Company
5. Casida LE. (1991). Industrial Microbiology. 1st edition. Wiley Eastern Limited.
6. Crueger W and Crueger A. (2000). Biotechnology: A textbook of Industrial Microbiology. 2nd edition. Panima Publishing Co. New Delhi.
7. 7. Stanbury PF, Whitaker A and Hall SJ. (2006). Principles of Fermentation Technology. 2nd edition, Elsevier Science Ltd..

END SEMESTER EXAMINATION QUESTION PAPER PATTERN

Max. Marks : 100 **Exam Duration : 3 Hrs.**

PART A : 10 questions of 2 marks each - No choice **20 Marks**

PART B : 2 questions from each UNIT of internal choice; each carrying 16 marks **80 Marks**

UNIT:1

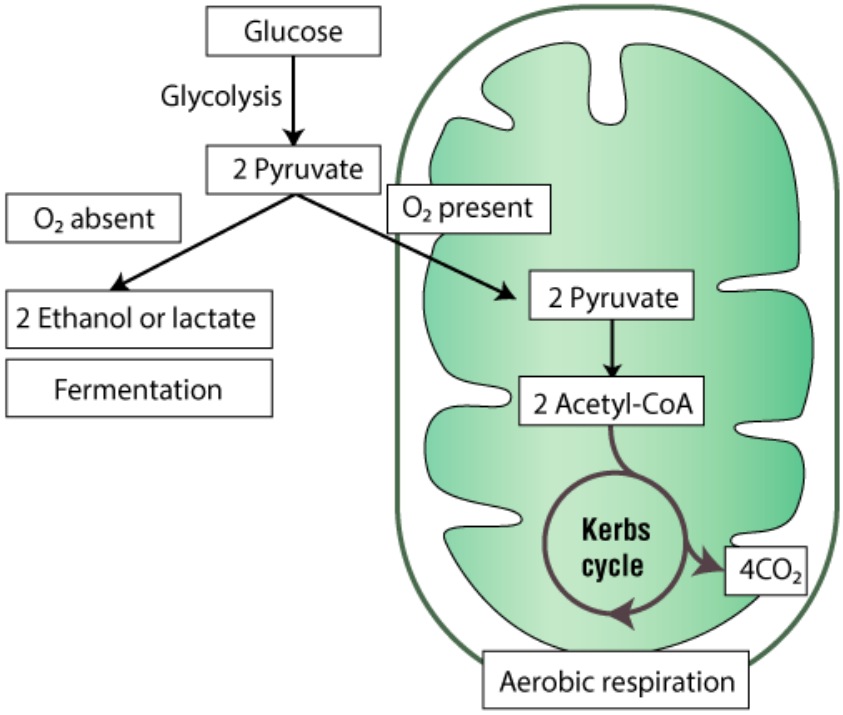
HISTORY OF INDUSTRIAL MICROBIOLOGY

Early biotechnology (BT) had its roots in fascinating discoveries, such as yeast as living matter being responsible for the fermentation of beer and wine. Serious controversies arose between vitalists and chemists, resulting in the reversal of theories and paradigms, but prompting continuing research and progress. Pasteur's work led to the establishment of the science of microbiology by developing pure monoculture in sterile medium, and together with the work of Robert Koch to the recognition that a single pathogenic organism is the causative agent for a particular disease. Pasteur also achieved innovations for industrial processes of high economic relevance, including beer, wine and alcohol. Several decades later Buchner, disproved the hypothesis that processes in living cells required a metaphysical 'vis vitalis' in addition to pure chemical laws. Enzymes were shown to be the chemical basis of bioconversions. Studies on the formation of products in microbial fermentations, resulted in the manufacture of citric acid, and chemical components required for explosives particularly in war time, acetone and butanol, and further products through fermentation. The requirements for penicillin during the Second World War lead to the industrial manufacture of penicillin, and to the era of antibiotics with further antibiotics, like streptomycin, becoming available. This was followed by a new class of high value-added products, mainly secondary metabolites, e.g. steroids obtained by biotransformation. By the mid-twentieth century, biotechnology was becoming an accepted specialty with courses being established in the life sciences departments of several universities. Starting in the 1970s and 1980s, BT gained the attention of governmental agencies in Germany, the UK, Japan, the USA, and others as a field of innovative potential and economic growth, leading to expansion of the field. Basic research in

Biochemistry and Molecular Biology dramatically widened the field of life sciences and at the same time unified them considerably by the study of genes and their relatedness throughout the evolutionary process. The scope of accessible products and services expanded significantly. Economic input accelerated research and development, by encouraging and financing the development of new methods, tools, machines and the foundation of new companies. The discipline of 'New Biotechnology' became one of the lead sciences. Although biotechnology has historical roots, it continues to influence diverse industrial fields of activity, including food, feed and other commodities, for example polymer manufacture, biofuels and energy production, providing services such as environmental protection, and the development and production of many of the most effective drugs. The understanding of biology down to the molecular level opens the way to create novel products and efficient environmentally acceptable methods for their production.

INTRODUCTION ABOUT FERMENTATION,

Fermentation refers to the metabolic process by which organic molecules (normally glucose) are converted into acids, gases, or alcohol in the absence of oxygen or any electron transport chain. Fermentation pathways regenerate the coenzyme nicotinamide adenine dinucleotide (NAD^+), which is used in glycolysis to release energy in the form of adenosine triphosphate (ATP). Fermentation only yields a net of 2 ATP per glucose molecule (through glycolysis), while aerobic respiration yields as many as 32 molecules of ATP per glucose molecule with the aid of the electron transport chain.

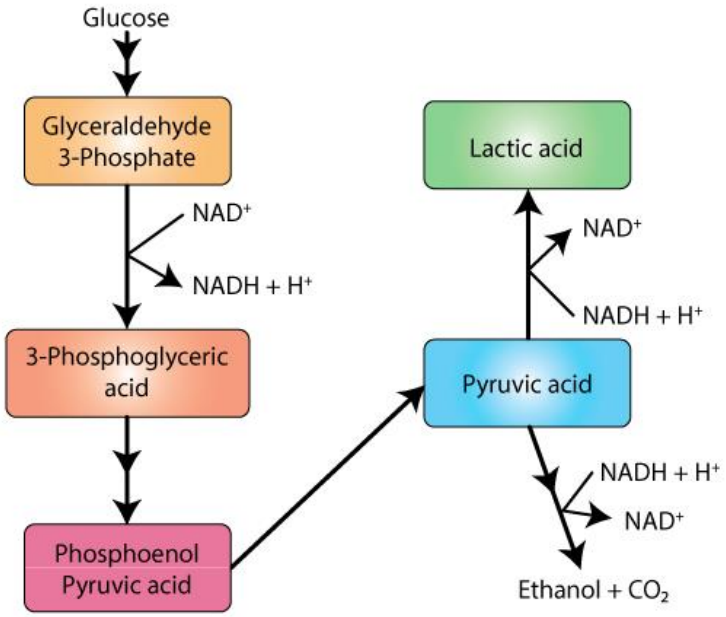


The study of fermentation and its practical uses is named zymology and originated in 1856 when French chemist Louis Pasteur demonstrated that fermentation was caused by yeast. Fermentation occurs in certain types of bacteria and fungi that require an oxygen-free environment to live (known as obligate anaerobes), in facultative anaerobes such as yeast, and also in muscle cells when oxygen is in short supply (as in strenuous exercise). The processes of fermentation are valuable to the food and beverage industries, with the conversion of sugars into ethanol used to produce alcoholic beverages, the release of CO_2 by yeast used in the leavening of bread, and with the production of organic acids to preserve and flavor vegetables and dairy products.

PROCESS OF FERMENTATION

Fermentation is an anaerobic biochemical process. In the process of fermentation, the first step is the same as cellular respiration, which is the formation of pyruvic acid by glycolysis where net 2 ATP molecules are synthesised. In the next step, pyruvate is reduced to lactic acid, ethanol or other

products. Here NAD+ is formed which is re-utilized back in the glycolysis process.



FUNCTION OF FERMENTATION

The main function of fermentation is to convert NADH back into the coenzyme NAD^+ so that it can be used again for glycolysis. During fermentation, an organic electron acceptor (such as pyruvate or acetaldehyde) reacts with NADH to form NAD^+, generating products such as carbon dioxide and ethanol (ethanol fermentation) or lactate (lactic acid fermentation) in the process.

PRODUCTS OF FERMENTATION

While there are a number of products from fermentation, the most common are ethanol, lactic acid, carbon dioxide, and hydrogen gas (H_2). These products are used commercially in foods, vitamins, pharmaceuticals, or as industrial chemicals. In addition, many less common products still offer commercial value. For example, the production of acetone via the acetone – butanol –

ethanol fermentation was first developed by the Jewish chemist Chaim Weizmann and was important to the British war industry during Word War I.

TYPES OF FERMENTATION BASED ON END PRODUCTS

- **Homo fermentation:** When only one type of product is formed
- **Hetero fermentation:** When more than one products are formed

Depending upon the end product formed, fermentation can be categorised into various types

There are many types of fermentation that are distinguished by the end products formed from pyruvate or its derivatives. The two fermentations most commonly used by humans to produce commercial foods are ethanol fermentation (used in beer and bread) and lactic acid fermentation (used to flavor and preserve dairy and vegetables).

Lactic Acid Fermentation

Lactic acid is formed from pyruvate produced in glycolysis. NAD+ is generated from NADH. Enzyme lactate dehydrogenase catalyses this reaction. Lactobacillus bacteria prepare curd from milk by this type of fermentation. During intense exercise when oxygen supply is inadequate, muscles derive energy by producing lactic acid, which gets accumulated in the cells causing fatigu

$$C_6H_{12}O_6(Glucose) \xrightarrow[Lactate.dehydrogenase]{Lactobacillus} 2CH_3CHOHCOOH(LacticAcid)$$

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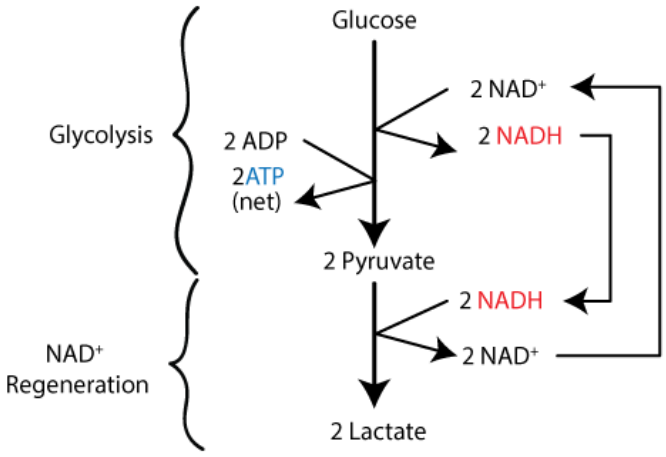
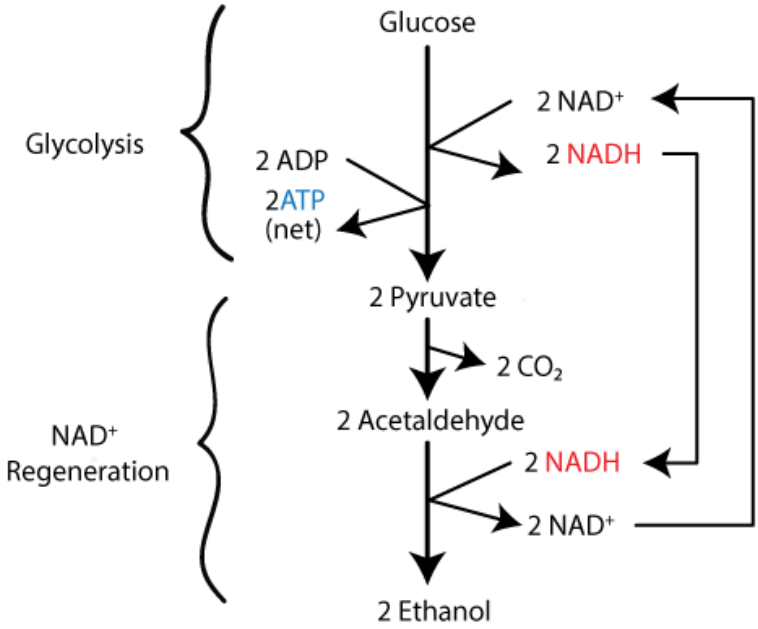


2. Alcohol Fermentation

This is used in the industrial production of wine, beer, biofuel, etc. The end product is alcohol and CO_2. Pyruvic acid breaks down into acetaldehyde and CO_2 is released. In the next step, ethanol is formed from acetaldehyde. NAD+ is also formed from NADH which is reused in glycolysis. Yeast and some bacteria carry out this type of fermentation. Enzyme pyruvic acid decarboxylase and alcohol dehydrogenase catalyse these reactions.

$$C_6H_{12}O_6(Glucose) \xrightarrow[Decarboxylase]{Dehydrogenase} 2CH_3CHO(Acetaldehyde) + 2CO_2 \rightarrow 2C_2H_5OH(Ethanol)$$



3. Acetic acid Fermentation

Vinegar is produced by this process. This is a two-step process.

The first step is the formation of ethyl alcohol from sugar anaerobically using yeast.

In the second step, ethyl alcohol is further oxidised to form acetic acid using acetobacter bacteria. Microbial oxidation of alcohol to acid is an aerobic process.

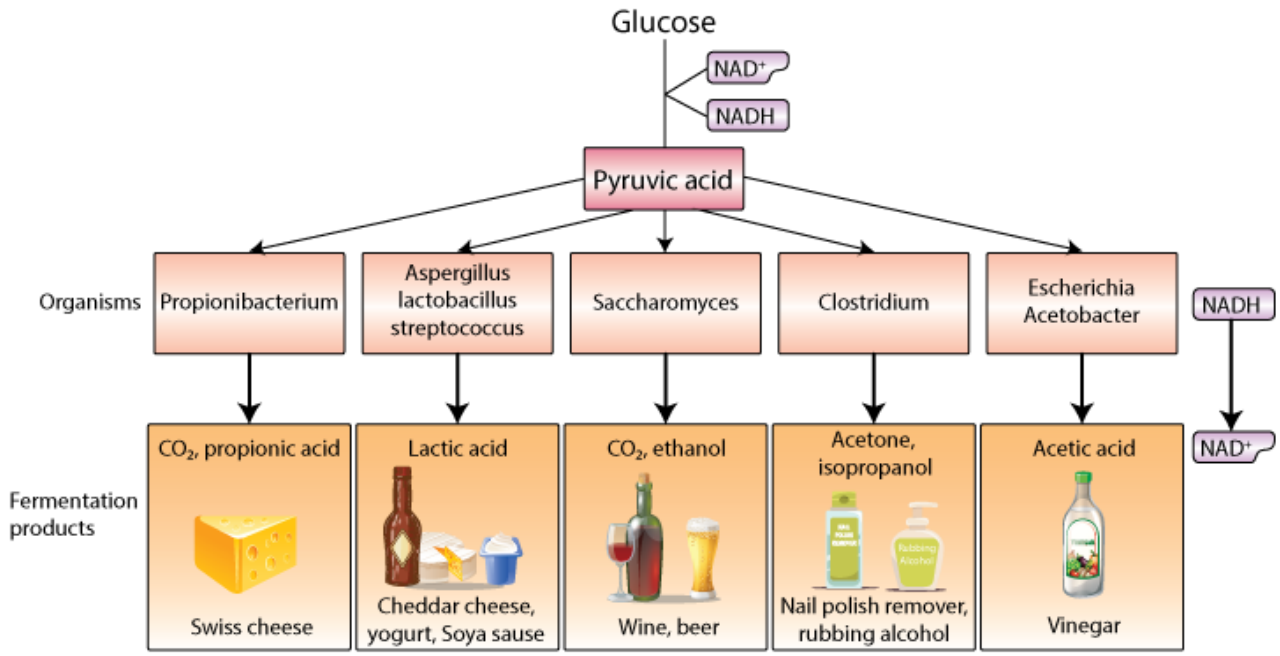
$$C_2H_5OH(ethanol) + O_2 \xrightarrow{acetobacter} CH_3COOH(acetic\,acid) + H_2O$$

4. Butyric acid Fermentation

This type of fermentation is characteristic of obligate anaerobic bacteria of genus clostridium. This occurs in retting of jute fibre, rancid butter, tobacco processing and tanning of leather. Butyric acid is produced in the human colon as a product of dietary fibre fermentation. It is an important source of energy for colorectal epithelium. Sugar is first oxidised to pyruvate by the process of glycolysis and then pyruvate is further oxidised to form acetyl-CoA by the oxidoreductase enzyme system with the production of CO_2 and H_2. acetyl-CoA is further reduced to form butyric acid. This type of fermentation leads to a relatively higher yield of energy. 3 molecules of ATP are formed.

$$C_6H_{12}O_2 \xrightarrow[butyricum]{clostridium} C_4H_8O_2 + 2CO_2 + 2H_2$$

ADVANTAGES OF FERMENTATION:



Fermentation is suitable for all kinds of environments. It is one of the oldest metabolic processes which is common to prokaryotes and eukaryotes. Fermentation is widely used in various industries.

Using suitable microorganisms and specified conditions different kinds of products are formed namely:-

- Wine
- Beer
- Biofuels
- Yogurt
- Pickles
- Bread
- Sour foods containing lactic acid
- Certain antibiotics and vitamins

Fermentation can make food nutritious, digestible and flavoured. There are many benefits of consuming fermented food.

- It improves digestion and helps to maintain intestinal bacteria
- It has an anti-cancer effect.
- Improves immune system
- Reduces lactose intolerance

Other than the food industry there are many other areas where the fermentation process is used. Methane is produced by fermentation in the sewage treatment plants and freshwater sediments.

STAGES OF FERMENTATION:

Fermentation is usually divided into three stages: primary, secondary, and conditioning (or lagering). Fermentation is when yeast produce all of the alcohol and aroma and flavor compounds found in beer. Manipulation of temperature, oxygen levels, and pitch rate as well as yeast strain selection will all dramatically affect the production of aroma and flavor compounds produced during fermentation.

PRIMARY FERMENTATION

The primary stage of fermentation begins when the yeast is introduced into cooled, aerated wort. The yeast quickly utilize the available oxygen to produce sterols, a vital compound for culture expansion. When the oxygen is gone, the yeast switch to the anaerobic phase where the majority of wort sugars are reduced to ethanol and CO_2. Yeast growth occurs during primary fermentation. The extent and rate of yeast growth is directly related to the production of aroma and flavor compounds.

PRIMARY FERMENTATION SUMMARY:

- Depletion of dissolved oxygen
- Acidification/reduction in pH
- Yeast growth or culture expansion
- Ethanol and CO_2 production
- Production of flavor compounds such as esters, diacetyl, sulfur containing compounds, etc.
- Consumption of most wort sugars

The temperature of the primary fermentation should be regulated according to the desired flavor and aroma profile. The following is a guideline:

PRIMARY FERMENTATION TEMPERATURES:

- Ales: 62-75 °F (17-24 °C)
- Lagers: 46-58 °F (8-14 °C) *Note: Lager fermentations can be started warmer (~60 °F/15.5 °C) until signs of fermentation (gravity drop, CO_2 production, head formation) are evident. Cool to desired fermentation temperature once signs of fermentation are observed.
- Wheat and Belgian styles: 62-85 °F (17-29 °C)

SECONDARY FERMENTATION

The secondary stage of fermentation refers to the stage of fermentation after the majority of the wort sugars have been consumed and there is a sharp decrease in the rate of fermentation. During this period, most of the final sugars are depleted and some secondary metabolites are converted by the yeast. Yeast flocculation and settling begins to occur due to the increase in alcohol content and the depletion of sugar and nutrients. Diacetyl reduction takes place during secondary fermentation and during the diacetyl rest that some brewers incorporate into the secondary stage of fermentation.

SECONDARY FERMENTATION SUMMARY:

- Decreased rate of ethanol and CO_2 production
- Diacetyl Conversion
- Reduction of some flavor compounds by yeast metabolism or CO_2 scrubbing
- Terminal gravity is reached
- Yeast flocculation and settling begins

SECONDARY FERMENTATION TEMPERATURES:

- Ales: Same as primary fermentation (higher temperatures will increase diacetyl reduction rates)
- Lagers: 40-60 °F (4-15 °C). Some brewers allow the beer to increase in temperature to speed the diacetyl reduction. This increased temperature is usually only sustained for 24 to 48 hours.
- Wheat and Belgian Beers: Same as primary fermentation (higher temperatures will increase diacetyl reduction rates).

CONDITIONING

The conditioning stage takes place when the terminal gravity has been reached and the tank is cooled to refrigeration temperatures (31-38 °F, 0-3 °C). During this time the yeast continues to flocculate and settle. The yeast also conditions the beer by reducing various undesirable flavor compounds. Ales do not benefit from long conditioning times like lagers do. The desirable flavors in ales will decrease with age and therefore it is recommended that conditioning be as short as possible before packaging. Exposure to oxygen at this stage is extremely detrimental to beer quality.

CONDITIONING SUMMARY:

- Most of the yeast is removed from beer
- Formation and precipitation of haze forming proteins
- Reduction and mellowing of harsh flavors
- Reduction of sulfur compounds, diacetyl, and acetaldehyde
- Flavor stabilization

TYPES OF FERMENTATION PROCESS

SOLID STATE (STATIONARY) (SSF)

- Solid state (substratum) fermentation (SSF) is generally defined as the growth of the microorganism on moist solid materials in the absence or near the absence of free water.
- In recent years SSF has shown much promise in the development of several bioprocesses and products
- SSF has been ambiguously used as solid-state fermentation or solid-substrate fermentation.
- However, it is proper to distinguish between two processes. Solid substrate fermentation should be used to define only those processes in which the substrate itself acts as carbon source occurring in absence or near absence of free water.
- On the other hand, the solid state fermentation is that fermentation which employs a natural substrate as above or an inert substrate used as solid support. Solid substrate fermentation are normally many step process involving.

Table 2.5: Comparison of characteristics of SSF and submerged fermentation

Characteristics	Solid State	Submerged
Microorganism, substrate	Static	Agitated
Water usage	Limited	Unlimited
Oxygen supply	Diffusion	Aeration
Volume of fermentation mash	Smaller	Larger
Liquid waste produced	Negligible	Significant volume
Physical energy requirement	Low	High
Human energy requirement	High	Low
Capital investment	Low	High

Table 2.4: History and development of solid-state fermentation

Period	Development
2000 BC	Bread making by Egyptians
(Recorded history 1000BC)	Cheese making by *penicillium roquefortii*
2500 BC	Fish fermentation/preservation with sugar, starch, salts, etc.
2500 BC	Koji process
7th Century	Koji process from China to Japan
18th Century	Vinegar from pomace
18th Century	Use of gallic acid tanning, printing, etc.
1860-1900	Sewage treatment
1900-1920	Production of fungal enzymes, kojic acid
1940-1950	Fantastic development in fermentation industry, penicillin production
1950-1960	Steroid transformation
1980-1990	Production of various primary and secondary metabolites, development of column type of fermenter, work on kinetics and modeling aspects of SSF
1990-present	Developments on fundamental aspects of SSF, bioprocesses/products developments: A. *Bioprocess:* Bioremediation and biodegradation of hazardous compounds, biological detoxification of agroindustrial residues, biotransformations, biopulping etc. B. *Products:* Bioactive compounds: Aflatoxins, Ocharatoxin A, bacterial endotoxins, gibberellic acid, zearalenone, ergot alkaloids, penicillin, cephalo-sporin, cephamycin C, tetracycline, chlorotetracycline, oxytetracycline, actinohodin, methylenomycin, surfactin, monorden, cyclosporin A, ustiloxins, antifungal volatiles. Calvulanic acid, mycophenolic acid.
	C. *Enzymes:* Cellulase, β-glucosidase, CMCase, laccase, xylanase, polygalacturonase, ligninase, xylanases, β-xylosidase, α-arabinofurano-sidase, L-peroxidase, Mn-peroxidase, arylalachol oxidase, catalase, phenol oxidase, proteases (acidic, neutral and alkaline), lipases, α-galactosidase, β-galactosidase, α-amylase, β-amylase, glucoamylase, glutaminase, inulinase, hytase, tannase, feruloyl para-coumaroyl esterase. D. *Organic acids:* Citric acid, fumaric acid, lactic acid, oxalic acid, gallic acid. E. *Other products:* L-glutamic acid, pigments, carotenoid, xanthan gum, succino- glycan, ethanol, aroma compounds, vitamins : B-12, B-6, riboflavin, thiamine, nicotinic acid, nicotinamide, gammalinolenic acid,

Based on the need for aeration and agitation, SSF can be divided into two groups:

- (a) Fermentation without agitation.
- (b) Fermentation with occasional or continuous agitation.

Second group can be further divided into:

- (i) Fermentation with occasional agitation, without forced aeration.
- (ii) Fermentation with slow continuous agitation with forced aeration.

STEPS FOR SUBSTRATE FERMENTATION

- Pretreatment of a substratum that often requires either mechanical, chemical or biological processing.
- Hydrolysis of polymeric substrates such as polysaccharides and proteins.
- Utilization of hydrolysis products.
- Separation and purification of end products.
- Fermentation with occasional agitation without forced aeration.
- Fermentation with slow continuous agitation and forced aeration.

Types of fermenters for solid state fermentation

1. Laboratory studies have generally been carried out in flasks, beakers, Roux bottles, petri dishes, glass jars and columns. Inoculum is added after substrate autoclaving and incubated without any agitation and aeration.

Roux bottles

2. Large-scale SSF bioprocess, three types of fermenters are in operation:

(a) Drum Fermenter:

It basically consists of drum type vessel usually equipped with a rotating device and arrangements for air circulation (Fig. 2.15a). The air inlet pipe may run parallel to the bottom or center or it may branch at several points over the whole length of the drum to facilitate air distribution which is normally attained by forced aeration, thus achieving the mixing of the fermenting substratum. Growth of the microorganism in this type of fermenter is considered to be better and more uniform than the tray fermenter.

Types of solid substrate fermenters

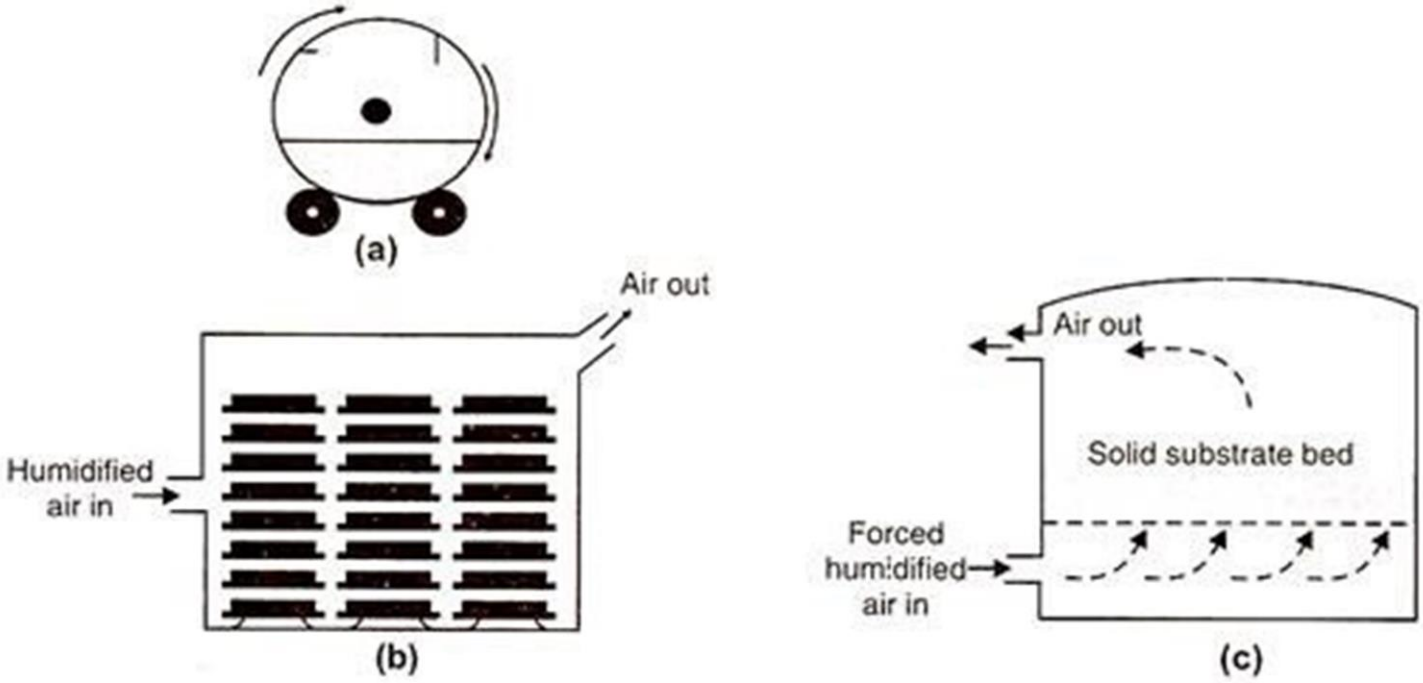


Fig. 2.15 (a), (b), (c): Three types of solid-substrate fermenters, (a) Rotating-drum fermenter, (b)Tray fermenter, and (c) Column fermenter

(b) Tray Fermenter:

- **Tray fermenters are the simplest and can be constructed using wood, metals or plastic material. The bottom of tray is perforated in such a way that it holds substrate and allows aeration (Fig. 2.15b).**
- Kofi fermentation has traditionally been carried out in tray fermenter. Tray fermenter, however, require a large operational area and labour intensive. Their design does not lead readily to mechanical handling. The substrate requires separate sterilization.

(c) Column Fermenter:

- Column fermenter consists of a glass or plastic column with lids at both ends. It may be fitted with a jacket for the circulation of water to control the temperature of fermenting substrate. Alternatively, the whole column may be placed in temperature controlled water bath. Usually air is circulated from bottom to top (Fig. 2.15c).
- The column may be vertical or horizontal as per convenience. Bed reactor is simple in design in which humidified air is pumped into substratum and the used waste gases goes out through the outlet provided continuous agitation with forced air to prevent adhesion and aggregation of substrate particles. These systems are very useful for biomass production for animal feed.

- Microorganisms associated with solid substrate fermentation are those that tolerate relatively low water activity down to 0.7. They may be employed in the form of monocultures as in mushroom production e.g. Agaricus bisporus.
- Dual cultures e.g. straw conversion using Chaetomium cellulolyticum and Candida tropicalis. Mixed cultures as used in compositing and the preparation of silage where the microorganisms may be indigenous or added as mixed starter cultures.

LIQUID OR SUBMERGED FERMENTATIONS:

- Submerged Fermentations are those in which the nutrient substratum is liquid and the organism grows inside the substratum. The culture conditions are made uniform with the help of spargers and impeller blades. Most of the industrial fermentations are of this type. The substratum which is in a liquid state and such medium is also called as broth.

Batch Fermentation:

- **A batch fermentation is a closed culture system, because initial and limited amount of sterilized nutrient medium is introduced into the fermenter.**
- The medium is inoculated with a suitable microorganism and incubated for a definite period for fermentation to proceed under optimal physiological conditions.
- Oxygen in the form of air, an antifoam agent and acid or base, to control the pH, are being added during the course of fermentation process

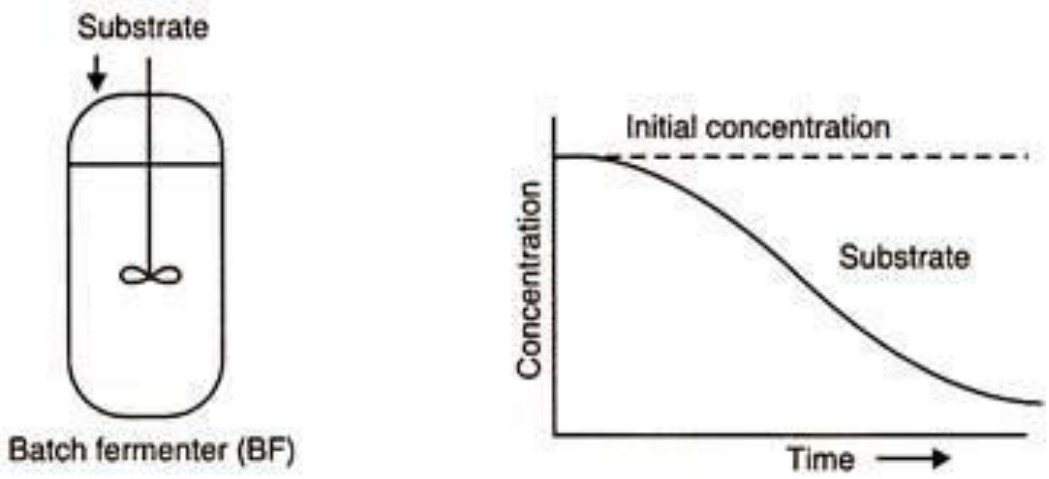


Fig. 2.11: A typical batch fermenter

- During the course of incubation, the cells of the microorganism undergo multiplication and pass through different phases of growth and metabolism due to which there will be change in the composition of culture medium, the biomass and metabolites. The fermentation is run for a definite period or until the nutrients are exhausted. The culture broth is harvested and the product is separated.
- Batch fermentation may be used to produce biomass, primary metabolites and secondary metabolites under cultural conditions supporting the fastest growth rate and maximum growth would be used for biomass production. The exponential phase of growth should be prolonged to get optimum yield of primary metabolite, while it should be reduced to get optimum yield of secondary metabolites.

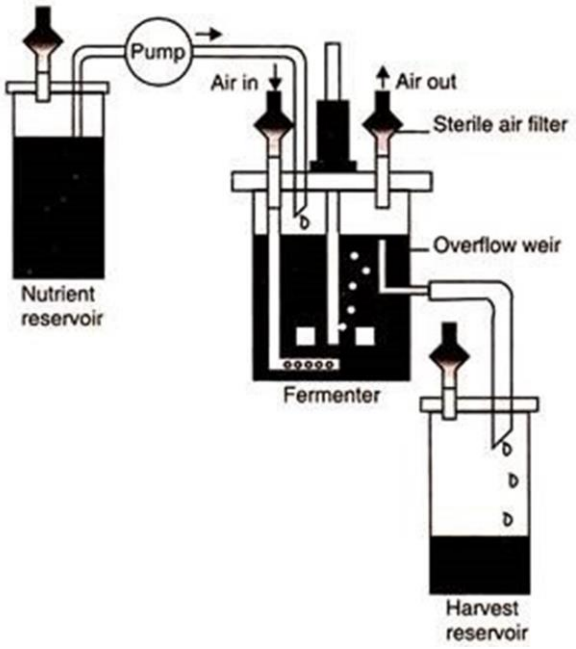
•The used medium along with cells of microorganism and the product is drawn out from the fermenter. When the desired product is formed in optimum quantities, the product is separated from the microorganism and purified later on.

- **It has both advantages and disadvantages which are detailed below:**
- **(i) Merits:**
- (a) The possibility of contamination and mutation is very less.
- (b) Simplicity of operation and reduced risk of contamination.
- **(ii) Demerits:**
- (a) For every fermentation process, the fermenter and other equipment are to be cleaned and sterilized.
- (b) Only fraction of each batch fermentation cycle is productive.
- (c) It is useful in fermentation with high yield per unit substratum and cultures that can tolerate initial high substrate concentration.
- (d) It can be run in repeated mode with small portion of the previous batch left in the fermenter for inoculum.
- (e) Use of fermenter is increased by eliminating turn round time or down time.
- (f) Running costs are greater for preparing and maintaining stock cultures.
- (g) Increased, frequency of sterilization may also cause greater stress on instrumentation and probes.
- (h) Fresh sterilized medium and pure culture are to be made for every fermentation process.
- (i) Yield of the desired product may also vary.
- (j) There will be a non-productive period of shutdown between one batch productive fermentation to the other,

- (k) More personal are required.

Continuous Fermentation:

It is an open system of fermentation, run for indefinite period. In this method, fresh nutrient medium is added continuously or intermittently to the fermenter and equivalent amount of used medium with microorganisms is withdrawn continuously or intermittently for the recovery of cells or fermentation products.



- ***As a result, volume of the medium and concentration of nutrients at optimum level are being maintained. This has been operated in an automatic manner. The continuousfermenter has its maximum use that take long time to reach high productivity, reduces down time and lowers the operating costs.***
- In continuous mode, starting medium and inoculum are added to the fermenter. After the culture is grown the fermenter is fed with nutrients and broth is withdrawn at the same rate maintaining a constant volume of broth in the fermenter. In continuous mode with cell cycle, the cell mass is returned to the fermenter using micro filtrations with bacteria or screens with fungal mycelium.

- **A continuous fermentation is generally carried out in the followingways:**
- (a) Single stage fermentation
- (b) Recycle fermentation
- (c) Multiple stage fermentation
- **(a) Single Stage Fermentation:**
- In this process, a single fermenter is inoculated and the nutrient medium and culture are kept in continuous operation by balancing the input and output of nutrient medium and harvested culture, respectively.
- **(b) Recycle Fermentation:**
- In this method, a portion of the medium is withdrawn and added to the culture vessel. Thus, the culture is recycled to the fermentation vessel. This method is generally adopted in the hydrocarbon fermentation process. The recycling of cells provides a higher population of cells in the fermenter which results in greater productivity of the desired product.

- (c) Multiple StageFermentation:
- In this process, two or more fermenters are employed simultaneously and the fermentation is operated in a sequence. Different phases of fermentation process like growth phase and synthetic phase are carried out in different fermenters. Generally, growth phase is allowed in the first fermenter, synthetic phase in the second and subsequent fermenters.
- This process is adapted particularly to those fermentations in which growth and synthetic activities of the microorganisms are not simultaneous. Synthesis is not growth related but occurs when cell multiplication rate has slowed down.

- **The process of continuous fermentation is monitored either by microbial growth activity or by product formation and these methods are called:**
- (i) Turbidostat method, and
- (ii) Chemostat method.
- **(i) Turbidostat Method:**
- In this method the total cell content is kept constant by measuring the culture turbidity at a regular interval of fermentation process. By turbidity measurement it is possible to the fermenter to regulate both the nutrient feed rate and the culture withdrawal rate.
- Fermentation, in which this method is employed, must be carried out at a low maximum cell population which leads to the usage of less amount of substrate and wastage of greater amount of substrate as unused and residual medium, which is removed from the fermenter along with the harvested culture

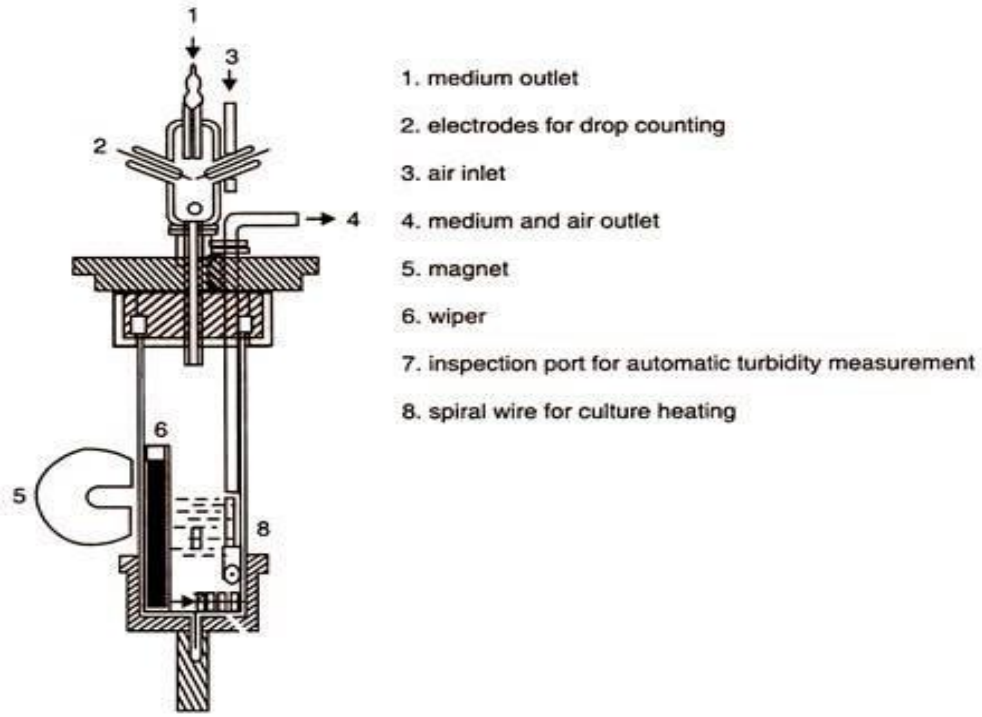


Fig. 2.13: Turbidostat

Chemostat Method

In this method nutrient feed rate and harvest culture withdrawal rate are maintained at constant value. This is achieved by controlling the growth rate of the microorganism by adjusting the concentration of any one of the chemicals of the medium, like carbon source, nitrogen source, salts, O_2 etc. which acts as a growth limiting factor.

- Apart from the above chemicals, sometimes the concentration of the toxic product generated in the fermentation process, the pH values and even temperature also act as growth limiting factors. This method is employed more often than turbidostat method because of fewer mechanical problems and presence of less amount of unused medium in the harvested culture **However, continuous fermentations have certain advantages and limitations which are as follows: (a) Merits:**
- 1. The fermenter is continuously used with little or no shutdown time.
- 2. Only little quantity of initial inoculum is needed and there is no need of additional inoculum.
- 3. It facilitates maximum and continuous production of the desired product.
- 4. There is optimum utilization of even slow utilizable substances like hydrocarbons.
- **(b) Demerits:**

- 1. Possibility of contamination and mutation because of prolonged incubation and continuous fermentation, are more.
- 2.

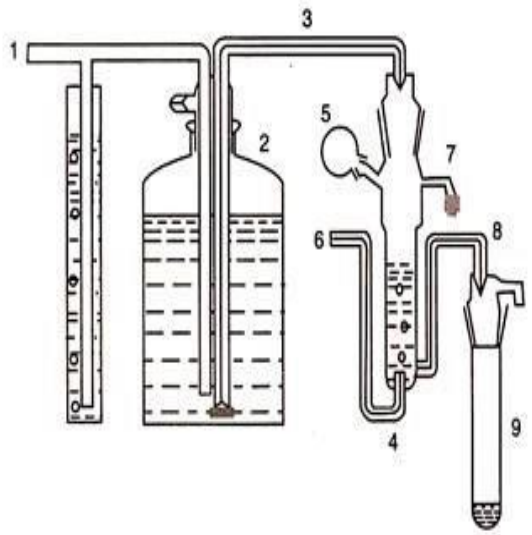


Fig. 2.14: Chemostat

1. Air inlet	2. Mariotte's bottle	3. Capillary for medium inlet
4. Culture vessel	5. Inoculation port	6. Air inlet
7. Air outlet	8. Overflow capillary	9. Sampling tube

Possibility of wastage of nutrient medium because of continuous withdrawal for product isolation.

- 3. The process becomes more complex and difficult to accomplish when the desired products are antibiotics rather than a microbial cells.
- 4. Lack of knowledge of dynamic aspects of growth and synthesis of product by microorganism used in fermentation.

Applications:

- Continuous culture fermentation has been used for the production of single cell protein, antibiotics, organic solvents, starter cultures etc. (table 2.2). Pilot plants or production plants have been installed for production of beer, fodder yeast, vinegar, baker's yeast. A wide variety of microorganisms are used for this type of fermentation (table 2.3).

Fed Batch Fermentation:

- It is a modification to the batch fermentation. In this process substrate is added periodically in instalments as the fermentation progresses, due to which the substratum is always at an optimal concentration.
- This is essential as some secondary metabolites are subjected to catabolite repression by high concentration of either glucose, or other carbohydrate or nitrogen compounds present in the medium.
- For this reason, the critical elements of the nutrient medium are added in low amount in the beginning of the fermentation and these substrates continue to be added in small doses during the production phase.
- This method is generally employed for the production of substances such as penicillin. Yoshida (1973) introduced this term for the first time for feeding the substrates to the medium as the nutrients are exhausted, so as to maintain the nutrients at an optimum level.

- **The fed-batch fermentation may be of three types:**
- **(i) Variable Volume Fed Batch Culture:**
- The same medium is added resulting in an increase in volume.
- **(ii) Fixed Volume Fed Batch Culture:**
- A very concentrated solution of the limiting substrate is added at a very little amount resulting in an insignificant increase in the volume of medium.
- **(iii) Cyclic Fed Batch Culture:**
- As it is not possible to measure the substrate concentration by following direct methods during fermentation, which is necessary for controlling the feeding process, generally indirect methods are employed. For example – in the production of organic acids, the pH value may be used to determine the rate of glucose utilization.

Table 2.3: Microorganisms used in continuous fermentation

Group of organism	Genera
Actinomycetes	*Streptomyces,*
Algae	*Chlorella, Euglena and Scenedesmus.*
Bacteria	*Aerobacter, Azotobacter, Bacillus, Brucella, Clostridium and Salmonella.*
Fungi	*Ophiostoma and Penicillium.*
Protozoa	*Tetrahymena*
Yeast	*Saccharomyces, Torula*

Advantages:

1. Production of high cell densities due to extension of working time (particularly growth associated products).
2. Controlled conditions in the provision of substrates during fermentation, particularly regarding the concentration of specific substrates for e.g. the carbon source.
3. Control over the production of, by products or catabolite repression, effects due to limited provision of substrates solely required for product formation.
4. The mode of operation can overcome and control deviations in the organism's growth pattern as found in batch fermentation.
5. Allows the replacement of water loss, by evaporation.
6. Alternative mode of operation for fermentations dealing with toxic substances or low solubility compounds.
7. Increase of antibiotic marked plasmid stability by producing the correspondent antibiotic during the time span of the fermentation.
8. No additional special piece of equipment is required as compared with the batch fermentation.
9. It is an effective method for the production of certain chemicals, which are produced at optimum level when the medium is exhausted like penicillin.

Disadvantages:

1. It is not possible to measure the concentration of feeding substrate by following direct methods like chromatography.
2. It requires precious analysis of the microorganism. Its requirements and the understanding of its physiology with productivity is essential.
3. It requires a substantial amount of operator skill for the set-up of fermentation and development of the process.
4. In a cyclic fed batch culture, care should be taken in the design of the process to ensure that toxins do not accumulate to inhibitory levels and that nutrients other than those incorporated into the fed medium become limited also, if many cycles are run. The accumulation of non-producing or low producing variants may result.
5. The quantities of components to control must be above the detection limits of the available measuring equipment.

Fed-batch with recycle of cells can also be used for specific purpose such as ethanol fermentation and waste water treatment.

- **At present following products are being produced under fed batch culture:**
- 1. Production of baker's yeast.
- 2. Penicillin production.
- 3. Production of Thiostrepton by Streptomyces laurentii
- 4. Production of industrial enzymes, histidine, glutathione (Brevibacterium flavum), Lysine (Corynebacterium glutamicum)

I.

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SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

UNIT – II – INDUSTRIAL MICROBIOLOGY – SMB2204

SMB2204	INDUSTRIAL MICROBIOLOGY	L	T	P	Credits	Total Marks
		3	1	0	4	100

COURSE OBJECTIVES

- The objective of this course to enable students to correlate metabolic aspects of industrially relevant microorganisms with the corresponding biotechnological products, and learn the basics of simple strategies for strain improvements encompassing both classical and metabolic engineering methods, design medium and downstream processing.

UNIT 1 HISTORY **12 Hrs.**

Brief history and developments in industrial microbiology Types of fermentation processes - Solid-state and liquid-state (stationary and submerged) fermentations; batch, fed-batch (Eg. Baker's yeast) and continuous fermentations

UNIT 2 BIO-REACTOR **12 Hrs.**

Components of a typical bio-reactor, Types of bioreactors-Laboratory, pilot- scale and production fermenters, constantly stirred tank and air-lift fermenters, Measurement and control of fermentation parameters - pH, temperature, dissolved oxygen, foaming and aeration

UNIT 3 UPSTREAM PROCESSING **12 Hrs.**

Sources of industrially important microbes and methods for their isolation, preservation and maintenance of industrial strains, strain improvement, Crude and synthetic media; molasses, corn steep liquor, sulphite waste liquor, whey, yeast extract and protein hydrolysates

UNIT 4 DOWNSTREAM PROCESSING **12 Hrs.**

Cell disruption, filtration, centrifugation, solvent extraction, precipitation, lyophilization and spray drying. Citric acid, ethanol, penicillin, glutamic acid, Vitamin B12 Enzymes (amylase, protease, lipase)
Wine, beer

UNIT 5 IMMOBILIZATION **12 Hrs.**

Methods of immobilization, advantages and applications of immobilization, large scale applications of immobilized enzymes (glucose isomerase and penicillin acylase)

Max Hours.60

TEXT / REFERENCE BOOKS

1. Patel A.H. (1996). Industrial Microbiology. 1st edition, Macmillan India Limited
2. Okafor N. (2007). Modern Industrial Microbiology and Biotechnology. 1st edition. Bios Scientific Publishers Limited. USA
3. Waites M.J., Morgan N.L., Rockey J.S. and Higton G. (2001). Industrial Microbiology: An Introduction. 1st edition. Wiley – Blackwell
4. Glaze A.N. and Nikaido H. (1995). Microbial Biotechnology: Fundamentals of Applied Microbiology. 1st edition. W.H. Freeman and Company
5. Casida LE. (1991). Industrial Microbiology. 1st edition. Wiley Eastern Limited.
6. Crueger W and Crueger A. (2000). Biotechnology: A textbook of Industrial Microbiology. 2nd edition. Panima Publishing Co. New Delhi.
7. 7. Stanbury PF, Whitaker A and Hall SJ. (2006). Principles of Fermentation Technology. 2nd edition, Elsevier Science Ltd..

END SEMESTER EXAMINATION QUESTION PAPER PATTERN

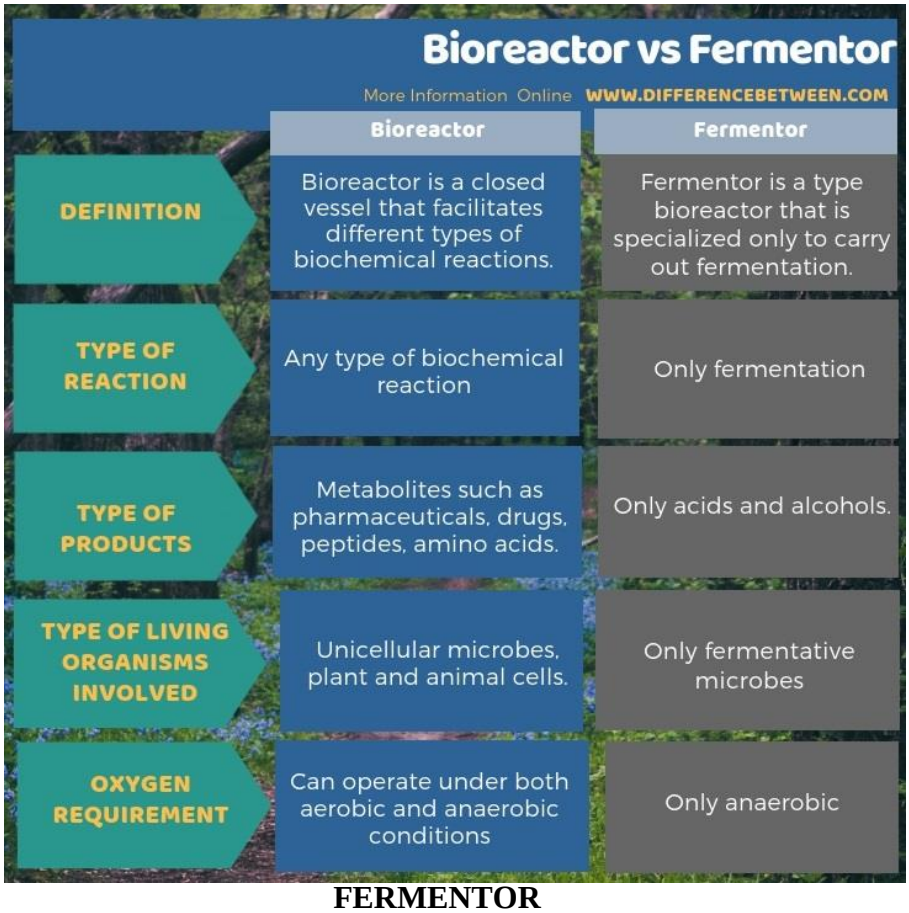
Max. Marks : 100 **Exam Duration : 3 Hrs.**
PART A : 10 questions of 2 marks each - No choice **20 Marks**
PART B : 2 questions from each UNIT of internal choice; each carrying 16 marks **80 Marks**

UNIT:II

BIOREACTOR

Bioreactor is a closed vessel that has the ability to process and facilitate all types of biochemical reactions. Thus, these bioreactors are important in various cell culturing techniques to facilitate cellular growth. The cells which grow inside the bioreactors can vary from single-celled microorganisms to multicellular plant and animal cells. At the end of the process, the desired products can be extracted or separated easily. Hence, these bioreactors utilize routinely in industries to produce secondary metabolites such as pharmaceuticals, vitamins and proteins.

There are different types of bioreactors based on the reactions they facilitate. The main types of bioreactors are stirred tank bioreactors, airlift bioreactors, column bioreactors and packed bed bioreactors. Apart from that, there are several kinds of bioreactors based on the types of culturing mechanism used in the bioreactor. Thus, if the bioreactors carry out suspension culturing, they are known as suspended growth bioreactors. In contrast, if the bioreactors form biofilms for producing metabolites, they are termed as biofilm bioreactors.

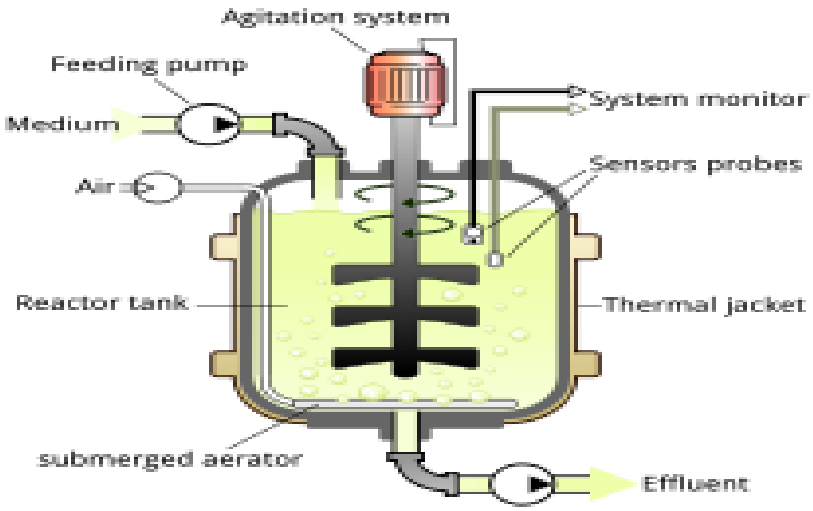


FERMENTOR

Fermentor is a specialized bioreactor. Thus, it only carries out fermentation reactions. Fermentation is the process that produces acids and alcohols from sugar sources under anaerobic conditions. Most industries such as wine industry etc widely use fermentation of sugars to produce lactic acid and ethanol. Thus, fermentors use microbial

sources that are capable of fermentation. They include fungi such as *Saccharomyces cerevisiae* and bacteria such as *Acetobacter*.

Fermentation takes place under anaerobic conditions and the regulation of the temperature and the pH of the system. Hence, fermentor has an inlet and an outlet to add raw materials and to remove the product respectively. Within the fermentors, two main types of fermentations can perform such as submerged fermentation and surface fermentation. Accordingly, submerged fermentation where the cells submerge in the media and surface fermentation where the microbial cultures lie loosely on the surface of the fermentor media.



A typical bioreactor consists of following parts:

1. **Head plate:**
   to cover the top of vessel of bioreactor
2. **Antifoam probe:**
   to prevent contamination in fermentation process
3. **Stirrer shaft seal:**
   To hold the stirrer and head plate
4. **Acid/base probe:**
   A flow to add acid and bases during fermentation process
5. **Shaft:**
   Not allow the medium to escape or microorganism enter
6. **Agitator** – used for the mixing of the contents of the reactor which keeps the “cells” in the perfect homogenous condition for better transport of nutrients and oxygen to the desired product(s).
7. **Baffle** – used to break the vortex formation in the vessel, which is usually highly undesirable as it changes the center of gravity of the system and consumes additional power.
8. **Sparger** – In aerobic cultivation process, the purpose of the sparger is to supply adequate oxygen to the growing cells.

9. **Jacket** – The jacket provides the annular area for circulation of constant temperature of water which keeps the temperature of the bioreactor at a constant value.

10. **2.5 L glass jar bioreactor:**
    Material of the vessel of bioreactor

11. **Sampling port:**
    A valve to get the sample of the fermentation

12. **6- bladed disk impeller:**
    a. To diminish the size of air bubbles to give a bigger interfacial area for O_2 transfer and to decrease diffusion path.

    b. To maintain uniform environment throughout the vessel content.

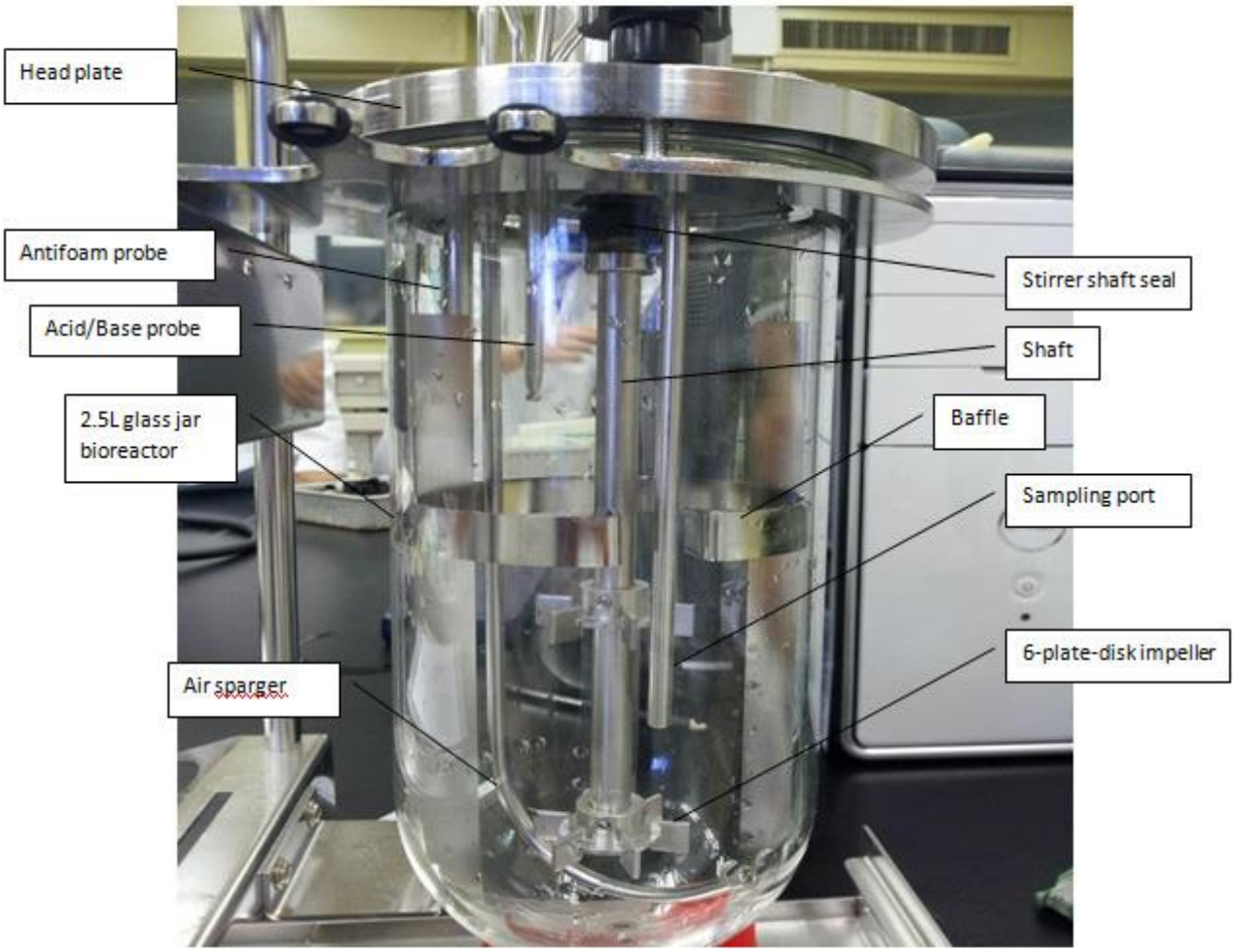


Image 1: compartments inside the vessel of bioreactor

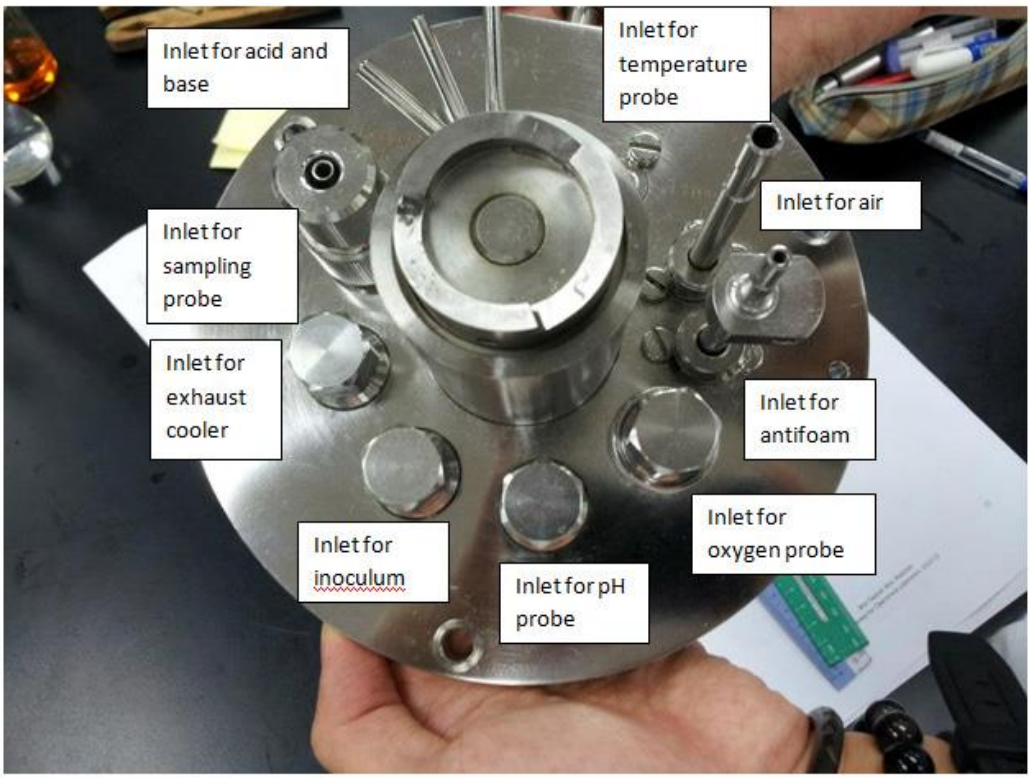


Image 2: All fittings on head plate of the bioreactor

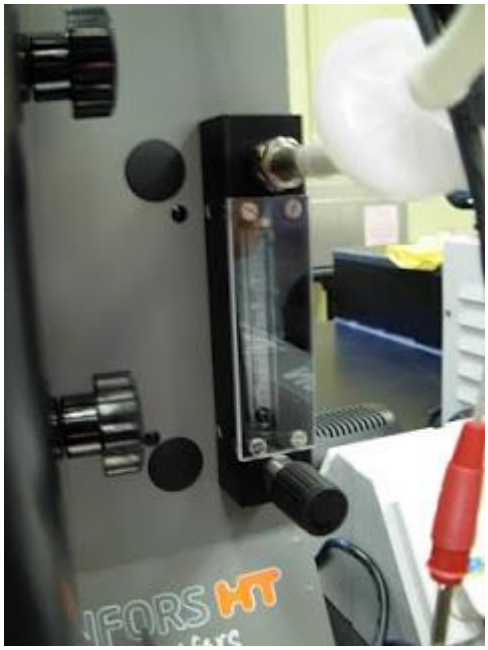


Image 3: rotameter

The rotameter is the means of manually controlling the amount of air entering the vessel and for controlling the gas flow rate.

Image 4: holder of reagent bottles

The function of this holder is where the reagent bottles are usually placed during the fermentation.

Image 5: reagent bottles

The reagent bottles that usually needed during the fermentation take place are, reagent bottle for base, antifoam and acid, but in this experiment we do not need acid because we dealing with microorganism, which is yeast (*Saccharomyces Cerevisiae*) which usually will produce acid trough out the process.

Image 6: pO2 electrode

Image 7: Air compressor

The pO2 electrode should already be connected to the bioreactor base unit which has been powered for at least 2 hours to ensure the electrode is polarized.

The function of air compressor is used to provide air during fermentation process.

BIOREACTORS TYPES

Type # 1. Continuous Stirred Tank Bioreactors:

A continuous stirred tank bioreactor consists of a cylindrical vessel with motor driven central shaft that supports one or more agitators (impellers). The shaft is fitted at the bottom of the bioreactor head plate (Fig. 19.1 A). The number of impellers is variable and depends on the size of the bioreactor i.e., height to diameter ratio, referred to as aspect ratio.

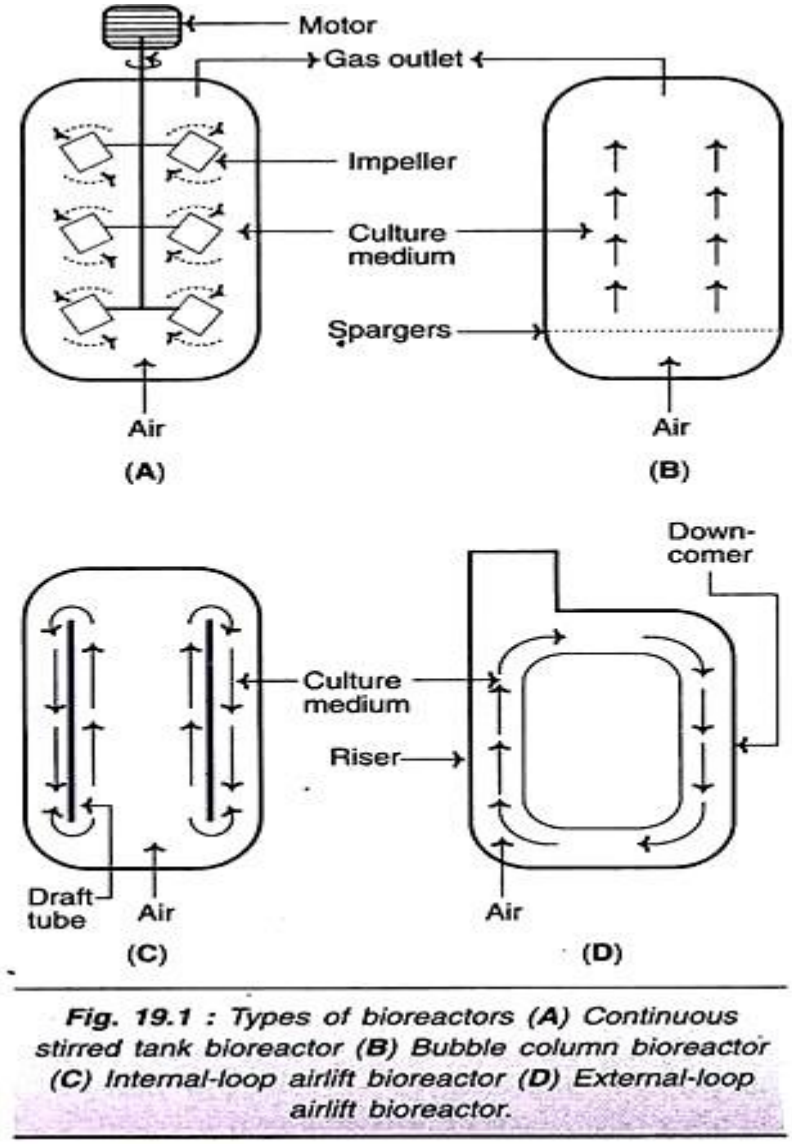


Fig. 19.1 : Types of bioreactors (A) Continuous stirred tank bioreactor (B) Bubble column bioreactor (C) Internal-loop airlift bioreactor (D) External-loop airlift bioreactor.

The aspect ratio of a stirred tank bioreactor is usually between 3-5. However, for animal cell culture applications, the aspect ratio is less than 2. The diameter of the impeller is usually 1/3 rd of the vessel diameter. The distance between two impellers is approximately 1.2 impeller diameter. Different types of impellers (Rustom disc, concave bladed, marine propeller etc.) are in use.

In stirred tank bioreactors or in short stirred tank reactors (STRs), the air is added to the culture medium under pressure through a device called sparger. The sparger may be a ring with many holes or a tube with a single orifice. The sparger along with impellers (agitators) enables better gas distribution system throughout the vessel.

The bubbles generated by sparger are broken down to smaller ones by impellers and dispersed throughout the medium. This enables the creation of a uniform and homogeneous environment throughout the bioreactor.

Advantages of STRs:

There are many advantages of STRs over other types. These include the efficient gas transfer to growing cells, good mixing of the contents and flexible operating conditions, besides the commercial availability of the bioreactors.

Type # 2. Bubble Column Bioreactors:

In the bubble column bioreactor, the air or gas is introduced at the base of the column through perforated pipes or plates, or metal micro porous spargers (Fig. 19.1B). The flow rate of the air/gas influences the performance factors —O_2 transfer, mixing. The bubble column bioreactors may be fitted with perforated plates to improve performance. The vessel used for bubble column bioreactors is usually cylindrical with an aspect ratio of 4-6 (i.e., height to diameter ratio).

Type # 3. Airlift Bioreactors:

In the airlift bioreactors, the medium of the vessel is divided into two interconnected zones by means of a baffle or draft tube. In one of the two zones referred to a riser, the air/gas is pumped. The other zone that receives no gas is the down comer. The dispersion flows up the riser zone while the down flow occurs in the down comer. There are two types of airlift bioreactors.

Internal-loop airlift bioreactor (Fig. 11.1C) has a single container with a central draft tube that creates interior liquid circulation channels. These bioreactors are simple in design, with volume and circulation at a fixed rate for fermentation.

External loop airlift bioreactor (Fig. 19.1D) possesses an external loop so that the liquid circulates through separate independent channels. These reactors can be suitably modified to suit the requirements of different fermentations. In general, the airlift bioreactors are more efficient than bubble columns, particularly for more denser suspensions of microorganisms. This is mainly because in these bioreactors, the mixing of the contents is better compared to bubble columns.

Airlift bioreactors are commonly employed for aerobic bioprocessing technology. They ensure a controlled liquid flow in a recycle system by pumping. Due to high efficiency, airlift bioreactors are sometimes preferred e.g., methanol production, waste water treatment, single-cell protein production. In general, the performance of the airlift bioreactors is dependent on the pumping (injection) of air and the liquid circulation.

Two-stage airlift bioreactors:

Two-stage airlift bioreactors are used for the temperature dependent formation of products. Growing cells from one bioreactor (maintained at temperature 30°C) are pumped into another

bioreactor (at temperature 42°C). There is a necessity for the two-stage airlift bioreactor, since it is very difficult to raise the temperature quickly from 30°C to 42°C in the same vessel. Each one of the bioreactors is fitted with valves and they are connected by a transfer tube and pump (Fig. 19.2A). The cells are grown in the first bioreactor and the product production takes place in the second reactor.

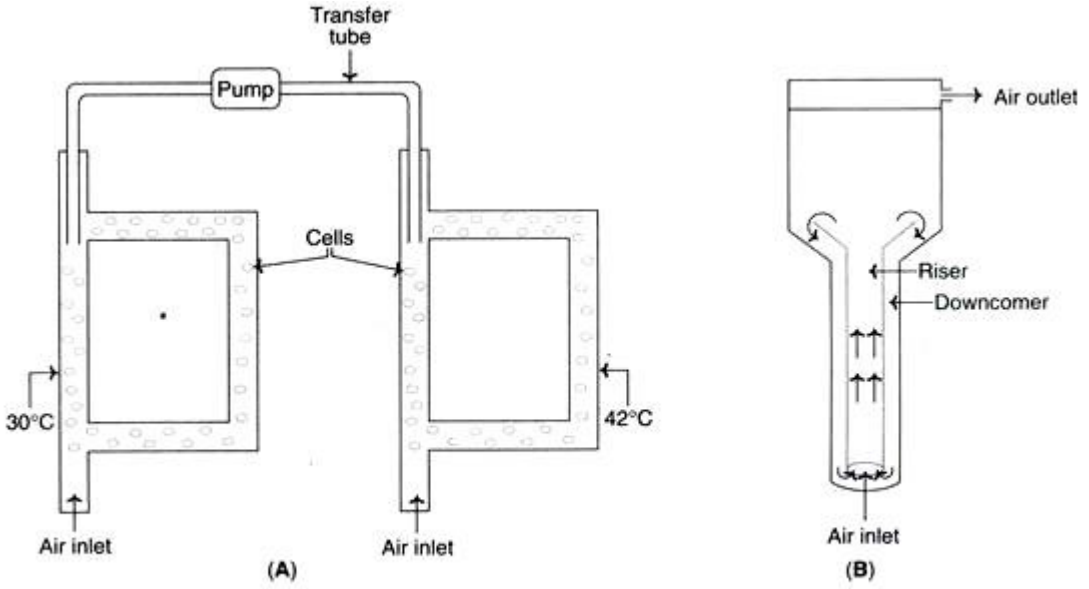


Fig. 19.2 : Types of bioreactors (A) Two–stage airlift bioreactor (B) Tower bioreactor.

Tower bioreactors:

A pressure-cycle fermenter with large dimensions constitutes a tower bioreactor (Fig. 19.2B). A high hydrostatic pressure generated at the bottom of the reactor increases the solubility of O_2 in the medium. At the top of the riser, (with expanded top) reduces pressure and facilitates expulsion of CO_2. The medium flows back in the down comer and completes the cycle. The advantage with tower bioreactor is that it has high aeration capacities without having moving parts.

Type # 4. Fluidized Bed Bioreactors:

Fluidized bed bioreactor is comparable to bubble column bioreactor except the top position is expanded to reduce the velocity of the fluid. The design of the fluidized bioreactors (expanded top and narrow reaction column) is such that the solids are retained in the reactor while the liquid flows out (Fig. 19.3A). These bioreactors are suitable for use to carry out reactions involving fluid suspended biocatalysts such as immobilized enzymes, immobilized cells, and microbial flocs.

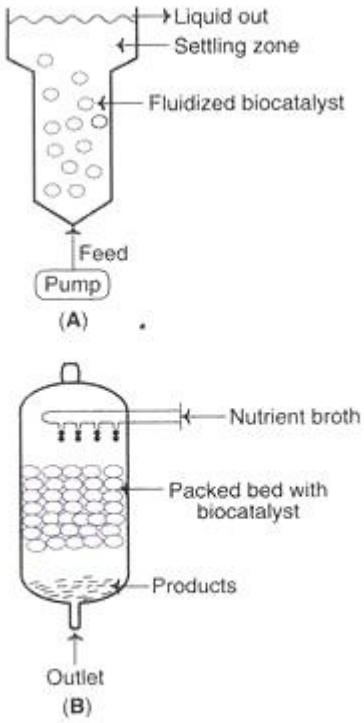


Fig. 19.3 : Types of bioreactors (A) Fluidized bed bioreactor (B) Packed bed bioreactor.

For an efficient operation of fluidized beds, gas is spared to create a suitable gas-liquid-solid fluid bed. It is also necessary to ensure that the suspended solid particles are not too light or too dense (too light ones may float whereas to dense ones may settle at the bottom), and they are in a good suspended state. Recycling of the liquid is important to maintain continuous contact between the reaction contents and biocatalysts. This enable good efficiency of bioprocessing.

Type # 5. Packed Bed Bioreactors:

A bed of solid particles, with biocatalysts on or within the matrix of solids, packed in a column constitutes a packed bed bioreactor (Fig. 19.3B). The solids used may be porous or non-porous gels, and they may be compressible or rigid in nature. A nutrient broth flows continuously over the immobilised biocatalyst. The products obtained in the packed bed bioreactor are released into the fluid and removed. While the flow of the fluid can be upward or downward, down flow under gravity is preferred.

The concentration of the nutrients (and therefore the products formed) can be increased by increasing the flow rate of the nutrient broth. Because of poor mixing, it is rather difficult to control the pH of packed bed bioreactors by the addition of acid or alkali. However, these bioreactors are preferred for bioprocessing technology involving product-inhibited reactions. The packed bed bioreactors do not allow accumulation of the products to any significant extent.

Type # 6. Photo-Bioreactors:

These are the bioreactors specialised for products from algae or algal biomass that can be carried out either by exposing to sunlight or artificial illumination. Since artificial illumination is expensive, only the outdoor photo-bioreactors are preferred. Certain important compounds are produced by employing photo-bioreactors e.g., p-carotene, asthaxanthin.

The different types of photo-bioreactors are depicted in Fig. 19.4. They are made up of glass or more commonly transparent plastic. The array of tubes or flat panels constitute light receiving systems (solar receivers). The culture can be circulated through the solar receivers by methods such as using centrifugal pumps or airlift pumps. It is essential that the cells are in continuous circulation without forming sediments. Further adequate penetration of sunlight should be maintained. The tubes should also be cooled to prevent rise in temperature.

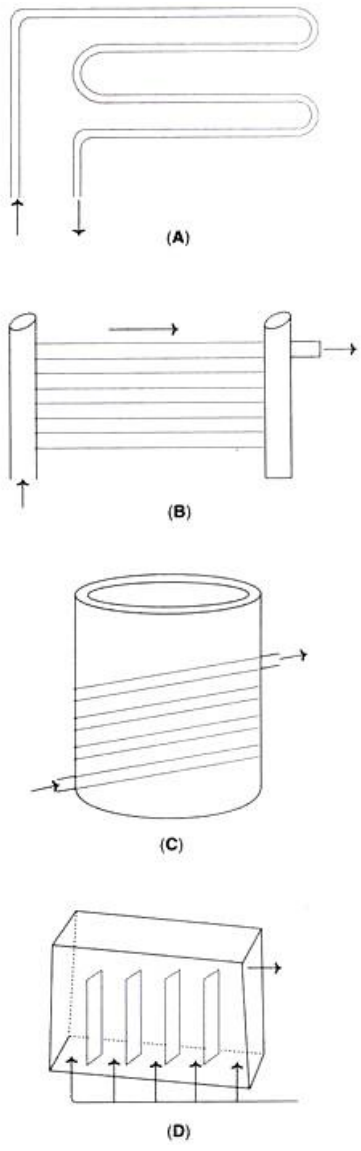


Fig. 19.4 : Types of photobioreactors (A) Continuous run tubular loop (B) Multiple parallel tube (C) Helical wound tubular loop (D) Flat panel configuration.

Photo-bioreactors are usually operated in a continuous mode at a temperature in the range of 25-40°C. Microalgae and cyanobacteria are normally used. The organisms grow during day light while the products are produced during night.

Construction of Fermentors:

Industrial fermentors can be divided into two major classes, anaerobic and aerobic. Anaerobic fermentors require little special equipment except for removal of heat generated during the fermentation process, whereas aerobic fermentors require much more elaborate equipment to ensure that mixing and adequate aeration are achieved.

Since most industrial fermentation process are aerobic, the construction of a typical aerobic fermentor is the following:

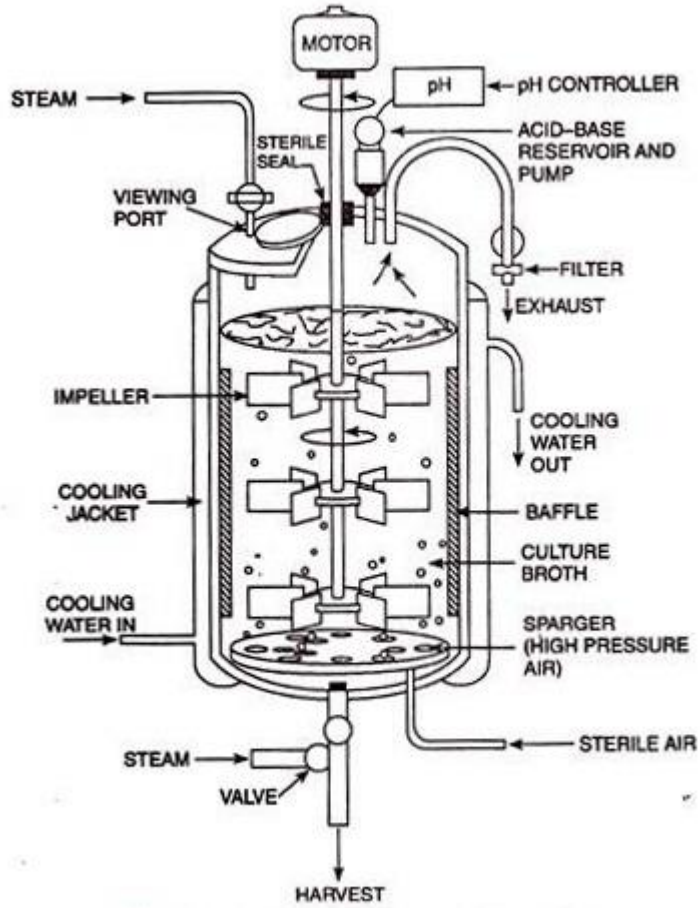


FIG. 39.1. An industrial aerobic fermentor (internal view)

1. Cooling Jacket:

Large-scale industrial fermentors are almost always constructed of stainless steel. A fermentor is a large cylinder closed at the top and the bottom and various pipes and valves are fitted into it. The fermentor is fitted externally with a cooling jacket through which steam (for sterilization) or cooling water (for cooling) is run.

Cooling jacket is necessary because sterilization of the nutrient medium and removal of the heat generated are obligatory for successful completion of the fermentation in the fermentor. For very large fermentors, insufficient heat transfer takes place through the jacket and therefore, internal coils are provided through which either steam or cooling water is run.

2. Aeration System:

Aeration system is one of the most critical part of a fermentor. In a fermentor with a high microbial population density, there is a tremendous oxygen demand by the culture, but oxygen being poorly soluble in water hardly transfers rapidly throughout the growth medium.

It is necessary, therefore, that elaborate precautions are taken using a good aeration system to ensure proper aeration an oxygen availability throughout the culture. However, two separate

aeration devices are used to ensure proper aeration in fermentor. These devices are sparger and impeller.

The sparger is typically just a series of holes in a metal ring or a nozzle through which filter-sterilized air (or oxygen-enriched air) passes into the fermentor under high pressure. The air enters the fermentor as a series of tiny bubbles from which the oxygen passes by diffusion into the liquid culture medium.

The impeller (also called agitator) is an agitating device necessary for stirring of the fermenter.

The stirring accomplishes two things:
(i) It mixes the gas bubbles through the liquid culture medium and

(ii) It mixes the microbial cells through the liquid culture medium. In this way, the stirring ensures uniform access of microbial cells to the nutrients.

The size and position of the impeller in the fermentor depends upon the size of the fermentor. In tall fermentors, more than one impeller is needed if adequate aeration and agitation is to be obtained. Ideally, the impeller should be 1/3 of the fermentors diameter fitted above the base of the fermentor. The number of impeller may vary from size to size to the fermentor.

3. Baffles:

The baffles are normally incorporated into fermentors of all sizes to prevent a vortex and to improve aeration efficiency. They are metal strips roughly one-tenth of the fermentors diameter and attached radially to the walls.

4. Controlling Devices for Environmental Factors:

In any microbial fermentation, it is necessary not only to measure growth and product formation but also to control the process by altering environmental parameters as the process proceeds. For this purpose, various devices are used in a fermentor. Environmental factors that are frequently controlled includes temperature, oxygen concentration, pH, cells mass, levels of key nutrients, and product concentration.

Use of Computer in Fermentor:
Computer technology has produced a remarkable impact in fermentation work in recent years and the computers are used to model fermentation processes in industrial fermentors. Integration of computers into fermentation systems is based on the computers capacity for process monitoring, data acquisition, data storage, and error-detection.

Some typical, on-line data analysis functions include the acquisition measurements, verification of data, filtering, unit conversion, calculations of indirect measurements, differential integration calculations of estimated variables, data reduction, tabulation of results, graphical presentation of results, process stimulation and storage of data.

Types of Fermentor:

The fermentor (bioreactor) types used extensively in industries are the stirred tank fermentor, airlift fermentor, and bubble column fermentor.

(i) Stirred Tank Fermentor:

Stirred tank fermentors consists of a cylindrical vessel with a motor driven central saft that supports one or more impellers.

(ii) Airlift Fermentor:

In airlift fermentor (Fig. 39.2) the liquid culture volume of the vessel is divided into two interconnected zones by means of a baffle or draft tube. Only one of the two zones is sparged with air or other gas and this sparged zone is known as the riser.

The other zone that receives no gas is called down-comer. The bulk density of the gas-liquid dispersion in the gas-sparged riser tends to be lower than the bulk density in the down-comer, consequently the dispersion flows up in the riser zone and down-flow occurs in the down-comer.

FERMENTATION PARAMETERS MEASUREMENT AND CONTROL

pH measurement::

There are pH electrodes that can withstand high temperature (sterilization) pressure and mechanical stresses, and yet measure the pH accurately. Combination electrodes (reference electrode, glass electrode) are being used. In fact, electrodes are also available for measuring several other inorganic ions.

O_2 and CO_2 measurement:

Oxygen electrodes and CO_2 electrodes can be used to measure O_2 and CO_2 concentrations respectively. The electrodes are amperometric in nature. They are however, susceptible for damage on sterilization. In a commonly used technique, O_2 and CO_2 respectively can be measured by the magnetic property of O_2 and the infrared absorption of CO_2. This can be done by using sensors.

Use of Mass Spectrometer:

The mass spectrometer is a versatile technique. It can be used to measure the concentrations of N_2, NH_3, ethanol and methanol simultaneously. In addition, mass spectrometer is also useful to obtain information on qualitative and quantitative exchange of O_2 and CO_2.

Use of Gas-permeable Membranes:

The measurement of dissolved gases, up to 8 simultaneously, can be done almost accurately by using gas-permeable membranes. The advantage is that such measurement is possible to carry out in the nutrient medium.

Use of Computers:

Computers are used in industrial biotechnology for data acquisition, data analysis and developing fermentation models.

Stirred fermenters are better suited than air-lift fermenters to produce better aeration capacities.

CONTROL SYSTEMS

It is essential to maintain optimal growth environment in the reaction vessel for maximum product formation. ***Maximal efficiency of the fermentation can be achieved by continuously monitoring the variables*** such as the pH, temperature, dissolved oxygen, adequate mixing, nutrient concentration and foam formation. Improved sensors are now available for continous and automated monitoring of these variables (i.e., on line measurement of pH).

Most of the microorganisms employed in fermentation grow optimally between pH 5.5 and 8.5. In the bioreactor, as the microorganisms grow, they release metabolites into the medium which change pH. Therefore, the pH of the medium should be continuously monitored and maintained at the optimal level. This can be done by the addition of acid or alkali base (as needed) and a thorough mixing of the fermentation contents. Sometimes, an acid or alkaline medium component can be used to correct pH, besides providing nutrients to the growing microorganisms.

Temperature

Temperature control is absolutely essential for a good fermentation process. Lower temperature causes reduced product formation while higher temperature adversely affects the growth of microorganisms. The bioreactors are normally equipped with heating and cooling systems that can be used as per the requirement, to maintain the reaction vessel at optimal temperature.

Dissolved oxygen

Oxygen is sparingly soluble in water (0.0084 g/1 at 25°C). Continuous supply of oxygen in the form of sterilized air is done to the culture medium. This is carried out by introducing air into the bioreactor in the form of bubbles. Continuous monitoring of dissolved oxygen concentration is done in the bioreactor for optimal product formation.

Adequate mixing

Continuous and adequate mixing of the microbial culture ensures optimal supply of nutrients and O_2, besides preventing the accumulation of toxic metabolic byproducts (if any). Good mixing (by agitation) also creates favourable environment for optimal and homogeneous growth environment, and good product formation. However, excessive agitation may damage microbial cells and increase the temperature of the medium, besides increased foam formation.

Nutrient concentration

The nutrient concentration in a bioreactor is limited so that its wastage is prevented. In addition, limiting concentrations of nutrients may be advantageous for optimal product formation, since high nutrient concentrations are often associated with inhibitory effect on microbial growth. It is now possible to do on-line monitoring of the nutrient concentration, and suitably modify as per the requirements.

Foam formation

The media used in industrial fermentation is generally rich in proteins. When agitated during aeration, it invariably results in froth or foam formation that builds in head space of the bioreactor. Antifoam chemicals are used to lower surface tension of the medium, besides causing foam bubbles to collapse. Mineral oils based on silicone or vegetable oils are commonly used as antifoam agents.

Mechanical foam control devices, referred to as mechanical foam breakers, can also be used. Such devices, fitted at the top of the bioreactor break the foam bubbles and the throw back into the fermentation medium.

CLEANING

As the fermentation is complete, the bioreactor is ***harvested*** i.e. the contents are removed for processing. The bioreactor is then prepared for the next round of fermentation after cleaning (technically called ***turn round***). The ***time taken for turn round***, referred to as ***down time***, should be as short as possible (since it is non-productive). Due to large size of the bioreactors, it is not possible to clean manually. The cleaning of the bioreactors is carried out by using high-pressure water jets from the nozzles fitted into the reaction vessel.

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SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

UNIT –III – INDUSTRIAL MICROBIOLOGY – SMB2204

SMB2204	INDUSTRIAL MICROBIOLOGY	L	T	P	Credits	Total Marks
		3	1	0	4	100

COURSE OBJECTIVES

- The objective of this course to enable students to correlate metabolic aspects of industrially relevant microorganisms with the corresponding biotechnological products, and learn the basics of simple strategies for strain improvements encompassing both classical and metabolic engineering methods, design medium and downstream processing.

UNIT 1 HISTORY **12 Hrs.**

Brief history and developments in industrial microbiology Types of fermentation processes - Solid-state and liquid-state (stationary and submerged) fermentations; batch, fed-batch (Eg. Baker's yeast) and continuous fermentations

UNIT 2 BIO-REACTOR **12 Hrs.**

Components of a typical bio-reactor, Types of bioreactors-Laboratory, pilot- scale and production fermenters, constantly stirred tank and air-lift fermenters, Measurement and control of fermentation parameters - pH, temperature, dissolved oxygen, foaming and aeration

UNIT 3 UPSTREAM PROCESSING **12 Hrs.**

Sources of industrially important microbes and methods for their isolation, preservation and maintenance of industrial strains, strain improvement, Crude and synthetic media; molasses, corn steep liquor, sulphite waste liquor, whey, yeast extract and protein hydrolysates

UNIT 4 DOWNSTREAM PROCESSING **12 Hrs.**

Cell disruption, filtration, centrifugation, solvent extraction, precipitation, lyophilization and spray drying. Citric acid, ethanol, penicillin, glutamic acid, Vitamin B12 Enzymes (amylase, protease, lipase)
Wine, beer

UNIT 5 IMMOBILIZATION **12 Hrs.**

Methods of immobilization, advantages and applications of immobilization, large scale applications of immobilized enzymes (glucose isomerase and penicillin acylase)

Max Hours.60

TEXT / REFERENCE BOOKS

1. Patel A.H. (1996). Industrial Microbiology. 1st edition, Macmillan India Limited
2. Okafor N. (2007). Modern Industrial Microbiology and Biotechnology. 1st edition. Bios Scientific Publishers Limited. USA
3. Waites M.J., Morgan N.L., Rockey J.S. and Higton G. (2001). Industrial Microbiology: An Introduction. 1st edition. Wiley – Blackwell
4. Glaze A.N. and Nikaido H. (1995). Microbial Biotechnology: Fundamentals of Applied Microbiology. 1st edition. W.H. Freeman and Company
5. Casida LE. (1991). Industrial Microbiology. 1st edition. Wiley Eastern Limited.
6. Crueger W and Crueger A. (2000). Biotechnology: A textbook of Industrial Microbiology. 2nd edition. Panima Publishing Co. New Delhi.
7. 7. Stanbury PF, Whitaker A and Hall SJ. (2006). Principles of Fermentation Technology. 2nd edition, Elsevier Science Ltd..

END SEMESTER EXAMINATION QUESTION PAPER PATTERN

Max. Marks : 100 **Exam Duration : 3 Hrs.**
PART A : 10 questions of 2 marks each - No choice **20 Marks**
PART B : 2 questions from each UNIT of internal choice; each carrying 16 marks **80 Marks**

UNIT:III

PRIMARY AND SECONDARY SCREENING OF INDUSTRIALLY IMPORTANT MICROBES SCREENING

"The use of highly selective procedures to allow the detection & isolation of only those microorganisms which are of interest from among a large microbial population"

Strategies for isolation of industrially important microbes

1. The diversity of microorganisms may be exploited still by searching for strains from the neutral environment able to produce products of commercial value

2. The first stage in the screening of microorganisms of potential industrial is their "isolation"

3. Isolation involves obtaining either pure or mixed cultures followed by their assessment to determine which carry out the desired reaction or produce the desired product

4. In some cases it is possible to design the isolation procedure in such a way that the growth of producers is encouraged or that they may be recognized at the isolation stage, whereas in other cases organisms must be isolated and producers recognized at a subsequent stage

5. It should be remembered that the isolate must carry out the process economically and therefore the selection of the culture to be used is a compromise between the productivity of the organism and the economic constraints of the process.

Criteria used for choice of organisms

1. The nutritional characteristics of the organism: Organism should be capable to utilize the ingredients present in the medium to produce interested product.
2. The optimum temperature of the organisms: For instance, the use of an organism having an optimistic temperature above 40o C considerably reduces the cooling costs of a large-scale fermentation, and therefore, the use of such a temperature in the isolation procedure may be beneficial
3. The reaction of the organism with the equipment to be employed
4. The stability of the organism and its amenability to genetic manipulation
5. The productivity of the organism, measured in its ability to convert substrate into product and to give a high yield of product per unit time.
6. The easy product recovery from the cultures.
7. It should be a high yielding strain
8. It should have stable biochemical characteristics
9. It should not produce undesirable substances
10. It should be easily cultivated on a large scale.
11. The ideal isolation procedure commences with an environmental source (frequently soil), which is highly profitable to be rich in the desired types.
12. Selective pressure may be used in the isolation of organism that will grow on particular substrates in the presence of certain compounds or under agricultural conditions adverse in their types
13. If it is not possible to apply selective pressure for the desired character it may be possible to design a procedure to select for a microbial taxon which is known to show the characteristics at a relatively high frequency. E.g. the production of antibiotic by Streptomycin.

14. Alternately, the isolation procedure may be designed to exclude certain microbial "weeds" and to encourage the growth of more novel types
15. The advantages in the taxonomic description of taxa have allowed the rational design of procedures for the isolation of strains that may have a high probability of being productive or are representatives of unusual groups.
16. The advances in pharmacology and molecular biology have also enabled the design of more effective screening tests to identify productive strains amongst the isolated organisms.

Screening of Industrially Important Microbes

Screening allows the discarding of many valueless microorganisms, at the same time it allows the easy detection of the useful microorganisms that are present in the population in very less number

Primary screening

"*Primary screening allows the detection & isolation of microorganisms that possess potentially interesting industrial application*"

- Primary screening separate out only a few microorganisms having real commercial value.

- Primary screening determines which microorganisms are able to produce a compound without providing much idea of the production or yield potential of the organisms

A. Primary screening of organic acid producing microorganisms

Use of an indicator dye:

- This method is used to detect microbes capable of producing organic acids or amines which changes the colour of the medium according to pH.
- Examples of such dye are neutral red, bromothymol blue, etc.

- Incorporation of a pH indicating dye such as neutral red or bromothymol blue into a poorly buffered agar medium.

- Greater buffer capacity of medium screen microbes having capability to produce considerable quantities of the acid

- Incorporation of calcium carbonate in the medium is also used to screen organic acid producing microbes on the basis cleared zone of dissolved calcium carbonate around the colony

- These screening approaches do not give idea that which organic acid has been produced

- Thus the colonies of microorganisms showing the potential to produce any fermentation product should immediately be purified and sub-cultured into appropriate medium to be maintained as stock cultures for further testing.

B. Primary screening of antibiotic producing microorganisms

Crowded Plate" technique

- The simplest screening technique for antibiotic producers is :Crowded Plate" technique

- The technique is used to find out the microorganisms that produce an antibiotic without giving much information of sensitivity towards other microorganisms.

- Procedure include dilution and spreading or pouring of soil samples that give 300 or 400 or more colonies per plate

- Colonies producing antibiotic activity are indicated by an area of agar around the colony

- Such a colony is sub-cultured to a similar medium and purified by streaking, before making stock cultures. The purified culture is then tested to find what types of microorganisms are sensitive in the presence of these the antibiotics i.e. "Microbial Inhibition Spectrum" (MIS).

- The crowded plate procedure also does not necessarily select an antibiotic producing microorganism, because the inhibition area around the colony sometimes can be due to other reason like….

(1) Marked change in the pH of the medium resulted due the metabolism of the colony.
(2) Rapid utilization of critical nutrients in the vicinity of the colony etc.

- Thus further testing is required to confirm the inhibitory activity associated with a microorganisms is whether attributed to the presence of an antibiotic or not

- Screening of antibiotic producing microorganisms can be improved by using a "test organism" and Wilkins method

C. Primary screening of extracellular metabolites (Vitamins, Amino acids and Growth factors) producing microorganisms

Auxanography:

This technique is largely employed to for detecting microbes able to produce growth factors extracellularly. The two major steps are:

a)Preparation of first plate:

A filter paper strip is put across the bottom of petridish.
The nutrient agar is prepared and poured on the paper disc and allowed to solidify.
Soil sample is diluted and proper dilutions are inoculated and incubated.

b)Preparation of second plate:

A minimal medium lacking the growth factor is prepared and seeded with the test organism.
The seeded medium is poured onto a fresh petridish and the plate is allowed to set.
The agar in the first plate is then carefully lifted with the spatula and placed on the second plate without inverting. The growth factors produced by the colonies present on the surface of the first layer of agar can diffuse into the lower layer containing the test organism. The zones of stimulated growth of the test organism around the colonies Is an indication that the organism produce growth factor extracellularly.

D. Primary screening of microorganisms utilizing specific Carbon and Nitrogen sources

Enrichment culture technique:

This was first designed by Beijerinck to isolate the desired microorganism from a heterogenous microbial population
Nutrient broth is inoculated with the microbial source material and incubated.
A small portion of the inoculums is plated on to the solid medium and well isolated colonies are obtained
Suspected colonies from the plate are subcultured on fresh media and subjected for further testing.

Secondary screening
Secondary screening allows further sorting out of microorganisms obtained from PS having real value for industrial processes and discarding of those lacking this potential
1. SS is conducted on agar plates, in flasks or small fermenter containing liquid media

2. SS can be qualitative or quantitative in its approach

3. Secondary screening should give information about the evaluation of the true potential of the microorganisms for industrial usage

4. SS should determine whether microorganisms are actually producing new chemical compounds not previously described

5. SS should reveal whether there is pH, aeration or other critical requirements associated with particular microorganisms, both for the growth of the organism and for the formation of chemical products

6. SS should also detect gross genetic instability in microbial cultures

7. SS should show whether certain medium constituents are missing or possibly, are toxic to the growth of the organisms or its ability to accumulate fermentation products
8. SS should determine whether the product has a simple, complex, or even a macromolecular structure, if this information is not already available

9. SS should show something of the chemical stability of the product and of the product's solubility picture on various organic solvents

10. SS should show whether the product possesses physical properties such as UV light absorption or fluorescence or chemical properties that can be employed to detect the compound during the use of paper chromatography or other analytical methods and which also might be of value in predicting the structure of the compound

11. In some case, for certain kinds of fermentation product determinations should be made as to whether gross animal, plant or human toxicity can be attributed to the fermentation product, particularly if it is utilized (as are antibiotics) in disease treatment

12. SS should reveal whether a product resulting from a microbial fermentation occurs in the culture broth in more than one chemical form and whether it is an optically or biologically active material

13. SS should reveal whether the microorganisms are able to chemically alter or even destroy their own fermentation products

14. Secondary screening helps in predicting the approaches to be utilized in conducting further research on the microorganisms and its fermentation processes.

Example: antibiotic producing *Streptomyces* sp is taken.
Streptomycal isolate is streaked as a narrow band on nutrient agar plates and plates are incubated.
Test organisms are then streaked from the edge of the plates without touching the streptomycal isolate and the plates are then incubated.
At the end of incubation, growth inhibitory zones for each organisms are measured in millimeters.
Such organisms are again subjected to further testing by growing the culture in sterilized liquid media and incubated at constant temperature in a mechanical shaker.
Samples are withdrawn at regular intervals under aseptic condition and are tested in quality control laboratory.
The tests to be done include:
i)checking for contamination
ii)cheking for pH
iii)estimation of critical nutrients
iv)assaying of the antibiotic.
Some other determinations include:
i)screening of fermentation media in which high yield is obtained.
ii)determining whether the antibiotic is new
iii)determination of number of antibiotics accumulated in the broth.
iv)toxicity tests are to be done in mice.
v)thestreptomycete is characterized and is classified into species.

The Preservation of Industrially Important Microbes

Introduction

Microbes are required for the production of fermentation products. They are very valuable for specific product. Not all the microbes will give one product produced efficiently by specific microbe.
The isolation of a desired organism for a fermentation process may be time consuming and very expensive procedure and it is therefore essential that it retain the desirable characteristics that led to its selection. In addition, the culture used for the fermentation process should remain viable and free from contamination. Thus, industrial cultures must be preserved and maintained in such way as to eliminate genetic change, protect against contamination, and retain viability.
Different techniques are used for maintenance and preservation of different organisms based on their properties. Selected method should also conserve the properties of the organisms.
Techniques for the Preservation of microbes broadly divided into two

1. Methods where organisms are in Continuous metabolic active state

2. Methods where organisms are in Suspended metabolic state

1. Continuous metabolic active state preservation technique

In this technique, organisms preserved on nutrient medium by repeated sub-culturing. In this technique, any organisms are stored by using general nutrient medium. Here repeated sub-

culturing is required due to depletion or drying of nutrient medium. This technique includes preservation by following methods.

1.1 Periodic transfer to fresh media

Organisms grown in general media on slant, incubated for particular period at particular temperature depending on the characteristics of the selected organisms, then it is stored in refrigerator. These cultures can be stored for certain interval of time depending on the organism and its growth conditions. After that time interval, again these organisms transferred to new fresh medium and stored in refrigerator.

1.2 Overlaying culture with mineral oil

Organisms are grown on agar slant then they are covered with sterile mineral oil to a depth of 1 cm. above the tip of the surface. This method is simple; one can remove some organisms in aseptic condition with the help of sterile wire loop and still preserving the initial culture. Some species preserved satisfactorily for 15 – 20 years by this method.

1.3 Storage in sterile soil

This method is widely used for preserving spore forming bacteria and fungi. In this method, organisms will remain in dormant stage in sterile soil. Soil sterilized then spore suspension added to it aseptically, this mixture dried at room temperature and stored in refrigerator. Viability of organisms found around 70 – 80 years.

1.4 Saline suspension

Normal Saline used to provide proper osmotic pressure to organism's otherwise high salt concentration is inhibitory for organisms. Organisms kept in screw cap bottles in normal saline, stored at room temperature, wherever required transfer made on agar slats, and incubated.

2 Methods where organisms are in Suspended metabolic state

Organisms preserved in suspended metabolic state by either drying or storing at low temperature. Microbes are dried or kept at low temperature carefully so that their revival is possible.

2.1 Drying in vacuum

In this technique, organisms dried over chemical instead of air dry. Cells passed over $CaCl_2$ in a vacuum and then stored in refrigerator. Organisms survive for longer period.

2.2 Lyophilization

Lyophilization is vacuum sublimation technique. Cells grown in nutritive media and then this culture distributed in small vials. These vials culture then immersed in a mixture of dry ice and alcohol at -78oC. These vials immediately connected to a high-vacuum line, and when they are completely dried, each vial sealed under vacuum. This is most effective and widely used technique due to long time survival, less opportunity for changes in characteristics of organisms and small storage area. Organisms can survive for period of 20 years or more.

2.3 Use of Liquid nitrogen

Microorganisms grown in nutritive media and then this culture frozen with Cryoprotective agents like Glycerol and Dimethyl Sulfoxide. Frozen culture kept in liquid Nitrogen refrigerator. Organisms can remain alive for longer period.

2.4 Storage in silica gel

Both bacteria and yeast stored by this method. By this technique, organisms can survive for 1 – 2 years. Finely Powdered Heat sterilized Silica powder mixed with thick suspension of cell at low temperature.

Note:

- Cells should be harvested when actively growing (mid logarithmic phase)
- One method used for few organisms or specific organism; not all the organisms preserved by any one technique mentioned above.

Quality control of the preserved stock culture

Whichever technique used for the preservation and maintenance of industrially important organisms it is essential to check the quality of the preserved organisms stocks. Each batch of newly preserved cultures routinely checked to ensure their quality. A single colony transferred into a shake-flask to ensure growth of particular kind of microorganism; further shake-flask subculture used for the preparation of huge quantity of vials. For the assessment of purity, viability, and productivity of cultures, few vials are tested. If samples fail any one of these tests, the entire batch destroyed. Thus, by the use of such a quality-control system stock cultures retain and used with confidence.

Strain Improvement

Use of high yielding strain is the most critical factor. Therefore strains require improvement and this is accomplished by producing mutant fermentation strains with the help of physical/chemical methods and by using recombinant DNA technology.

Mutation for strain improvement:

Mutants formed by mutation are grouped into 2 categories:

i) auxotrophic mutants &

ii)mutants resistant to analogues.

Microorganisms usually have regulatory mechanisms that control the amount of metabolites synthesized, therefore suppression of regulatory mechanisms is necessary to develop the strains for higher yields.

Microbial cultures which have multivalent mechanisms, concerted repression or feed-back inhibition may be used for strain development

Mutants which have lost the ability to synthesize one of the end product capable of feed-back inhibition or repression is selected.

Different types of industrially important mutants have been summarized: 1)A mutant strain of *Corynebacteriumglutamicum*can excrete about 60g of lysine/l in a medium based on glucose & minerals. This mutant strain needs homoserine. On the other hand wild strain does not need homoserine& fails to excrete lysine.

2)There are some mutant strains with enzymes that offer resistance to feed back control. A mutant strain produced, may have the enzyme with an altered regulatory site, such altered regulatory site fails to interact with the inhibitor.

3)Use of an analogue in selection of industrially important strains:

An analogue can interact with the regulatory site associated with feed back inhibition. Such an analogue exerts toxic effect. This toxicity eliminates all sensitive mutant cells in population. Eg: α-amino, β hydroxyl valeric acid is analogue of threonine. Selection of a mutant strain using this antimetabolite is done in 2 stages:

a)The analogue of an aminoacid, threonine is added during preparation of a nutrient agar & poured onto a sterile petridish& allowed to solidify & a wedge has been set. When the wedge has set, a second layer of the same medium, without analogue is poured onto it & allowed to

set. After sometime, diffusion of an analogue into upper layer of the medium takes place. As a result a concentration gradient is developed at the surface. Now a culture previously treated with a mutagen is spread on the surface of this medium and selection of any mutants offering resistance to high concentrations is done.

b)It is also important to find out resistant mutants capable of producing threonine. This is accomplished by inoculating the mutants as point cultures, onto an agar medium seeded with a threonine dependent culture. Growth of seeded culture (ie, threonine requiring culture) around each colony of threonine excreting mutant strain may occur. Diameter of the zone of seeded culture growth depends on the quantity of threonine produced by mutants. Thus analogue resistant mutants excreting higher yields of threonine may be obtained. Using this technique, mutant strain of *Brevibacteriumflavum*capable of excreting threonine upto 12.6g/l is obtained.

4)Revertants from non-producing strains are high producers. Eg: a reversion mutant of *Streptomyces viridifaciens*showed 6 fold increase in the production of chlortetracycline over the original strain.

5)Reversion mutants of appropriate auxotrophs may be high producers. Eg: in case of *S.viridifaciens*reversion mutants of an auxotrophic mutant requiring homocysteine showed 28% more chlortetracycline.

6)Selection for resistance to the antibiotic produced by the organism itself may lead to increased yields. Eg: *Streptomyces aureofaciens*mutants selected for resistance to 200-400 mg/l chlortetracycline showed a 4 fold increase in production of this antibiotic.

7)Mutants with altered cell membrane permeability show high production of some metabolites. A mutant *E.coli*strain has defective lysine transport; it actively excretes L-lysine into the medium to 5-times high in concentration.

8)Mutants have been selected to produce altered metabolites, especially in case of aminoglycoside antibiotics. For eg: *Pseudomonas aureofaciens*produces the antibiotic pyrrolnitrin; a mutant of this organism yields 4'-fluoropyrrolnitrin.

Mutant selection has been the most successful approach for strain improvement, but major advances are made in r-DNA technology.

Recombinant DNA technology for strain improvement:

This technique has been used to achieve the following 2 broad objectives:

(i)production of recombinant proteins, and

(ii)modification of the organism's metabolic pattern for the production of new, modified or more quantity of metabolites.

Recombinant Proteins: These are the proteins produced by the transferred gene or transgene; they themselves are of commercial value. Eg: insulin, interferon, etc.,

Metabolic engineering: When metabolic activities of an organism are modified by introducing transgene; it affects enzymatic, transport and/or regulatory function of its cells, it is known as "metabolic engineering". Various approaches are summarized below:

1)A transgene may be added, which encodes an enzyme to modify a metabolite produced by the organism to yield a new product of interest. Eg: *Acremoniumchrysogenum*produces cephalosporin C. The gene encoding D-amino acid oxidase from *Fusarium*was introduced into the former. This enzyme converts cephalosporin C into 7-amino cephalosporanic acid, a precursor of several semisynthetic antibiotics.

2)The enzyme encoded by transgene may enable a better utilization of the substrate or even the previously inaccessible components of the substrate. Eg: normal yeasts are unable to utilize cyclodextrinspresent in malt; this increases the calorie content of the beer. Transgenic yeasts capable of utilizing cyclodextrins are now commercially used to produce low calorie beer with 1% more alcohol content.

3)All the genes of an entirely new biosynthetic pathway may be transferred to generate new products. Eg: 2 genes are involved in conversion of acetyl-CoA to PHB, which is used to produce biodegradable plastic. The 2 genes were transferred into *E.coli*from *Alcaligeneseutrophus.* Transgenic *E.coli,* under appropriate conditions, accumulate PHB to upto 50% of their dry weight.
4)Several gene transfers have enhanced growth rates of the organisms, reduced their nutrient requirements and enabled their growth to higher cell densities. Eg: transfer of gene encoding glutamate dehydrogenase from *E.coli*to glutamate synthase deficient mutants of *Methylophilusmethylotrophus*increased the efficiency of carbon conversion from *4% - 7%*.
5)In some cases conversion of an intermediate product to the end product is slow due to low activity of the rate-limiting enzyme. In such cases the activity of rate limiting enzyme can be increased by increasing its dosage. Eg: in case of C.acremonium the enzyme (encoded by the gene *cef*EF) that converts penicillin N intermediate in the cephalosporin C biosynthesisis rate limiting. The dosage of *cef*EF was increased leading to a 15% higher cephalosporin C yield.

Fermentation Media

In a fermentation process, the choice of the most optimum micro-organisms and fermentation media is very important for high yield of product. The quality of fermentation media is important as it provides nutrients and energy for growth of micro-organisms. This medium provides substrate for product synthesis in a fermentor.

Fermentation media consists of major and minor components.

- Major components include Carbon and Nitrogen source.
- Minor components include inorganic salts, vitamins, growth factors, anti-foaming agents, buffers, dissolved oxygen, other dissolved gases, growth inhibitors and enzymes.

Nutrients required for fermentation media also depend upon the type of fermentation organisms as well as the type of fermentation process to be used. Poor choice of fermentation media might result in poor yield of output. Types of nutrients present in the fermentation media always determine the yield of the product.

There are two uses of fermentation media

1. Growth media
2. Fermentation media

Growth medium contains low amounts of nutrients.It is useful in creating raw material for further fermentation processes.

Fermentation media contains high amounts of nutrients. It is used in creating final products using fermentation.

For example, growth of yeast requires 1% carbon. But during fermentation of alcohol, yeast requires 12 to 13 % carbon in the medium.

What is the role of Fermentation Media?

During the fermentation process, media contains high amounts of nutrients, micro-organism and optimum conditions. When these micro-organisms are incubated at the desired optimum conditions, they enjoy luxurious metabolism. Here, the fermentation organisms become hyperactive due to presence of high quantities of nutrients, thus it results in consumption of excess nutrients and partial degradation of fermentation media. The waste effluents excreted by the microbes could be the desired output product of the fermentation process.

The amount of substrate given to microbes should not reach inhibitory concentration levels because excess substrate inhibits vital enzymes and may results in death of cells. Also, water present in cytoplasm is important for metabolism process. If excess sugar or salt is available in the fermentation media, it would tie up cytoplasm water and may result in lack of water for metabolism and cause death of microbes, thus affecting fermentation output.

Excess substrate may increase osmotic pressure and effect enzyme activities in a cell. Microbes excrete this excess substrate in the form of partially digested fermentation media. It is converted to an insoluble inert compound in the form of reserve food material and this reserve food material is harmless to cells.

The media used for the growth of microorganisms in industrial fermentation must contain all the elements in a suitable form for the synthesis of cellular substances as well as the metabolic products. While designing a medium, several factors must be taken into consideration. The most important among them is the ultimate product desired in the fermentation.

For growth-linked products (primary metabolites e.g. ethanol, citric acid), the product formations is directly dependent on the growth of the organisms, hence the medium should be such that it supports good growth. On the other hand, for products which are not directly linked to the growth (secondary metabolites e.g. antibiotics, alkaloids, gibberellins), the substrate requirements for product formation must also be considered.

In the laboratory, pure defined chemicals may be used for culturing microorganisms. However, for industrial fermentations, undefined and complex substrates are frequently used for economic reasons. Cheaper substrates are advantageous since they minimize the production cost of the fermented products.

Wastes from agriculture, and byproducts of other industries are generally preferred, although they are highly variable in composition. Raw materials used in fermentation largely depend on their cost at a particular time, since there are seasonal variations.

The choice of the medium is very critical for successful product formation. For industrial fermentation, the microorganisms, in general, utilize a luxury metabolism. Therefore, good production yields are expected with an abundant supply of carbon and nitrogen sources, besides requisite growth factors. The media used in fermentation processes may be synthetic or crude.

There are two types of fermentation media used in industries.

1. Synthetic media
2. Crude media

1. **Synthetic media**

Media with all the requisite constituents in a pure form in the desired proportion represents synthetic media. Use of this type of media in fermentations is not practicable.

Synthetic media is useful in the field of research as each and every component is chemically known and the exact composition of nutrients is predetermined. So, in case of synthetic media, variation in levels and concentration of nutrients can be controlled. Here, by experimentation with synthetic media, the effect of nutrients on growth and yield of product

can be analysed. We can redesign the synthetic media as per our needs. It is very useful in controlling the growth and yield of product in a lab environment. We can also use it to determine the metabolic pathway used in the synthesis of products.

With the help of radio-isotope labelling technique, we can determine the main ingredients that gets used up to create the final desired product. In this way, we can know the exact proportions of ingredients required for our process. We can optimise this process by using alternative sources of carbon or nitrogen, and creating a fermentation media which is the most optimum for our needs. The use of Synthetic media allows us to experiment with various sources of fermentation media in the lab as the results are accurately reproducible for a given composition.

An advantage of a welldesigned synthetic media is that it lacks sources of protein and peptides. Hence, there is no foam formation, and chances of contamination are very less. Product recovery is easier because synthetic media contains pure components.

Although there is a big list of advantages of synthetic media, there are some disadvantages. A major disadvantage is the cost of media. The most important aspect of fermentation is that it should be economic and profitable. Synthetic media is never used on industrial scale because it is expensive. This process in only suitable for experimentation in a lab on a small scale.

2. **Crude Media**

The non-synthetic media with naturally available sources are better suited for fermentation.

In practice, crude media with an addition of requisite synthetic constituents is ideal for good product yield in fermentation.

The most frequently used substrates for industrial fermentation with special reference to the supply of carbon and nitrogen sources and growth factors are briefly described below.

Crude media is generally used on an industrial scale for fermentation process. Crude media contains a rough composition of media required for fermentation. It gives high yield of product and contains undefined sources of ingredients. Crude media contains high level of nutrients, vitamins, proteins, growth factors, anti-foaming agents and precursors. It is important to ensure that crude media should not contain toxic substances that could effect the growth of bacteria and yield of product.

Ingredients of Crude Media

1) Inorganic nutrients

Crude media contains inorganic salts containing cations and anion along with a carbon source. Sometimes, fermentation micro-organisms have a specific requirement of ions like magnesium ions, phosphates or sulphates. These requirements are fulfilled by addition of these ions to balance the crude media.

2) Carbon source

Simple to complex carbohydrates can be added to media as a source of carbon. We can add different sugars like mannitol, sorbitol, organic acids, fatty acids, proteins, peptides we can choose any of these as a source of carbon. The selection of carbon source depends upon the

availability as well as the cost of raw material. In most of the fermentation media, crude source of carbon is added.

1. **Simple carbohydrates** – simple sugars are semi purified polysaccharides and sugar alcohol are added. Sources of simple carbohydrates are Black strap molasses, Corn molasses, Beet molasses, sulphite waste liquor, Hydrol (corn sugar molasses), Cannery waste.
2. **Complex carbohydrates** – Source of complex carbohydrates are Starch, Corn, Rice, Rye, Milo, wheat potatoes etc. Source of starch cellulose are corn cobs, straws, wood waste, saw meal etc.

Substrates Used As Carbon Sources:

Carbohydrates constitute the most predominant source of energy in fermentation industry. Refined and pure carbohydrates such as glucose or sucrose are rarely used for economic reasons.

Molasses:

Molasses is a byproduct of sugar industry and is one of the cheapest sources of carbohydrates. Molasses are concentrated syrups or mother liquors recovered at any one of several steps in sugar refining process with different names depending on the step from which it is recovered. Blaskstrap molasses from sugar cane is normally the cheapest and most used sugar source for industrial fermentation. Sugar cane molasses (sucrose around 48%) and sugar beet molasses (sucrose around 33%) are commonly used. Besides being rich in sugar, molasses also contain nitrogenous substances, vitamins and trace elements.

There occurs variation in the composition of the molasses which mostly depends on the climatic conditions and production process. Hydrol molasses, a byproduct in glucose production from corn, is also used as a fermentation substrate.

Malt extract:

Malt extract, an aqueous extract of malted barley, contains about 80% carbohydrates (glucose, fructose, sucrose, and maltose). Nitrogen compounds constitute around 4.5% (proteins, peptides, amino acids, purines, pyrimidine's). Malt extract are useful carbon sources for the cultivation of filamentous fungi, yeast and actinomycetes.

Starch, dextrin and cellulose:

The polysaccharides-starch, dextrin and cellulose are not as readily utilized as monosaccharides and disaccharides, but can be directly metabolised by amylase producing microorganisms, particularly filamentous fungi. Their extracelluar enzymes hydrolyze the substrate to a mixture of glucose, maltose or maltotriose to produce a sugar spectrum similar to that found in many malt extracts. They are frequently used for the industrial production of alcohol. Due to its wide availability and low cost, the use of cellulose for alcohol production is extensively studied.

Sulfite Waste Liquor**or spent pulping** liquor**:**

Sulfite waste liquor is derived from paper pulping industry after wood for paper manufacture is digested to cellulose pulp.The Sulfurous acid (SO_2 in water) and its salts of Ca, Na, Mg, and NH_4 used in the **sulfite** pulping cause bydrolysis of the more easily hydrolyzable components of raw materials like hemi cellulose It can be used as a dilute fermentation medium for ethanol production by S.cerivisiae and the growth of Torulautilis

for feed. Waste liquors from coniferous trees contain 2-3 sugar, which is a mixture of hexoses (80%) and pentoses (20%)..

Whey:
Whey is a byproduct of dairy industry and is produced worldwide. Most of it is consumed by- humans and animals. Whey is a reasonably good source of carbon for the production of alcohol, single-cell protein, vitamin B_{12}, lactic acid and gibberellic acid. Storage of whey is a limiting factor for its widespread use in fermentation industry.

Methanol and ethanol:
Some of the microorganisms are capable of utilizing methanol and/or ethanol as carbon source. Methanol is the cheapest substrate for fermentation. However, it can be utilized by only a few bacteria and yeasts. Methanol is commonly used for the production of single-cell protein. Ethanol is rather expensive. However, at present it is used for the production of acetic acid.

3) Nitrogen source
Salts of urea, ammonia, and nitrate can be used as a nitrogen source. When fermentation organisms are non-proteolytic in nature, pure form of urea, ammonia and nitrate are used as a source of nitrogen. When fermentation organisms are proteolytic in nature, animal and plant raw material is used; like distillery dried solubles, Casein, Cereal grains, peptones, yeast extract, hydrolysate, and soybean meal etc.

Substrates Used As Nitrogen Sources:
The nitrogen supply to the fermentation microorganisms may come from inorganic or organic sources.

Inorganic nitrogen sources:
Ammonium salts (ammonium sulphate and diammonium hydrogen phosphate) and free ammonia are cheap inorganic nitrogen sources, particularly in industrialised countries. Ammonia can also be used to adjust pH of the fermentation. However, not all the microorganisms are capable of utilizing them, hence their use is limited.

Organic nitrogen sources:
Urea is fairly a good source of nitrogen. However, other cheaper organic forms of nitrogen sources aminoacids and proteins are preferred.

Corn steep liquor:
This is formed during starch production from corn. Corn steep liquor is rich in nitrogen (about 4%) and is very efficiently utilized by microorganisms. Concentrated extracts generally contain about 4%(w/v) nitrogen, includingseveral amino acids (alanine, valine, methionine, arginine, threonine, glutamate). Its first use in fermentations was for penicillin production in the 1940s. The extract composition of the liquor varies depending on the quality of the corn and the processing conditions.

Yeast extracts:
They contain about 8% nitrogen and are rich in amino acids, peptides and vitamins. Glucose formed from glycogen and trehalose during yeast extraction is a good carbon source. Yeast extracts are produced from baker’s yeast through autolysis (at 50-55°C) or through

plasmolysis (high concentration of NaCI). Yeast extracts are very good sources for many industrially important microorganisms.

Soy meal:
After extracting the soy bean oil from the soy bean seeds, the left out residue is soy meal. It is rich in proteins (about 50%) as well as carbohydrates (about 30%) contents. Soy meal is often used in antibiotic production.

Peptones or protein hydrolysates:
The protein hydro-lysates are collectively referred to as peptones, and they are good sources for many microorganisms. They are prepared by acid or enzyme hydrolysis of peptones include meat, soy meal, peanut seeds, cotton seeds and sunflower seeds, casein, gelatin, keratin.

The proteins namely casein, gelatin and keratin can also be hydrolysed to yield peptones. In general, peptones derived from animal sources have more nitrogen content while those from plant sources have more carbohydrate content. Peptones are relatively more expensive, hence not widely used in industries.

4) Growth factors
Crude media constituents provides enough amount of growth factors so no extra addition of growth factor is required. If there is a lack of any kind if vitamins or nutrients, growth factors can be added to media. Examples are yeast extract, and beef extract.

Sources of Growth Factors:
Some of the microorganisms are not capable of synthesizing one or more growth factors such as vitamins. These growth factors are very expensive in pure form, hence crude sources are preferred. Yeast extract is a rich source of almost all growth factors. Generally, the substrates derived from plant or animal sources in a crude form are reasonably rich in mineral content. Sometimes, however mineral (phosphate, sulfate) supplementation may be required.

5) Precursors
Precursors are generally present in the media as crude constituents. Precursors are added in the fermentation media at time of fermentation as it get incorporated in the molecules of product without bringing any kind of change to the final product. This helps in improving yield and quality of product. Sometimes, precursors are added in pure form depending upon the need of product. For example, Cobalt chloride is added less than 10 ppm in fermentation of vitamin B12.

6) Buffers
Buffers are used to control drastic changes of pH. Sometimes, media components may act as buffers. For example, protein, peptides, mino-acids act as good buffers at neutral pH. Sometimes inorganic buffers like K_2HPO_4, KH_2PO_4, and $CaCO_3$ etc, can be added as required. Generally, during the fermentation process, pH changes to acidic or alkaline pH. The cheapest and easily available buffer is $CaCO_3$.

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SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

UNIT –IV – INDUSTRIAL MICROBIOLOGY – SMB2204

SMB2204	INDUSTRIAL MICROBIOLOGY	L	T	P	Credits	Total Marks
		3	1	0	4	100

COURSE OBJECTIVES

- The objective of this course to enable students to correlate metabolic aspects of industrially relevant microorganisms with the corresponding biotechnological products, and learn the basics of simple strategies for strain improvements encompassing both classical and metabolic engineering methods, design medium and downstream processing.

UNIT 1 HISTORY **12 Hrs.**

Brief history and developments in industrial microbiology Types of fermentation processes - Solid-state and liquid-state (stationary and submerged) fermentations; batch, fed-batch (Eg. Baker's yeast) and continuous fermentations

UNIT 2 BIO-REACTOR **12 Hrs.**

Components of a typical bio-reactor, Types of bioreactors-Laboratory, pilot- scale and production fermenters, constantly stirred tank and air-lift fermenters, Measurement and control of fermentation parameters - pH, temperature, dissolved oxygen, foaming and aeration

UNIT 3 UPSTREAM PROCESSING **12 Hrs.**

Sources of industrially important microbes and methods for their isolation, preservation and maintenance of industrial strains, strain improvement, Crude and synthetic media; molasses, corn steep liquor, sulphite waste liquor, whey, yeast extract and protein hydrolysates

UNIT 4 DOWNSTREAM PROCESSING **12 Hrs.**

Cell disruption, filtration, centrifugation, solvent extraction, precipitation, lyophilization and spray drying. Citric acid, ethanol, penicillin, glutamic acid, Vitamin B12 Enzymes (amylase, protease, lipase)
Wine, beer

UNIT 5 IMMOBILIZATION **12 Hrs.**

Methods of immobilization, advantages and applications of immobilization, large scale applications of immobilized enzymes (glucose isomerase and penicillin acylase)

Max Hours.60

TEXT / REFERENCE BOOKS

1. Patel A.H. (1996). Industrial Microbiology. 1st edition, Macmillan India Limited
2. Okafor N. (2007). Modern Industrial Microbiology and Biotechnology. 1st edition. Bios Scientific Publishers Limited. USA
3. Waites M.J., Morgan N.L., Rockey J.S. and Higton G. (2001). Industrial Microbiology: An Introduction. 1st edition. Wiley – Blackwell
4. Glaze A.N. and Nikaido H. (1995). Microbial Biotechnology: Fundamentals of Applied Microbiology. 1st edition. W.H. Freeman and Company
5. Casida LE. (1991). Industrial Microbiology. 1st edition. Wiley Eastern Limited.
6. Crueger W and Crueger A. (2000). Biotechnology: A textbook of Industrial Microbiology. 2nd edition. Panima Publishing Co. New Delhi.
7. 7. Stanbury PF, Whitaker A and Hall SJ. (2006). Principles of Fermentation Technology. 2nd edition, Elsevier Science Ltd..

END SEMESTER EXAMINATION QUESTION PAPER PATTERN

Max. Marks : 100 **Exam Duration : 3 Hrs.**
PART A : 10 questions of 2 marks each - No choice **20 Marks**
PART B : 2 questions from each UNIT of internal choice; each carrying 16 marks **80 Marks**

UNIT: IV
DOWNSTREAM PROCESSING

The five stages are:
(1) Solid-Liquid Separation (2) Release of Intracellular Products (3) Concentration (4) Purification by Chromatography and (5) Formulation

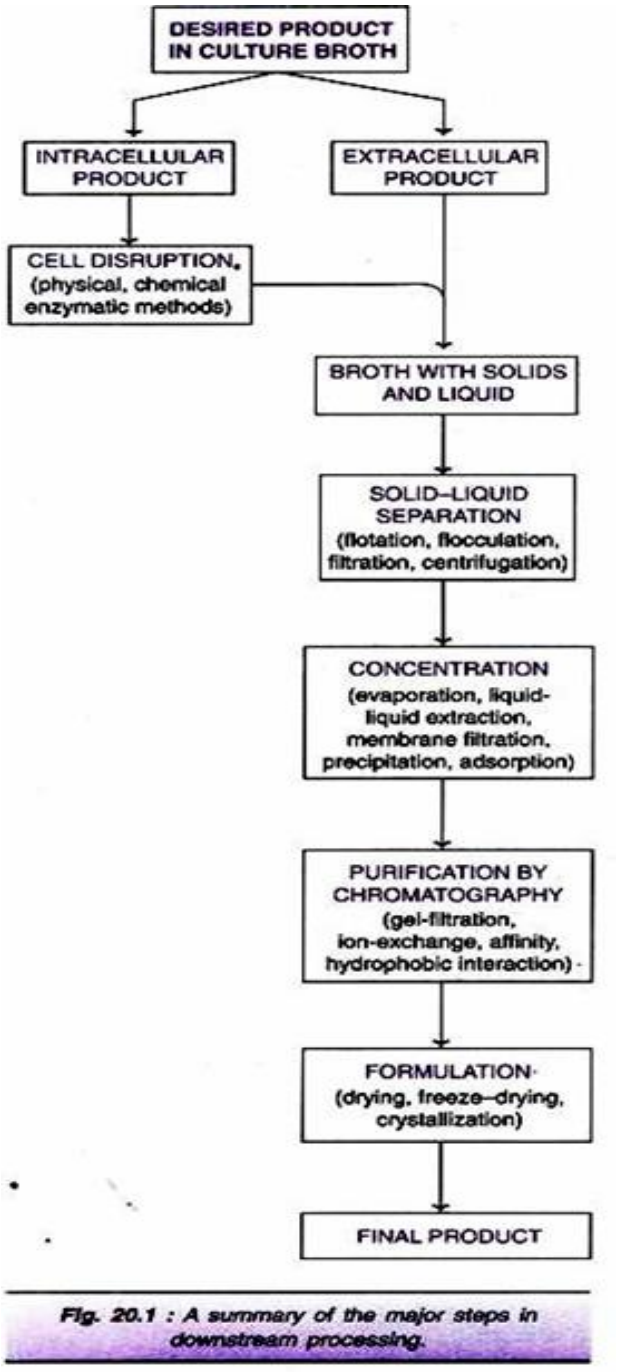


Fig. 20.1 : A summary of the major steps in downstream processing.

Stage # 1. Solid-Liquid Separation:

The first step in product recovery is the separation of whole cells (cell biomass) and other insoluble ingredients from the culture broth (Note: If the desired

product is an intracellular metabolite, it must be released from the cells before subjecting to solid-liquid separation). Some authors use the term harvesting of microbial cells for the separation of cells from the culture medium. Several methods are in use for solid-liquid separation. These include flotation, flocculation, filtration and centrifugation.

Flotation:

When a gas is introduced into the liquid broth, it forms bubbles. The cells and other solid particles get adsorbed on gas bubbles. These bubbles rise to the foam layer which can be collected and removed. The presence of certain substances, referred to as collector substances, facilitates stable foam formation e.g., long chain fatty acids, amines.

Flocculation:

In flocculation, the cells (or cell debris) form large aggregates to settle down for easy removal. The process of flocculation depends on the nature of cells and the ionic constituents of the medium. Addition of flocculating agents (inorganic salt, organic polyelectrolyte, mineral hydrocolloid) is often necessary to achieve appropriate flocculation.

CELL DISRUPTION METHODS

- As already stated, there are several biotechnological products (vitamins, enzymes) which are located within the cells. Such compounds have to be first released (maximally and in an active form) for their further processing and final isolation. The microorganisms or other cells can be disintegrated or disrupted by physical, chemical or enzymatic methods

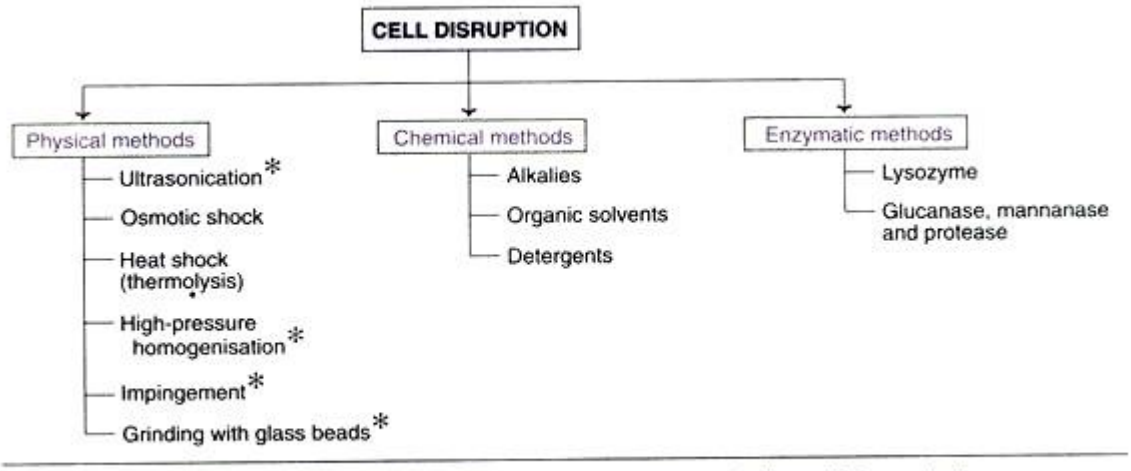


Fig. 20.5 : Major methods for cell disruption to release the intracellular products (indicate mechanical methods while all the remaining are non-mechanical).*

The selection of a particular method depends on the nature of the cells, since there is a wide variation in the property of cell disruption or breakage. For

instance, Gram-negative bacteria and filamentous fungi can be more easily broken compared to Gram-positive bacteria arid yeasts.

Cell Disruption:

Physical methods of cell disruption:

The microorganisms or cells can be disrupted by certain physical methods to release the intracellular products.

Ultra sonication:

Ultrasonic disintegration is widely employed in the laboratory. However, due to high cost, it is not suitable for large-scale use in industries.

Osmotic shock:

This method involves the suspension of cells (free from growth medium) in 20% buffered sucrose. The cells are then transferred to water at about 4°C. Osmotic shock is used for the release of hydrolytic enzymes and binding proteins from Gram-negative bacteria.

Heat shock (thermolysis):

Breakage of cells by subjecting them to heat is relatively easy and cheap. But this technique can be used only for a very few heat-stable intracellular products.

High pressure homogenization:

This technique involves forcing of cell suspension at high pressure through a very narrow orifice to come out to atmospheric pressure. This sudden release of high pressure creates a liquid shear that can break the cells.

Impingement:

In this procedure, a stream of suspended cells at high velocity and pressure are forced to hit either a stationary surface or a second stream of suspended cells (impinge literally means to strike or hit). The cells are disrupted by the forces created at the point of contact. Micro fluidizer is a device developed based on the principle of impingement. It has been successfully used for breaking E. coli cells. The advantage with impingement technique is that it can be effectively used for disrupting cells even at a low concentration.

Grinding with glass beads:

The cells mixed with glass beads are subjected to a very high speed in a reaction vessel. The cells break as they are forced against the wall of the vessel by the beads. Several factors influence the cell breakage-size and quantity of the glass beads, concentration and age of cells, temperature and agitator speed. Under optimal conditions, one can expect a maximal breakage of about 80% of the cells.

A diagrammatic representation of a cell disrupter employing glass beeds is shown in Fig. 20.6. It contains a cylindrical body with an inlet, outlet and a central motor-driven shaft. To this shaft are fitted radial agitators. The cylinder is fitted with glass beads. The cell suspension is added through the inlet and the disrupted cells come out through the outlet. The body of the cell disrupter is kept cool while the operation is on.

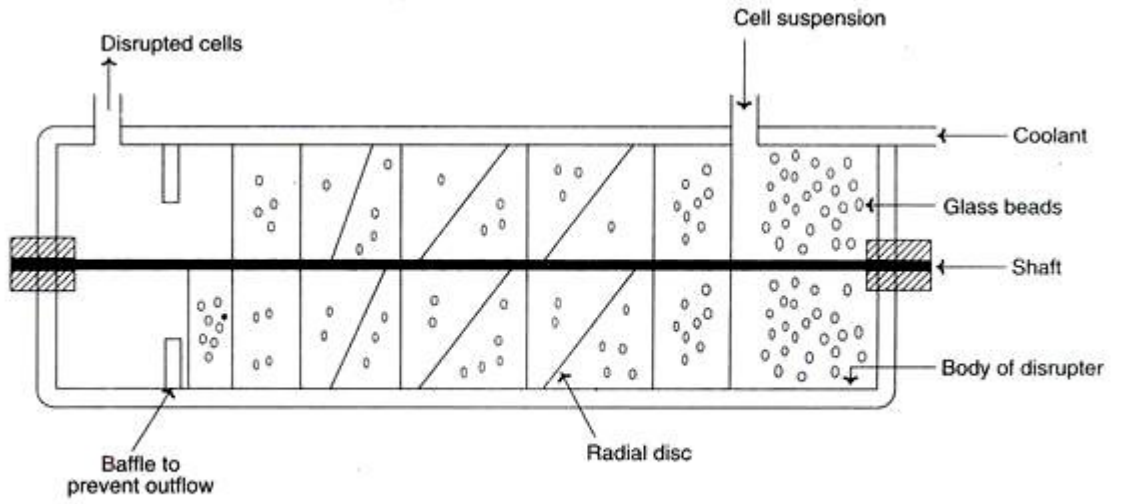


Fig. 20.6 : Diagrammatic representation of a cell disrupter.

Mechanical and non-mechanical methods:

Among the physical methods of cell disruption described above, ultra sonication, high-pressure homogenization, impingement and grinding with glass beads are mechanical while osmotic shock and heat shock are non-mechanical. The chemical and enzymatic methods (described below) are non- mechanical in nature.

Chemical methods of cell disruption:

Treatment with alkalies, organic solvents and detergents can lyse the cells to release the contents.

Alkalies:

Alkali treatment has been used for the extraction of some bacterial proteins. However, the alkali stability of the desired product is very crucial for the success of this method e.g., recombinant growth hormone can be efficiently released from E. coli by treatment with sodium hydroxide at pH 11.

Organic solvents:

Several water miscible organic solvents can be used to disrupt the cells e.g., methanol, ethanol, isopropanol, butanol. These compounds are inflammable; hence require specialised equipment for fire safety. The organic solvent toluene is frequently used. It is believed that toluene dissolves membrane phospholipids and creates membrane pores for release of intracellular contents.

Detergents:
Detergents that are ionic in nature, cationic-cetyltrimethyl ammonium bromide or anionic-sodium lauryl sulfate can denature membrane proteins and lyse the cells. Non-ionic detergents (although less reactive than ionic ones) are also used to some extent e.g., Triton X-100 or Tween. The problem with the use of detergents is that they affect purification steps, particularly the salt precipitation. This limitation can be overcome by using ultrafiltration or ion-exchange chromatography for purification.

Enzymatic methods of cell disruption:
Cell disruption by enzymatic methods has certain advantages i.e., lysis of cells occurs under mild conditions in a selective manner. This is quite advantageous for product recovery. Lysozyme is the most frequently used enzyme and is commercially available (produced from hen egg white). It hydrolyses β-1, 4-glycosidic bonds of the mucopeptide in bacterial cell walls. The Gram- positive bacteria (with high content of cell wall mucopeptides) are more susceptible for the action of lysozyme.

For Gram-negative bacteria, lysozyme in association with EDTA can break the cells. As the cell wall gets digested by lysozyme, the osmotic effects break the periplasmic membrane to release the intracellular contents. Certain other enzymes are also used, although less frequently, for cell disruption. For the lysis of yeast cell walls, glucanase and mannanase in combination with proteases are used.

Combination of methods:
In order to increase the efficiency of cell disintegration in a cost-effective manner, a combination of physical, chemical and enzymatic methods is employed.

FILTRATION
Filtration is the most commonly used technique for separating the biomass and culture filtrate. The efficiency of filtration depends on many factors— the size of the organism, presence of other organisms, viscosity of the medium, and temperature. Several filters such as depth filters, absolute filters, rotary drum vacuum filters and membrane filters are in use.

Depth Filters:
They are composed of a filamentous matrix such as glass wool, asbestos or filter paper. The particles are trapped within the matrix and the fluid passes out. Filamentous fungi can be removed by using depth filters.

Absolute Filters:

These filters are with specific pore sizes that are smaller than the particles to be removed. Bacteria from culture medium can be removed by absolute filters.

Rotary Drum Vacuum Filters:

These filters are frequently used for separation of broth containing 10-40% solids (by volume) and particles in the size of 0.5-10µm. Rotary drum vacuum filters have been successfully used for filtration of yeast cells and filamentous fungi. The equipment is simple with low power consumption and is easy to operate. The filtration unit consists of a rotating drum partially immersed in a tank of broth (Fig. 20.2). As the drum rotates, it picks up the biomass which gets deposited as a cake on the drum surface. This filter cake can be easily removed.

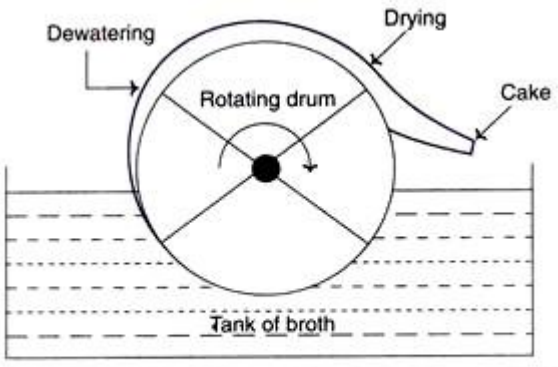


Fig. 20.2 : Diagrammatic representation of a rotary drum vacuum filter.

Membrane Filters:

In this type of filtration, membranes with specific pore sizes can be used. However, clogging of filters is a major limitation. There are two types of membrane filtrations—static filtration and cross-flow filtration (Fig. 20.3). In cross-flow filtration, the culture broth is pumped in a crosswise fashion across the membrane. This reduces the clogging process and hence better than the static filtration.

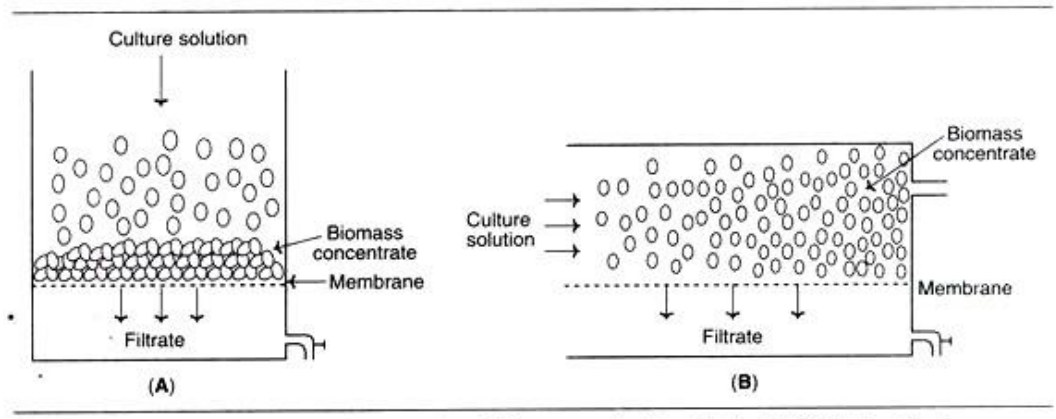


Fig. 20.3 : Filter systems for separation of biomass and culture filtrate (A) Static–flow filtration (B) Cross–flow filtration.

Types of filtration processes:

There are 3 major types of filtrations based on the particle sizes and other characters (Table 20.1). These are microfiltration, ultrafiltration and reverse osmosis.

TABLE 20.1 Major types of filtration processes with characteristic features

Type	Sizes of particles separated	Compound or particle separated
1. Microfiltration	0.1–10 μm	Cells or cell fractions, viruses.
2. Ultrafiltration	0.001–0.1 μm	Compounds with molecular weights greater than 1000 (e.g. enzymes).
3. Reverse osmosis (hyperfiltration)	0.0001–0.001 μm	Compounds with molecular weights less than 1000 (e.g. lactose).

CENTRIFUGATION

The technique of centrifugation is based on the principle of density differences between the particles to be separated and the medium. Thus, centrifugation is mostly used for separating solid particles from liquid phase (fluid/particle separation). Unlike the centrifugation that is conveniently carried out in the laboratory scale, there are certain limitations for large scale industrial centrifugation.

However, in recent years, continuous flow industrial centrifuges have been developed. There is a continuous feeding of the slurry and collection of clarified fluid, while the solids deposited can be removed intermittently. The different types of centrifuges are depicted in Fig. 20.4, and briefly described hereunder.

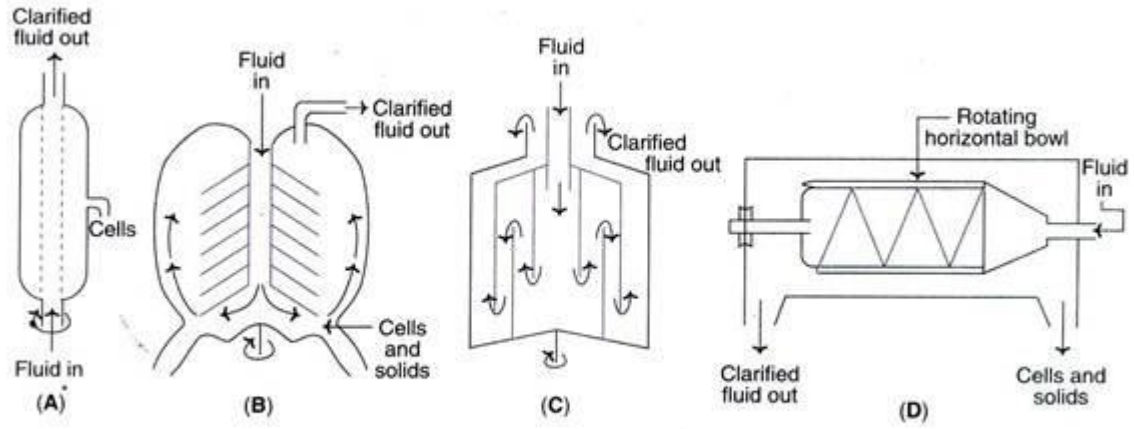


Fig. 20.4 : Centrifuges commonly used in downstream processing (A) Tubular bowl centrifuge (B) Disc centrifuge (C) Multichamber centrifuge (D) Scroll centrifuge (decanter).

Tubular bowl centrifuge (Fig. 20.4A):
This is a simple and a small centrifuge, commonly used in pilot plants. Tubular bowl centrifuge can be operated at a high centrifugal speed, and can be run in both batch or continuous mode. The solids are removed manually.

Disc centrifuge (Fig. 20.4B):
It consists of several discs that separate the bowl into settling zones. The feed/slurry is fed through a central tube. The clarified fluid moves upwards while the solids settle at the lower surface.

Multi-chamber centrifuge (Fig. 20.4C):
This is basically a modification of tubular bowl type of centrifuge. It consists of several chambers connected in such a way that the feed flows in a zigzag fashion. There is a variation in the centrifugal force in different chambers. The force is much higher in the periphery chambers, as a result smallest particles settle down in the outermost chamber.

Scroll centrifuge or decanter (Fig. 20.4D):
It is composed of a rotating horizontal bowl tapered at one end. The decanter is generally used to concentrate fluids with high solid concentration (biomass content 5-80%). The solids are deposited on the wall of the bowl which can be scrapped and removed from the narrow end.

CONCENTRATION

The filtrate that is free from suspended particles (cells, cell debris etc.) usually contains 80-98% of water. The desired product is a very minor constituent. The water has to be removed to achieve the product concentration. The commonly used techniques for concentrating biological products are evaporation, liquid-

liquid extraction, membrane filtration, precipitation and adsorption. The actual procedure adopted depends on the nature of the desired product (quality and quantity to be retained as far as possible) and the cost factor.

EVAPORATION*:*

Water in the broth filtrate can be removed by a simple evaporation process. The evaporators, in general, have a heating device for supply of steam, and unit for the separation of concentrated product and vapour, a condenser for condensing vapour, accessories and control equipment. The capacity of the equipment is variable that may range from small laboratory scale to industrial scale. Some of the important types of evaporators in common use are briefly described.

Plate evaporators:

The liquid to be concentrated flows over plates. As the steam is supplied, the liquid gets concentrated and becomes viscous.

Falling film evaporators:

In this case, the liquid flows down long tubes which gets distributed as a thin film over the heating surface. Falling film evaporators are suitable for removing water from viscous products of fermentation.

Forced film evaporators:

The liquid films are mechanically driven and these devices are suitable for producing dry product concentrates.

Centrifugal forced film evaporators:

These equipment evaporate the liquid very quickly (in seconds), hence suitable for concentrating even heat-labile substances. In these evaporators, a centrifugal force is used to pass on the liquid over heated plates or conical surfaces for instantaneous evaporation.

LIQUID-LIQUID EXTRACTION OR SOLVENT EXTRACTION

The concentration of biological products can be achieved by transferring the desired product (solute) from one liquid phase to another liquid phase, a phenomenon referred to as liquid-liquid extraction. Besides concentration, this technique is also useful for partial purification of a product.

The efficiency of extraction is dependent on the partition coefficient i.e. the relative distribution of a substance between the two liquid phases. The process of liquid-liquid extraction may be broadly categorized as extraction of low molecular weight products and extraction of high molecular weight products.

Extraction of low molecular weight products:

By using organic solvents, the lipophilic compounds can be conveniently extracted. However, it is quite difficult to extract hydrophilic compounds. Extraction of lipophilic products can be done by the following techniques.

Physical extraction:
The compound gets itself distributed between two liquid phases based on the physical properties. This technique is used for extraction of non-ionising compounds.

Dissociation extraction:
This technique is suitable for the extraction of ionisable compounds. Certain antibiotics can be extracted by this procedure.

Reactive extraction:
In this case, the desired product is made to react with a carrier molecule (e.g., phosphorus compound, aliphatic amine) and extracted into organic solvent. Reactive extraction procedure is quite useful for the extraction of certain compounds that are highly soluble in water (aqueous phase) e.g., organic acids.

Supercritical fluid (SCF) extraction:
This technique differs from the above procedures, since the materials used for extraction are supercritical fluids (SCFs). SCFs are intermediates between gases and liquids and exist as fluids above their critical temperature and pressure. Supercritical CO_2, with a low critical temperature and pressure is commonly used in the extraction. Supercritical fluid extraction is rather expensive, hence not widely used (SCF has been used for the extraction of caffeine from coffee beans, and pigments and flavor ingredients from biological materials).

Extraction of high molecular weight compounds:
Proteins are the most predominant high molecular weight products produced in fermentation industries. Organic solvents cannot be used for protein extraction, as they lose their biological activities. They are extracted by using an aqueous two-phase systems or reverse micelles formation.

Aqueous two-phase systems (ATPS):
They can be prepared by mixing a polymer (e.g., polyethylene glycol) and a salt solution (ammonium sulfate) or two different polymers. Water is the main component in ATPS, but the two phases are not miscible. Cells and other solids remain in one phase while the proteins are transferred to other phase. The distribution of the desired product is based on its surface and ionic character and the nature of phases. The separation takes much longer time by ATPS.

Reverse miceller systems:
Reverse micelles are stable aggregates of surfactant molecules and water in organic solvents. The proteins can be extracted from the aqueous medium by forming reverse micelles. In fact, the enzymes can be extracted by this procedure without loss of biological activity.

Membrane Filtration:
Membrane filtration has become a common separation technique in industrial biotechnology. It can be conveniently used for the separation of biomolecules and particles, and for the concentration of fluids. The membrane filtration technique basically involves the use of a semipermeable membrane that selectively retains the particles/molecules that are bigger than the pore size while the smaller molecules pass through the membrane pores.

Membranes used in filtration are made up of polymeric materials such as polyethersulfone and polyvinyl di-fluoride. It is rather difficult to sterilize membrane filters. In recent years, micro-filters and ultrafiIters composed of ceramics and steel are available. Cleaning and sterilization of such filters are easy. The other types of membrane filtration techniques are described briefly.

Membrane adsorbers:
They are micro- or macro porous membranes with ion exchange groups and/or affinity ligands. Membrane adsorbers can bind to proteins and retain them. Such proteins can be eluted by employing solutions in chromatography.

Pervaporation:
This is a technique in which volatile products can be separated by a process of permeation through a membrane coupled with evaporation. Pervaporation is quite useful for the extraction, recovery and concentration of volatile products. However, this procedure has a limitation since it cannot be used for large scale separation of volatile products due to cost factor.

Perstraction:
This is an advanced technique working on the principle of membrane filtration coupled with solvent extraction. The hydrophobic compounds can be recovered/ concentrated by this method.

PRECIPITATION

Precipitation is the most commonly used technique in industry for the concentration of macromolecules such as proteins and polysaccharides. Further, precipitation technique can also be employed for the removal of certain unwanted byproducts e.g. nucleic acids, pigments.

Neutral salts, organic solvents, high molecular weight polymers (ionic or non-ionic), besides alteration in temperature and pH are used in precipitation. In addition to these non-specific protein precipitation reactions (i.e. the nature of the protein is unimportant), there are some protein specific precipitations e.g., affinity precipitation, ligand precipitation.

Neutral salts:
The most commonly used salt is ammonium sulfate, since it is highly soluble, nontoxic to proteins and low-priced. Ammonium sulfate increases hydrophobic interactions between protein molecules that result in their precipitation. The precipitation of proteins is dependent on several factors such as protein concentration, pH and temperature.

Organic solvents:
Ethanol, acetone and propanol are the commonly used organic solvents for protein precipitation. They reduce the dielectric constant of the medium and enhance electrostatic interaction between protein molecules that lead to precipitation. Since proteins are denatured by organic solvents, the precipitation process has to be carried out below 0°C.

Non-ionic polymers:
Polyethylene glycol (PEG) is a high molecular weight non-ionic polymer that can precipitate proteins. It reduces the quantity of water available for protein solvation and precipitates protein. PEG does not denature proteins, besides being non-toxic.

Ionic polymers:
The charged polymers such as polyacrylic acid and polyethyleneimine are used. They form complexes with oppositely charged protein molecules that causes charge neutralisation and precepitation.

Increase in temperature:
The heat sensitive proteins can be precipitated by increasing the temperature.

Change in pH:
Alterations in pH can also lead to protein precipitation.

Affinity precipitation:
The affinity interaction (e.g., between antigen and antibody) is exploited for precipitation of proteins.

Precipitation by ligands:
Ligands with specific binding sites for proteins have been successfully used for selective precipitation.

ADSORPTION

The biological products of fermentation can be concentrated by using solid adsorbent particles. In the early days, activated charcoal was used as the adsorbent material. In recent years, cellulose-based adsorbents are employed for protein concentration.

And for concentration of low molecular weight compounds (vitamins, antibiotics, peptides) polystyrene, methacrylate and acrylate based matrices are used. The process of adsorption can be carried out by making a bed of adsorbent column and passing the culture broth through it. The desired product, held by the adsorbent, can be eluted

FORMULATION

Formulation broadly refers to the maintenance of activity and stability of a biotechnological products during storage and distribution. The formulation of low molecular weight products (solvents, organic acids) can be achieved by concentrating them with removal of most of the water. For certain small molecules, (antibiotics, citric acid), formulation can be done by crystallization by adding salts.

Proteins are highly susceptible for loss of biological activity; hence their formulation requires special care. Certain stabilizing additives are added to prolong the shelf life of protein. The stabilizers of protein formulation include sugars (sucrose, lactose), salts (sodium chloride, ammonium sulfate), polymers (polyethylene glycol) and polyhydric alcohols (glycerol). Proteins may be formulated in the form of solutions, suspensions or dry powders.

DRYING

Drying is an essential component of product formulation. It basically involves the transfer of heat to a wet product for removal of moisture. Most of the biological products of fermentation are sensitive to heat, and therefore require gentle drying methods. Based on the method of heat transfer, drying devices may be categorized as contact, convection, radiation dryers. These three

TYPES OF DRYERS ARE COMMERCIALLY AVAILABLE.

SPRAY DRYING

Spray drying is used for drying large volumes of liquids. In spray drying, small droplets of liquid containing the product are passed through a nozzle directing it over a stream of hot gas. The water evaporates and the solid particles are left behind.

FREEZE-DRYING OR LYOPHILIZATION

Freeze-drying or lyophilization is the most preferred method for drying and formulation of a wide-range of products—pharmaceuticals, foodstuffs, diagnostics, bacteria, viruses. This is mainly because freeze-drying usually does not cause loss of biological activity of the desired product.

Lyophilization is based on the principle of sublimation of a liquid from a frozen state. In the actual technique, the liquid containing the product is frozen and then dried in a freeze-dryer under vacuum. The vacuum can now be released and the product containing vials can be sealed e.g., penicillin can be freeze dried directly in ampules.

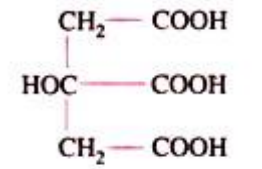
INTEGRATION OF DIFFERENT PROCESSES

It is ideal to integrate the fermentation and downstream processing to finally get the desired product. However, this has not been practicable for various reasons. Integration of certain stages in downstream processing for purification of product has met with some success. For instance, protein concentration by extraction into two phase systems combined with clarification and purification can be done together.

CITRIC ACID PRODUCTION

This acid was first produced commercially by John and Edmund Storage Company in UK in the year 1826. Scheele (1789) reported the isolation and crystallization of the four constituents of lemon juice. Grimoux and Adams (1880) synthesized citric acid from glycerol. Wehmner (1893) observed the occurrence of citric acid as a microbial product by using Penicillium and Citromyces.

It was Millard (1922) who recorded accumulation of citric acid in culture of Aspergillusniger under condition of nutrition deficiency. Meanwhile, Currie (1917) reported better yield while using A. niger. In 1923, Pfizer began operating fermentation based process in USA.



Citric acid (2-hydroxy-1,2, 3 propane tricarboxylic acid)

Fermentation of Citric Acid:

Aspergillusniger has been the choice for the production of this primary metabolite citric acid for several decades. A large number of other microorganisms (fungi and yeast) such as Aspergillusdavatus, A. wentii,

Penicilliumluteum, P. citrinum, Mucorpyriforms, Candida lipolytica, C. oleophila, C. guillermondis, Hensenulaspp. Torulopsis spp., Pichiaspp.,Debaromycesdaussenii etc. have also been used for citric acid production in industries.

The advantages of using yeast, rather than A. niger are the possibility of using very high initial sugar concentration together with a much faster fermentations. This combination gives a high productivity run to which must be added the reported insensitivity of the fermentation to variations in the heavy metal content of the crude carbohydrates.

From 1965 onwards, yeasts are used for citric acid production using carbohydrate and n- alkanes. In all the processes, a variety of carbohydrates such as beet molasses, cane molasses, sucrose, commercial glucose, starch hydrolysate etc. used in fermentation medium.

The starchy raw material is diluted to obtain 20-25% sugar concentration and mixed with a nitrogen source (ammonium salts or urea) and other salts. The pH of the medium is kept around 5 when molasses is used and at pH 3 when sucrose used.

The fermentation is carried out by any of the processes:

(a) Kofi process or solid state fermentation:

It is a Japanese process in which special strains of Aspergillusniger are used with the solid substrate such as sweet potato starch.

(b) Liquid surface culture process:

In this case, A. niger floats on the surface of a solution.

(c) Submerged fermentation process:

It is the process in which the fungal mycelium grows throughout a solution in a deep tank.

(a) Koji process:

Mold is used in the preparation called Koji to which wheat bran was substituted in the sweet potato material. The pH of the bran is adjusted between 4 and 5, and additional moisture is picked up during steaming so as to get the water content of the mash around 70-80%.

After cooling the bran to 30-60°C, the mass is inoculated with a koji which was made by a special strain of A. niger which is probably not as possible to the presence of ions of iron as the culture strains used in other process.

Since bran contains starch which on saccharification by the amylase enzyme of A. niger induces citric acid production. The bran after inoculation, is spread in trays to a depth of 3-5 cm and kept for incubation at 25-30°C. After 5-8 days, the koji is harvested and citric acid is extracted with water.

(b) Liquid surface culture process:
In this case aluminium or stainless steel shallow pans (5-20 cms deep) or trays are used. The sterilized medium usually contains molasses and salts. The fermentation is carried out by blowing the spores of A. niger over the surface of the solution for 5-6 days, after which dry air is used.

Spore germination occurs within 24 hours and a white mycelium grows over the surface of the solution, eight or ten days after inoculation, the initial sugar concentration (20-25%) reduced to the range of 1-3%.

The liquid can be drained off and any portion of mycelial mat left becomes submerged and inactivated. The small quantity of citric acid is produced during the growth phase. This is called primary metabolite. The mycelium can also be reused.

During the preparation of fermentable sugar from molasses, sucrose is the main carbohydrate along with some glucose as well as protein, peptide, amino acids, and inorganic ions. This is to be subjected to heat; so it contains saccharic acids and related compounds in traces.

The initial sugar concentration is about 20-25%. The removal of metallic ions or reduction in quantity of undesirable ions in sucrose syrup by adsorption with a combination of $CaCO_3$, colloidal silica, tricalcium phosphate and starch are other important steps, The iron is also precipitated by addition of calcium ferrocyanide.

Initially, the pH remains in the range of 5-6, but on spore germination, pH approaches the range of 1.5-2 as ammonium ions are removed from the solution. It is important to mention that at initial pH of 3-5 some oxalic acid is also produced.

The presence of iron also favours oxalic acid production, and of yellow or yellow green pigments in the mycelium sometimes secreted into the culture solution and is difficult to remove during product recovery and purification (Fig. 20.12).

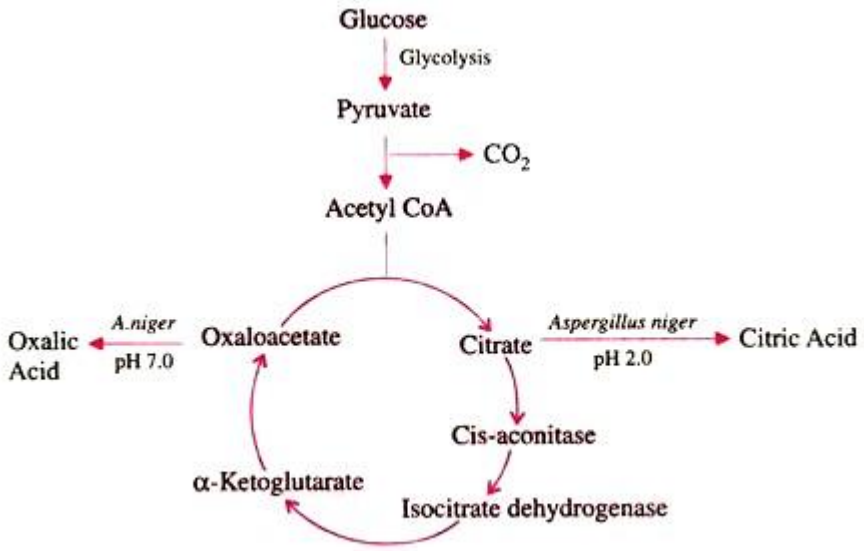


Fig. 20.12 : Biochemical pathway of oxalic acid and citric acid.

(c) Submerged culture process:

This process is quite economical. In this case, the organism (Aspergillusjaponicus) which is a black Aspergillus is slowly bubbled in a steam of air through a culture solution of 15 cm depth. Since the organism shows subsurface growth and produces citric acid in the culture solution, the yields are inferior in comparison to liquid surface culture fermentation.

The earlier workers used shaking culture and extracted Mollard's phosphate deficiency concept to submerged fermentation. But they could not realize the role of metallic ions which are commonly occurring as impurities in phosphate salts. Aeration is required for the continuous fermentation.

The addition of copper ion is must to ensure that the new growth is of the right biochemical type. The antifoam agents are necessary. Such agents must be free of iron, cobalt or nickel. Continuous culture techniques are not considered suitable for use in citric acid product.

Recovery of Citric Acid:

The culture filtrate used to be hazy due to the presence of residual antifoam agents, mycelia and oxalate. The $Ca(OH_2)$ slurry is added to precipitate calcium citrate. After filtrations the filterate is transferred and treated with H_2SO_4 to precipitate Ca as $CaSO_4$.

This is subjected to the treatment with activated carbon. It is demineralized by successive passages through ion exchange beds and the purified solution is evaporated in a circulating granulator or in a circulating crystallizers.

The crystals are removed by centrifugation. The remaining mother liquor is returned to the recovery stream. The solvent extraction can also be performed by adding 100 parts tri-n-butyl phosphate and 5-30 parts n-butyl acetate or

methyl isobutyl ketone which are to be mixed with the filterate. The solvent is then extracted with water at 70-90°C. Citric acid is further concentrated, decolorized and crystallized (Fig. 20.13).

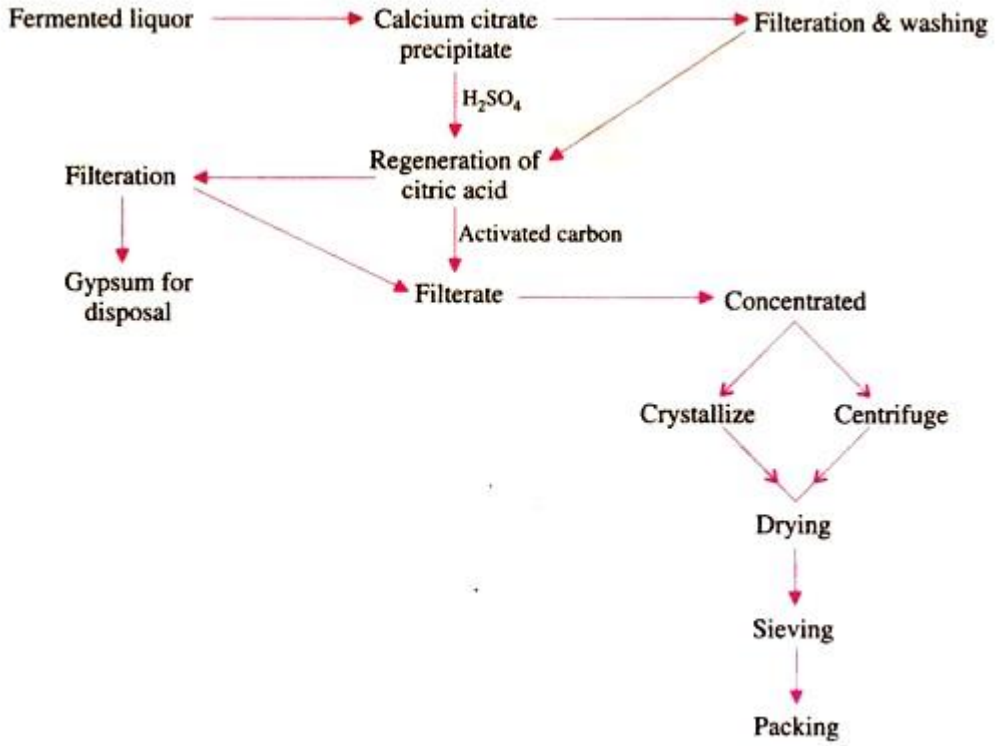


Fig. 20.13 : Process of recovery of citric acid.

ETHANOL PRODUCTION

Introduction

- Biological process in which sugars (glucose, fructose, sucrose) are converted into cellular energy.
- Ethanol is produced in the result of this process.
- It is colorless, volatile or flammable liquid.
- It is widely used as biofuel as well as an alcoholic beverage is increasingly being consumed globally.
- This is mainly because of the cheap raw materials available.

Raw material

- Ethanol can be derived from either sugar, starchy materials or lignocelluloses.
- The main feedstock for ethanol production includes sugarcane, sugar beet, corn, wheat.

Sugars:

- Sugarcane (molasses & juice)
- Cane sugar (clarified concentrated syrup)

- Sugar beet

Starchy materials:

- Corn
- Wheat
- Sweet sorghum
- Cassava

Lignocellulosic material:

- Sugarcane bagasse
- Corn stover
- Cereal straws

Feedstock conditioning and Pretreatment

Dilution:

- Molasses must be diluted to below to 25 °Bx (Brix) as yeast start to ferment quickly at this concentration.

Sedimentation:

- To prevent any incrustation in the pipelines or distillation towers due to ash content in molasses greater than 10%.
- The special chelating agent can also be used to remove the incrustation.

Addition of org & inorganic compounds:

- Done to offset the negative effect of salt which in turn increases the osmotic pressure.
- Yeast strains resistant to salts are also developed.

Microfiltration:

- To remove the impurities that stick to the surface of the biocatalyst when immobilize cells are used.

Microorganism involved

Bacteria used:

- *Zymomonas mobilis*
- *Clostridium acetobutylicum*
- *E.coli*

Yeast used:

- *Saccharomyces cerevisiae*
- *Saccharomyces uvarum*
- *Candida utilis*
- *Kluyveromyces fragilis*

Features of Microbes:

- Due to the small size, having a high surface ratio.
- Due to having a resistant cell wall, producing high concentration substances leads to a faster fermentation rate.
- The intense metabolism permits the development of a continuous fermentation process.

- Cells growth rate offsets at which cells are removed from the bioreactor.
- Have the ability to “predigest” the available food source and release both products and the intermediate metabolites.
- Using immobilized cells of microbes by treating with Calcium alginate to adsorbed on the surface of materials.
- Using genetically modified microbes to enhance the fermentation process.

Physical requirements:

- The ideal pH is around 4.0-4.5.
- The initial temperature is kept between 21-26 ºC.
- Ethanol gets evaporated at 27 ºC.
- Aeration is initially required for the growth of microbes.
- Later, anaerobic condition are created by withdrawing oxygen coupled with the production of carbon dioxide.

Chemical requirements

Nitrogen source:

- Urea is the most suitable source.
- Gaseous ammonium increases the pH of the medium.
- Ammonium sulfate can lead to incrustation.

Phosphorus source:

- Diammonium phosphate used as a source.

Hydrolytic enzymes:

- They can also be added to convert biopolymers and non-fermentable substances in the molasses to monosaccharides or amino acids.

Process flow:

Classical fermentation can be achieved in three steps:

- During the first phase (22-24 h), yeast cells multiply aerobically by consuming oxygen present in the mash.
- In the middle phase (24-48 h), alcohol production occurs with post saccharification of sugars and multiplication of yeast falls off.
- The decrease in alcohol formation along with insignificant yeast growth at the final stage (48-72 h).

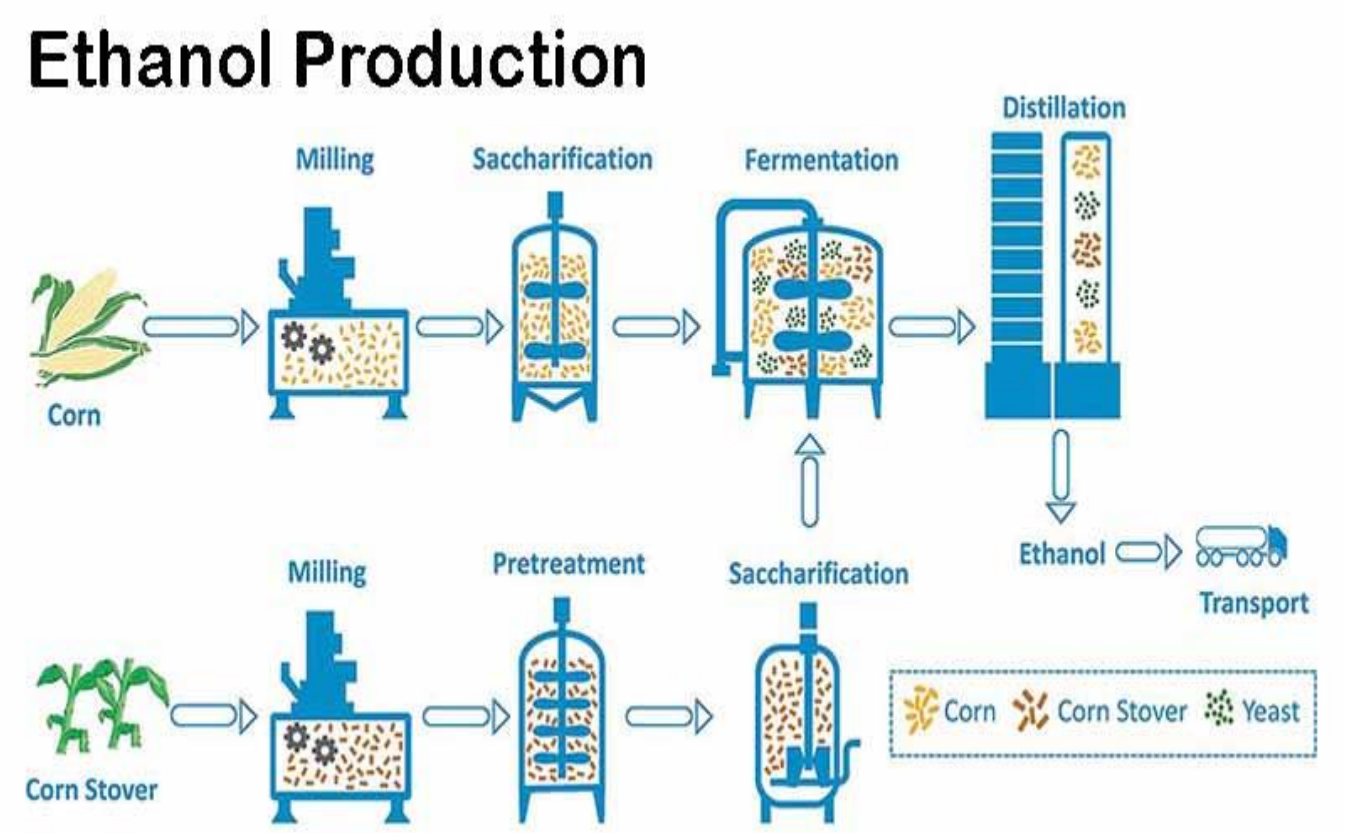


Image Source: https://doi.org/10.1016/j.biortech.2018.02.125

Production process:

There are following steps in ethanol production:

- Milling
- Liquefaction
- Saccharification
- Fermentation
- Distillation
- Dehydration

1. Milling:

- The feedstock is passed through a hammer mill which grinds it into a fine powder called a meal.

2. Liquefaction:

- The meal is mixed with water and alpha-amylase.
- Then passed through cookers where the starch is liquified and heat is applied here to enable liquefaction.
- Cookers with the high-temperature stage (120-150˚C) and lower temperature holding period (95˚C) are used.
- High temperatures reduce bacteria levels in the mash.

3. Saccharification:

- The mash from the cookers is cooled.

- And secondary enzyme glucoamylase is added.
- This converts the liquified starch into the fermentable sugars.

4. Fermentation:

- Yeast is added to ferment the sugars to ethanol and carbon dioxide.
- In a continuous process, the fermenting mash can flow through several fermenters until it is fully fermented and leaves the final tank.
- In a batch process, the mash stays in one fermenter for about 48 hours before distillation starts.

Batch fermentation

´Yeast reuse results in a decrease in new growth with no more sugar available for ethanol production and an increase in the yield from 2 to 7%.

´Traditional yield 1-3g/L.

Continuous fermentation:

- To ensure system homogeneity and reduce the concentration gradient in the culture broth, CSTRs are employed.
- Reduce construction costs of bioreactors
- Lower requirements of maintenance and operation
- Better control of the process
- Higher productivities
- Cultivation of yeast under anaerobic conditions for a long time diminish their ability to produce ethanol.
- Aeration is important which can enhance cell concentration.

Extraction of the final product:

5. Distillation:

- Mash is pumped to continuous flow.
- Multicolumn distillation system where the alcohol is removed from solid and water.
- The alcohol leaves the top of the final column at about 96% strength.
- The residue mash is called stillage which is transferred from the base of the column to the co-product processing area.

6. Dehydration:

- The alcohol from the top of the column is passed through a dehydration system where the remaining water will be removed.
- Most ethanol plants use a molecular sieve to capture the last bit of water in the ethanol.
- The alcohol product at this stage is called anhydrous alcohol.

Fermentation Byproduct

Dried distiller grains with soluble (DDGS):

- The form is available to the feed industry.
- The liquid is separated from mash during the distillation process.
- It is partially dehydrated into syrup.
- Then added back on to the dried distiller’s grain to create DDGS.

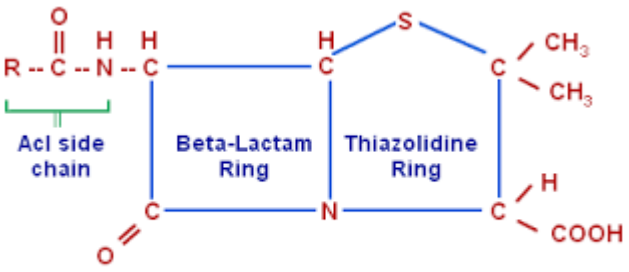
Carbon dioxide:

- Used to carbonate the beverages.
- Manufacture dry ice.
- Used to flash freeze meat.
- Used by paper mills and food industries.

PENICILLIN PRODUCTION COMMERCIALLY BY FERMENTATION BIOTECHNOLOGY

Structure of Penicillin:

- The basic structure of penicillin consists of a thiozolidine ring condensed with a B-lactum ring.
- Natural penicillin is 6-amino-penicillinic acid (6APA).



General Structure of Penicillins

Fermentation biotechnology for penicillin production:

- By fermentation technology penicillin is produced from Penicillium spp. If penicillin fermentation is carried out without addition of side chain precursor, the natural penicillins are produced. But fermentation can be better controlled by adding a side chain precursor to obtain derived penicillin. The synthetic penicillins are produced by enzymatic hydrolysis of 6APA by penicillin acylase enzyme and then addition of desired side chain by chemical means,
- B-lactumthiozolidine ring of penicillin is constructed from l-cystine and l-valine. These two amino acids when combined with L-α-aminoadipic acid (α-AAA) the tripeptide is formed which undergoes two step cyclization process to give isopenicillin.

Regulation of penicillin production:

- The amino acids lysine is synthesized from a pathway that involves L-α-AAA, so that penicillin and lysine share a common but branched biosynthetic pathway. Higher concentration of lysine causes feed back inhibition of homocitrate synthase, an enzyme involved in α-AAA synthesis. Either lysine level should keep low or α-AAA level should added during fermentation.

- Penicillin biosynthesis is affected by Po4—concentration and also shows a distinct catabolic repression by glucose. Therefore, either slowly metabolizable sugars such as lactose is used or fed continuously with glucose with small dose.

Penicillin Production process:

- Penicillin production is previously achieved by surface process ie. Solid state fermentation and surface liquid ferementation. Now a days acommercial production is carried out by fed batch process
- **Inoculum (Organism): *Penicilliumchrysogenum* (improved strain)**

i. Inoculum preparation:

- For inoculum preparation, spore from heavily sporulated working stocks are suspended inwater or non-toxic wetting agensts (sodium sulfonate 1: 10000)
- Theses spore are then added to flask containing wheat bran and nutrient solution for heavy sporulation
- Incubate for 5-7 days at 24C
- Spore are then transferred to seed tank and incubated for 24-48 hours at 24C with aeration and agitation for sufficient mycelial growth
- These mycelia can be used for production fermenter

ii. Production fermentation:

- **Method:** fed-batch or batch
- **Substrate:** glucose, phenoxyacetic acid (fed component used for production of side chain), Corn steep liquor, Additional nitrogen source ie, soyameal, yeast extract, Lactic acid, inorganic ions, growth factors
- **Fermenter:** stirred tank or air lift tank
- **pH**: set at 5.5 t0 6.0 which increased upto 7-7.5 (optimum) due to liberation of NH3 gas and consumptionof lactic acid. If pH is 8 or more, CaCO3 or MgCO3 or phosphate buffer is added
- **temperature:** 25-27 C
- **aeration:** 0.5-1 vvm (initially more, latter less O2)
- **agitation:** 120-150 rpm)
- **time:** 3-5 days
- **antiform:** edible oil (0.25%)

iii. Product recovery:

- harvest broth from fermenter tank by filtration (rotary vaccum filtration)
- chill to 5-10 C (because penicillin is highly reactive and destroyed by alkali and enzyme)

- acidify filtrate to pH 2.0-2.5 with H_2SO_4 (to convert penicillin to its anionic form)
- extract penicillin from aqueous filtrate into butyl acetate or amyl acetate (at this very low pH as soon as possible in centrifugal counter current extractor)
- discard aqueous fraction
- allow the organic solvent to pass through charcoal to remove impurities and extract penicillin from butylacetate to 2% aqueous phosphate buffer at pH 7.5
- acidify the aq. Fraction to pH 2-2.5 with mineral acid and re-extract penicillin into fresh butylacetate (it concentrated upto 80-100 times)
- add potassium acetate to the solvent extract in a crystallization tank to crystalize as potassium salt
- recover crystal in filter centrifuge
- sterilization
- further processing
- packaging

Application of penicillin:

- **clinical uses of penicillin:**
- naturally effective antibiotics against gram + bacteria
- used for treatment of bacterial endocarditis

GLUTAMIC ACID PRODUCTION

The history of the first amino acid production dates back to 1908 when Dr. K. Ikeda, a chemist in Japan, isolated glutamic acid from kelp, a marine alga, after acid hydrolysis and fractionation. He also discovered that glutamic acid, after neutralization with castic soda, developed an entirely new, delicious taste.

This was the birth of the use of monosodium glutamate (MSG) as a flavour-enhancing compound. The breakthrough in the production of MSG was the isolation of a specific soil-inhabiting gram-positive bacterium, Corynebacteriumglutamicum, by Dr. S. Ukada and Dr. S. Kinoshita in 1957. The successful commercialization of monosodium glutamate (MSG) with this bacterium provided a big boost for amino acid production and later with other bacteria like E. coli as well.

Commercial Production of Glutamic Acid:

Glutamic acid commercial production (Fig. 40.6) by microbial fermentation provides 90% of world's total demand, and remaining 10% is met through

chemical methods. For the actual fermentation the microbial strains are grown in fermentors as large as 500 m^3. The raw materials used include carbohydrate (glucose, molasses, sucrose, etc.), peptone, inorganic salts and biotin.
ADVERTISEMENTS:

Biotin concentration in the fermentation medium has a significant influence on the yield of glutamic acid. Fermentation completes within 2-4 days and, at the end of the fermentation, the broth contains glutamic acid in the form of its ammonium salt.

In a typical downstream process, the bacterial cells are separated and the broth is passed through a basic anion exchange resin. Glutamic acid anions get bound to the resin and ammonia is released. This ammonia can be recovered via distillation and reused in the fermentation.

Elution is performed with NaOH to directly form monosodium glutamate (MSG) in the solution and to regenerate the basic anion exchanger. From the elute, MSG may be crystallized directly followed by further conditioning steps like decolourization and serving to yield a food-grade quality of MSG.

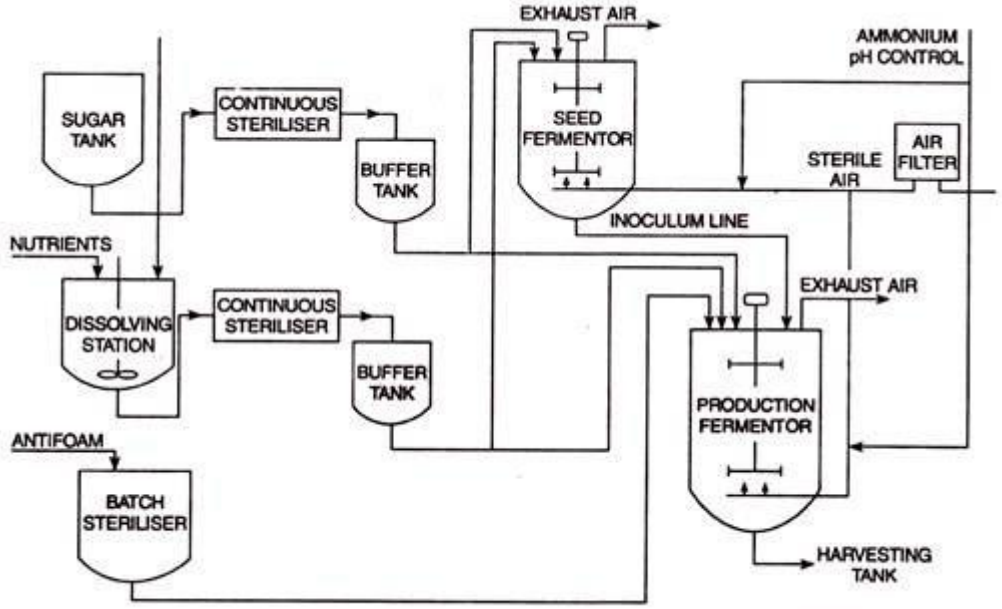
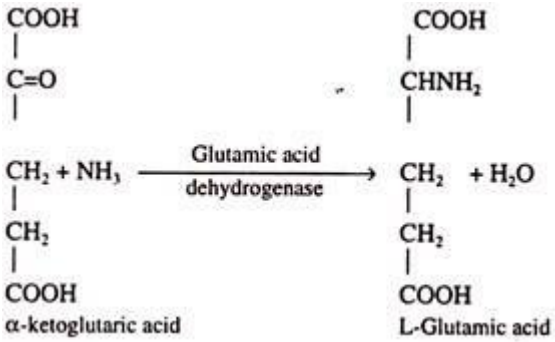


FIG. 40.6. A flow diagram of commercial production method of glutamic acid (glutamate).

α-ketoglutaric acid serves as the precursor of glutamic acid and the conversion of the α- ketoglutaric acid to glutamic acid occurs in presence of enzyme glutamic acid dehydrogenase. It has been found that if penicillin is added in he medium, the glutamic acid production can be increased manifold.



Uses of Glutamic Acid:

As stated earlier, glutamic acid is widely used in the production of monosodium glutamate (MSG) which is commonly known as the 'seasoning salt'. The world production of glutamic acid is to the tune of 800,000 tonnes/year. Monosodium glutamate is condiment and flavour-enhancing agent, it finds its greatest use as a common ingredient in convenient food-stuffs.

Lysine:

In recent years, a 'single state' fermentation process is being used for the commercial production of lysine. This process has replaced the "two-stage" fermentation process of lysine production in which Escherichia coli was used to produce diammopimelic acid (DAP) in the first stage, and Enterobacteraerogenes was used for the formation of lysine from diaminopimelic acid through decarboxylation by an enzyme called diaminopimelic decarboxylase (DAP-decarboxylasc) in the second stage.

The 'single-stage' process of fermentation of lysine involves mutants of Carynebacterium, Brevibacterium, etc. These bacteria are grown in a synthetic medium containing carbohydrate (glucose), an inorganic nitrogen source, small concentration of homoserine or methionine, and small concentration of biotin. The process of fermentation is completed within 48-70 hours and the yield of the amino acid is as high as 30 gm/litre.

Use:

Lysine is a vital amino acid for humans. Since cereal proteins are often deficient in lysine, it is generally used as a supplement for nutritional requirement such as bread and other food stuffs for human being.

Other Amino Acids:

Amino acid	*Microorganism*
Tryptophan	Mutants of *Escherichia coli, Claviceps purpurea*
Threonine	*Bacillus subtilis*, mutants of *E. coli, Brevibracterium flavus, Streptomyces racemosus*
Phenyl alanine	*Corynebacterium glutanicus*
L-aspartic acid	*Escherichia* mutants
L-isoleucine	*Bacillus subtilis, Pseudomonas* spp.
Valine	*Brevibacterium*, members of Enterobacteriaceae

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SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

UNIT –V – INDUSTRIAL MICROBIOLOGY – SMB2204

SMB2204	INDUSTRIAL MICROBIOLOGY	L	T	P	Credits	Total Marks
		3	1	0	4	100

COURSE OBJECTIVES

- The objective of this course to enable students to correlate metabolic aspects of industrially relevant microorganisms with the corresponding biotechnological products, and learn the basics of simple strategies for strain improvements encompassing both classical and metabolic engineering methods, design medium and downstream processing.

UNIT 1 HISTORY **12 Hrs.**

Brief history and developments in industrial microbiology Types of fermentation processes - Solid-state and liquid-state (stationary and submerged) fermentations; batch, fed-batch (Eg. Baker's yeast) and continuous fermentations

UNIT 2 BIO-REACTOR **12 Hrs.**

Components of a typical bio-reactor, Types of bioreactors-Laboratory, pilot- scale and production fermenters, constantly stirred tank and air-lift fermenters, Measurement and control of fermentation parameters - pH, temperature, dissolved oxygen, foaming and aeration

UNIT 3 UPSTREAM PROCESSING **12 Hrs.**

Sources of industrially important microbes and methods for their isolation, preservation and maintenance of industrial strains, strain improvement, Crude and synthetic media; molasses, corn steep liquor, sulphite waste liquor, whey, yeast extract and protein hydrolysates

UNIT 4 DOWNSTREAM PROCESSING **12 Hrs.**

Cell disruption, filtration, centrifugation, solvent extraction, precipitation, lyophilization and spray drying. Citric acid, ethanol, penicillin, glutamic acid, Vitamin B12 Enzymes (amylase, protease, lipase)
Wine, beer

UNIT 5 IMMOBILIZATION **12 Hrs.**

Methods of immobilization, advantages and applications of immobilization, large scale applications of immobilized enzymes (glucose isomerase and penicillin acylase)

Max Hours.60

TEXT / REFERENCE BOOKS

1. Patel A.H. (1996). Industrial Microbiology. 1st edition, Macmillan India Limited
2. Okafor N. (2007). Modern Industrial Microbiology and Biotechnology. 1st edition. Bios Scientific Publishers Limited. USA
3. Waites M.J., Morgan N.L., Rockey J.S. and Higton G. (2001). Industrial Microbiology: An Introduction. 1st edition. Wiley – Blackwell
4. Glaze A.N. and Nikaido H. (1995). Microbial Biotechnology: Fundamentals of Applied Microbiology. 1st edition. W.H. Freeman and Company
5. Casida LE. (1991). Industrial Microbiology. 1st edition. Wiley Eastern Limited.
6. Crueger W and Crueger A. (2000). Biotechnology: A textbook of Industrial Microbiology. 2nd edition. Panima Publishing Co. New Delhi.
7. 7. Stanbury PF, Whitaker A and Hall SJ. (2006). Principles of Fermentation Technology. 2nd edition, Elsevier Science Ltd..

END SEMESTER EXAMINATION QUESTION PAPER PATTERN

Max. Marks : 100 **Exam Duration : 3 Hrs.**
PART A : 10 questions of 2 marks each - No choice **20 Marks**
PART B : 2 questions from each UNIT of internal choice; each carrying 16 marks **80 Marks**

UNIT: V

IMMOBILIZATION

Immobilization of enzymes (or cells) refers to the technique of confining/anchoring the enzymes (or cells) in or on an inert support for their stability and functional reuse. By employing this technique, enzymes are made more efficient and cost-effective for their industrial use. Some workers regard immobilization as a goose with a golden egg in enzyme technology. Immobilized enzymes retain their structural conformation necessary for catalysis.

There are several advantages of immobilized enzymes:

a. Stable and more efficient in function.

b. Can be reused again and again.

c. Products are enzyme-free.

d. Ideal for multi-enzyme reaction systems.

e. Control of enzyme function is easy.

f. Suitable for industrial and medical use.

g. Minimize effluent disposal problems.

Disadvantages also associated with immobilization:

a. The possibility of loss of biological activity of an enzyme during immobilization or while it is in use.

b. Immobilization is an expensive affair often requiring sophisticated equipment.

Immobilized enzymes are generally preferred over immobilized cells due to specificity to yield the products in pure form. However, there are several advantages of using immobilized multi-enzyme systems such as organelles and whole cells over immobilized enzymes. The immobilized cells possess the natural environment with cofactor availability (and also its regeneration capability) and are particularly suitable for multiple enzymatic reactions.

Methods of Immobilization:

The commonly employed techniques for immobilization of enzymes are adsorption, entrapment, covalent binding and cross-linking.

Adsorption:

Adsorption involves the physical binding of enzymes (or cells) on the surface of an inert support. The support materials may be inorganic (e.g. alumina, silica gel, calcium phosphate gel, glass) or organic (starch, carboxymethyl cellulose, DEAE-cellulose, DEAE-sephadex).

Adsorption of enzyme molecules (on the inert support) involves weak forces such as van der Waals forces and hydrogen bonds (Fig. 21.3). Therefore, the adsorbed enzymes can be easily removed by minor changes in pH, ionic strength or temperature. This is a disadvantage for industrial use of enzymes.

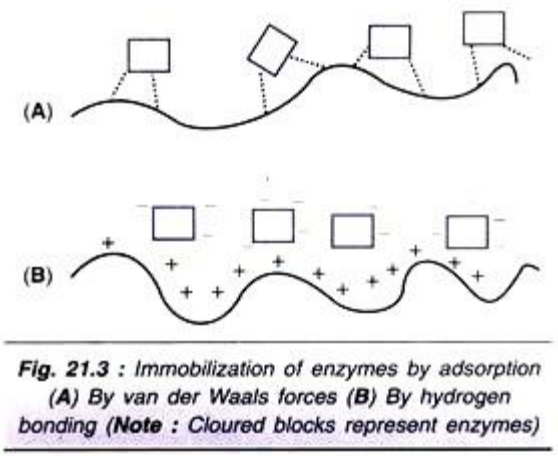

*Fig. 21.3 : Immobilization of enzymes by adsorption (A) By van der Waals forces (B) By hydrogen bonding (**Note** : Cloured blocks represent enzymes)*

Entrapment:

Enzymes can be immobilized by physical entrapment inside a polymer or a gel matrix. The size of the matrix pores is such that the enzyme is retained while the substrate and product molecules pass through. In this technique, commonly referred to as lattice entrapment, the enzyme (or cell) is not subjected to strong binding forces and structural distortions.

Some deactivation may however, occur during immobilization process due to changes in pH or temperature or addition of solvents. The matrices used for entrapping of enzymes include polyacrylamide gel, collagen, gelatin, starch, cellulose, silicone and rubber. Enzymes can be entrapped by several ways.

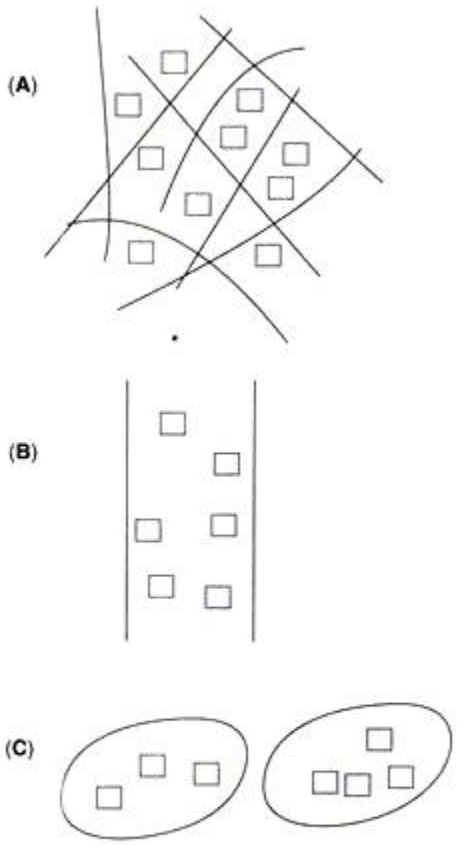

*Fig. 21.4 : Immobilization of enzymes by entrapment (A) Inclusion in gels (B) Inclusion in fibres (C) Inclusion in microcapsules (**Note** : Coloured blocks represent enzymes)*

1. Enzyme inclusion in gels:
This is an entrapment of enzymes inside the gels (Fig. 21.4A).

2. Enzyme inclusion in fibres:
The enzymes are trapped in a fibre format of the matrix (Fig. 21.4B).

3. Enzyme inclusion in microcapsules:
In this case, the enzymes are trapped inside a microcapsule matrix (Fig. 21.4C). The hydrophobic and hydrophilic forms of the matrix polymerise to form a microcapsule containing enzyme molecules inside. The major limitation for entrapment of enzymes is their leakage from the matrix. Most workers prefer to use the technique of entrapment for immobilization of whole cells. Entrapped cells are in use for industrial production of amino acids (L-isoleucine, L-aspartic acid), L-malic acid and hydroquinone.

Microencapsulation:

Microencapsulation is a type of entrapment. It refers to the process of spherical particle formation wherein a liquid or suspension is enclosed in a semipermeable membrane. The membrane may be polymeric, lipoidal, lipoprotein-based or non-ionic in nature. There are three distinct ways of microencapsulation.

1. Building of special membrane reactors.

2. Formation of emulsions.

3. Stabilization of emulsions to form microcapsules.

Microencapsulation is recently being used for immobilization of enzymes and mammalian cells. For instance, pancreatic cells grown in cultures can be immobilized by microencapsulation. Hybridoma cells have also been immobilized successfully by this technique.

Covalent Binding:

Immobilization of the enzymes can be achieved by creation of covalent bonds between the chemical groups of enzymes and the chemical groups of the support (Fig. 21.5). This technique is widely used. However, covalent binding is often associated with loss of some enzyme activity. The inert support usually requires pretreatment (to form pre-activated support) before it binds to enzyme. The following are the common methods of covalent binding.

*Fig. 21.5 : A general representation of immobilization of enzymes by covalent binding (**Note** : coloured blocks represent enzymes).*

1. Cyanogen bromide activation:

The inert support materials (cellulose, sepharose, sephadex) containing glycol groups are activated by CNBr, which then bind to enzymes and immobilize them (Fig. 21.6A).

2. Diazotation:

Some of the support materials (amino benzyl cellulose, amino derivatives of polystyrene, aminosilanized porous glass) are subjected to diazotation on

treatment with $NaNO_2$ and HCI. They, in turn, bind covalently to tyrosyl or histidyl groups of enzymes (Fig. 21.6B).

3. Peptide bond formation:

Enzyme immobilization can also be achieved by the formation of peptide bonds between the amino (or carboxyl) groups of the support and the carboxyl (or amino) groups of enzymes (Fig. 21.6C). The support material is first chemically treated to form active functional groups.

4. Activation by bi- or poly-functional reagents:

Some of the reagents such as glutaraldehyde can be used to create bonds between amino groups of enzymes and amino groups of support (e.g. aminoethylcellulose, albumin, amino alkylated porous glass). This is depicted in Fig. 21.6D.

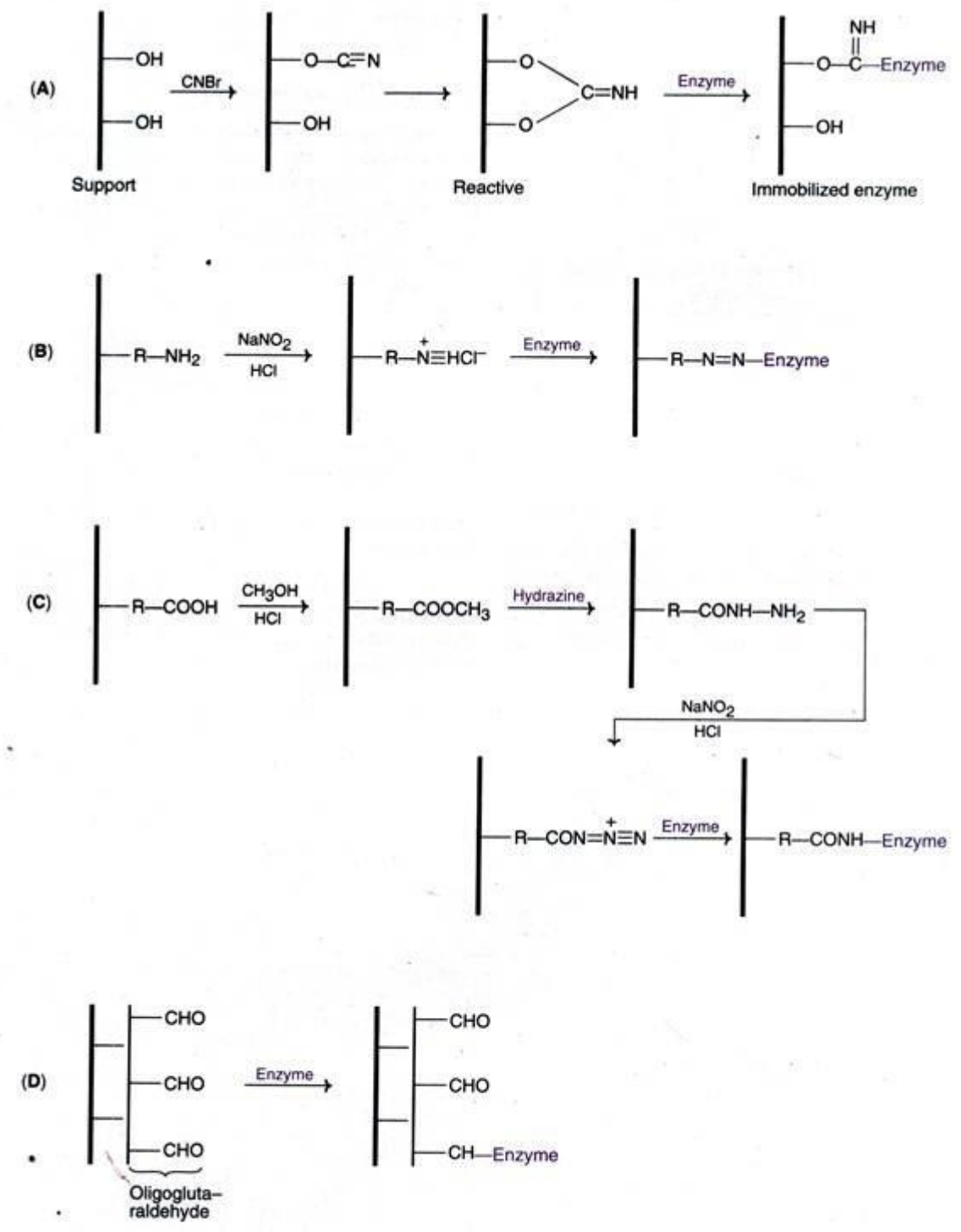

Fig. 21.6 : Immobilization of enzymes by covalent binding (A) Cyanogen bromide activation, (B) Diazotation, (C) Peptide bond formation, (D) Activation by bifunctional agent.

Cross-Linking:

The absence of a solid support is a characteristic feature of immobilization of enzymes by cross- linking. The enzyme molecules are immobilized by creating cross-links between them, through the involvement of poly-functional reagents. These reagents in fact react with the enzyme molecules and create bridges which form the backbone to hold enzyme molecules (Fig. 21.7). There are several reagents in use for cross-linking. These include glutaraldehyde, diazobenzidine, hexamethylene diisocyanate and toluene di- isothiocyanate.

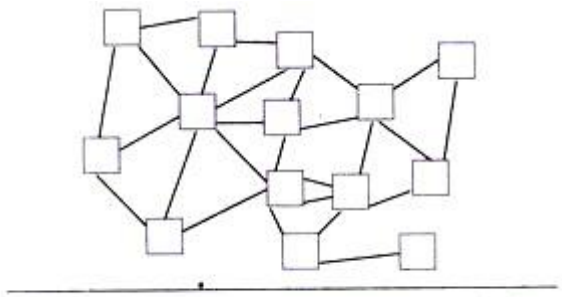

Fig. 21.7 : Immobilization of enzyme molecules by cross linking.

Glutaraldehyde is the most extensively used cross-linking reagent. It reacts with lysyl residues of the enzymes and forms a Schiff's base. The cross links formed between the enzyme and glutaraldehyde are irreversible and can withstand extreme pH and temperature. Glutaraldehyde cross- linking has been successfully used to immobilize several industrial enzymes e.g. glucose isomerase, penicillin amidase. The technique of cross-linking is quite simple and cost-effective. But the disadvantage is that it involves the risk of denaturation of the enzyme by the poly-functional reagent.

Choice of Immobilization Technique:

The selection of a particular method for immobilization of enzymes is based on a trial and error approach to choose the ideal one. Among the factors that decide a technique, the enzyme catalytic activity, stability, regenerability and cost factor are important.

Immobilization of L-amino acid acylase:

L-Amino acid acylase was the first enzyme to be immobilized by a group of Japanese workers (Chibata and Tosa, 1969). More than 40 different immobilization methods were attempted by this group. Only three of them were found be useful. They were covalent binding to iodoacetyl cellulose, ionic binding to DEAE-Sephadex and entrapment within polyacrylamide.

Stabilization of Soluble Enzymes:

Some of the enzymes cannot be immobilized and they have to be used in soluble form e.g. enzymes used in liquid detergents, some diagnostic reagents and food additives. Such enzymes can be stabilized by using certain additives or by chemical modifications. The stabilized enzymes have longer half-lives, although they cannot be recycled. Some important methods of enzyme stabilization are briefly described.

Solvent Stabilization:
Certain solvents at low concentrations stabilize the enzymes, while at high concentrations the enzymes get denatured e.g. acetone (5%) and ethanol (5%) can stabilize benzyl alcohol dehydrogenase.

Substrate Stabilization:
The active site of an enzyme can be stabilized by adding substrates e.g. starch stabilizes a-amylase; glucose stabilizes glucose isomerase.

Stabilization by Polymers:
Enzymes can be stabilized, particularly against increased temperature, by addition of polymers such as gelatin, albumin and polyethylene glycol.

Stabilization by Salts:
Stability of metalloenzymes can be achieved by adding salts such as Ca, Fe, Mn, Cu and Zn e.g. proteases can be stabilized by adding calcium.

Stabilization by Chemical Modifications:
Enzymes can be stabilized by suitable chemical modifications without loss of biological activity. There are several types of chemical modifications.

a. Addition of poly-amino side chains e.g. polytyrosine, polyglycine.

b. Acylation of enzymes by adding groups such as acetyl, propionyl and succinyl.

Stabilization by Rebuilding:
Theoretically, the stability of the enzymes is due to hydrophobic interactions in the core of the enzyme. It is therefore, proposed that enzymes can be stabilized by enhancing hydrophobic interactions. For this purpose, the enzyme is first unfold and then rebuilt in one of the following ways (Fig. 21.8).

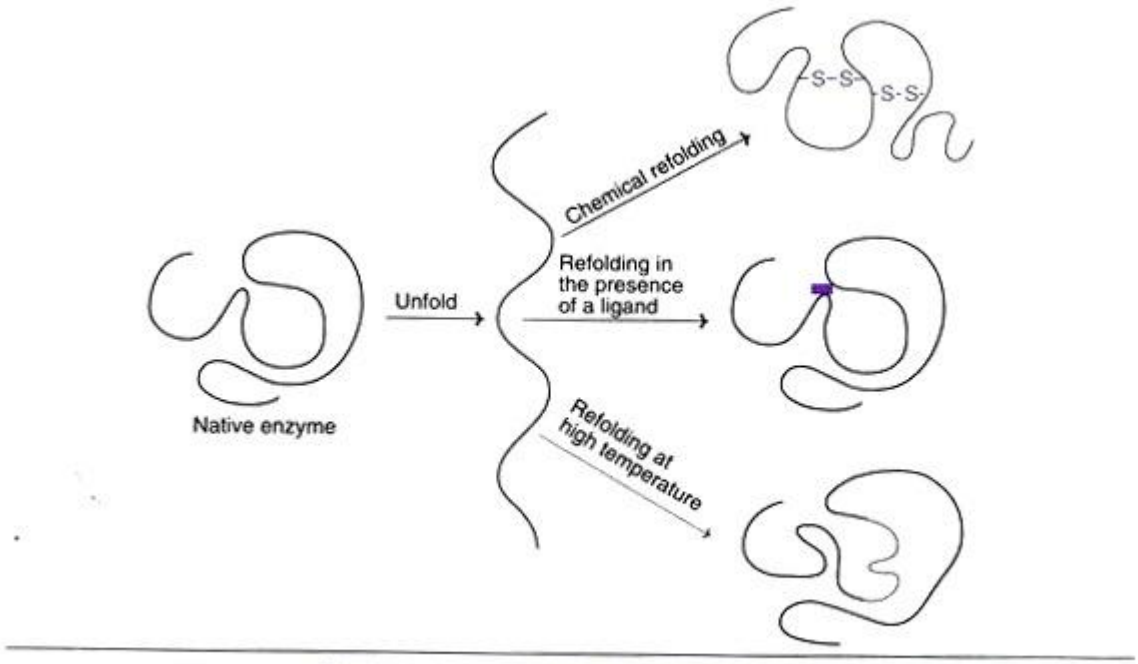

Fig. 21.8 : Stabilization of an enzyme by refolding.

1. The enzyme can be chemically treated (e.g. urea and a disulfide) and then refolded.

2. The refolding can be done in the presence of low molecular weight ligands.

3. For certain enzymes, refolding at higher temperatures (around 50°C) stabilize them.

Stabilization by Site-Directed Mutagenesis:

Site-directed mutagenesis has been successfully used to produce more stable and functionally more efficient enzymes e.g. subtilisin E.

Immobilization of Cells:

Immobilized individual enzymes can be successfully used for single-step reactions. They are, however, not suitable for multi-enzyme reactions and for the reactions requiring cofactors. The whole cells or cellular organelles can be immobilized to serve as multi-enzyme systems. In addition, immobilized cells rather than enzymes are sometimes preferred even for single reactions, due to cost factor in isolating enzymes. For the enzymes which depend on the special arrangement of the membrane, cell immobilization is preferred.

Immobilized cells have been traditionally used for the treatment of sewage. The techniques employed for immobilization of cells are almost the same as that used for immobilization of enzymes with appropriate modifications. Entrapment and surface attachment techniques are commonly used. Gels, and to some extent membranes, are also employed.

Immobilized Viable Cells:

The viability of the cells can be preserved by mild immobilization. Such immobilized cells are particularly useful for fermentations. Sometimes mammalian cell cultures are made to function as immobilized viable cells.

Immobilized Non-viable Cells:

In many instances, immobilized non-viable cells are preferred over the enzymes or even the viable cells. This is mainly because of the costly isolation and purification processes. The best example is the immobilization of cells containing glucose isomerase for the industrial production of high fructose syrup. Other important examples of microbial biocatalysts and their applications are given in Table 21.5.

Table 21.5 Selected examples of immobilized cells (to bring out one or two enzyme reactions) in industrial applications

Immobilized microorganism (microbial biocatalyst)	*Application(s)*
Escherichia coli	For the synthesis of L-aspartic acid from fumaric acid and NH_3
Escherichia coli	For the production of L-tryptophan from indole and serine
Pseudomonas sp	Production of L-serine from glycine and methanol
Saccharomyces cerevisiae	Hydrolysis of sucrose
Saccharomyces sp	Large scale production of alcohol
Zymomonas mobilis	Synthesis of sorbitol and gluconic acid from glucose and fructose
Anthrobacter simplex	Synthesis of prednisolone from hydrocortisone
Pseudomonas chlororaphis	Production of acrylamide from acrylonitrile
Humicola sp	For the conversion of rifamycin B to rifamycin S
Bacteria and yeasts (several sp)	In biosensors

Limitations of Immobilizing Eukaryotic Cells:

Prokaryotic cells (particularly bacterial) are mainly used for immobilization. It is also possible to immobilize eukaryotic plant and animal cells. Due to the presence of cellular organelles, the metabolism of eukaryotic cells is slow. Thus, for the industrial production of biochemical, prokaryotic cells are preferred. However, for the production of complex proteins (e.g.

immunoglobulin's) and for the proteins that undergo post- translational modifications, eukaryotic cells may be used.

Effect of Immobilization on Enzyme Properties:

Enzyme immobilization is frequently associated with alterations in enzyme properties, particularly the kinetic properties of enzymes.

Some of them are listed below:

1. There is a substantial decrease in the enzyme specificity. This may be due to conformational changes that occur when the enzyme gets immobilized.

2. The kinetic constants K_m and V_{max} of an immobilized enzyme differ from that of the native enzyme. This is because the conformational change of the enzyme will affect the affinity between enzyme and substrate.

Immobilized Enzyme Reactors:

The immobilized enzymes cells are utilized in the industrial processes in the form of enzyme reactors. They are broadly of two types — batch reactors and continuous reactors. The frequently used enzyme reactors are shown in Fig. 21.9.

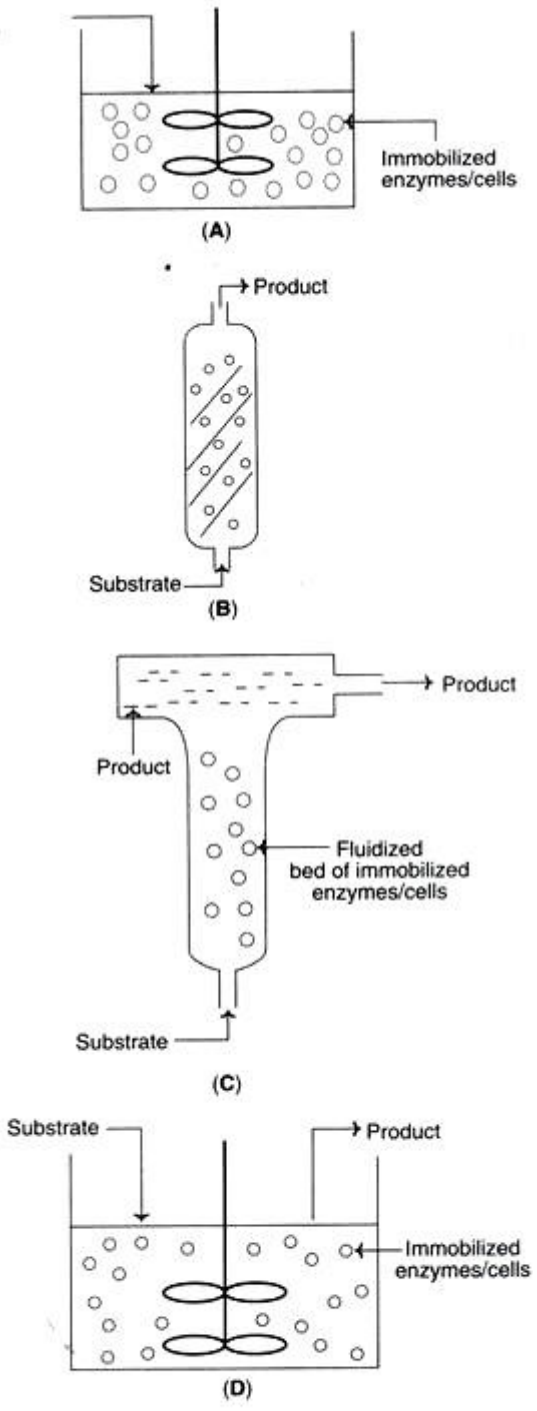

Fig. 21.9 : *Immobilized enzyme (cell) reactors*
(A) *Batch stirred tank reactor,* ***(B)*** *Packed bed reactc*
(C) *Fluidized bed reactor,* ***(D)*** *Continuous stirred tank reactor.*

Batch Reactors:

In batch reactors, the immobilized enzymes and substrates are placed, and the reaction is allowed to take place under constant stirring. As the reaction is completed, the product is separated from the enzyme (usually by denaturation).

Soluble enzymes are commonly used in batch reactors. It is rather difficult to separate the soluble enzymes from the products; hence there is a limitation of their reuse. However, special techniques have been developed for recovery of soluble enzymes, although this may result in loss of enzyme activity.

Stirred tank reactors:
The simplest form of batch reactor is the stirred tank reactor (Fig. 21.9A). It is composed of a reactor fitted with a stirrer that allows good mixing, and appropriate temperature and pH control. However, there may occur loss of some enzyme activity. A modification of stirred tank reactor is basket reactor. In this system, the enzyme is retained over the impeller blades. Both stirred tank reactor and basket reactor have a well-mixed flow pattern.

Plug flow type reactors:
These reactors are alternatives to flow pattern type of reactors. The flow rate of fluids controlled by a plug system. The plug flow type reactors may be in the form of packed bed or fluidized bed (Fig. 21.9B and 21.9C). These reactors are particularly useful when there occurs inadequate product formation in flow type reactors. Further, plug flow reactors are also useful for obtaining kinetic data on the reaction systems.

Continuous Reactors:
In continuous enzyme reactors, the substrate is added continuously while the product is removed simultaneously. Immobilized enzymes can also be used for continuous operation. Continuous reactors have certain advantages over batch reactors. These include control over the product formation, convenient operation of the system and easy automation of the entire process. There are mainly two types of continuous reactors-continuous stirred tank reactor (CSTR) and plug reactor (PR). A diagrammatic representation of CSTR is depicted in Fig. 21.9D. CSTR is ideal for good product formation.

Membrane Reactors:
Several membranes with a variety of chemical compositions can be used. The commonly used membrane materials include polysulfone, polyamide and cellulose acetate. The biocatalysts (enzymes or cells) are normally retained on

the membranes of the reactor. The substrate is introduced into reactor while the product passes out. Good mixing in the reactor can be achieved by using stirrer (Fig. 21.10A). In a continuous membrane reactor, the biocatalysts are held over membrane layers on to which substrate molecules are passed (Fig. 21.10B).

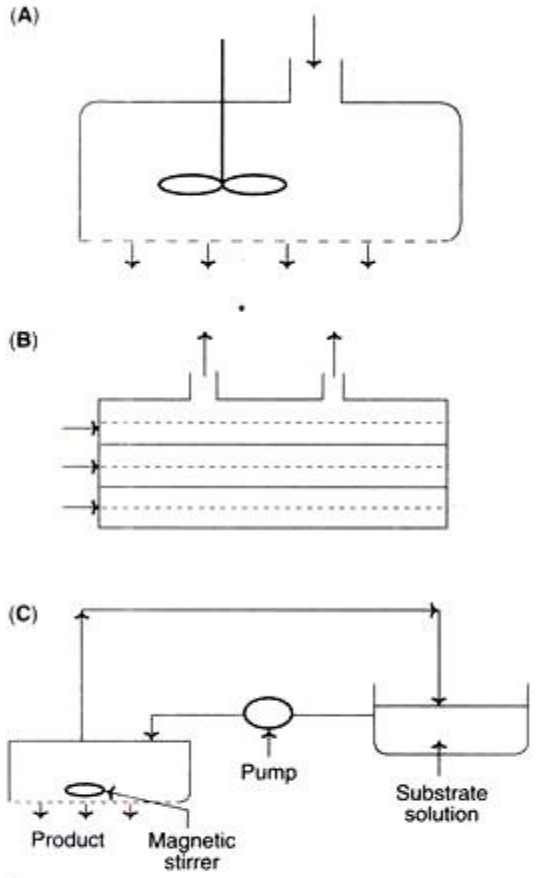

Fig. 21.10 : Membrane reactors (A) Batch membrane reactor, (B) Continuous membrane reactor, (C) Recycle membrane reactor (Coloured lines indicate membranes).

In a recycle model membrane reactor, the contents (i.e. the solution containing enzymes, cofactors, and substrates along with freshly released product are recycled by using a pump (Fig. 21.10C). The product passes out which can be recovered.

Applications of Immobilized Enzymes and Cells:

Immobilized enzymes and cells are very widely used for industrial, analytical and therapeutic purpose, besides their involvement in food production and exploring the knowledge of biochemistry, microbiology and other allied

specialties. A brief account of the industrial applications of immobilized cells is given in Table 21.6.

Manufacture of Commercial Products:

A selected list of important immobilized enzymes and their industrial applications is given in Table 21.6. Some details on the manufacture of L-amino acids and high fructose syrup are given hereunder.

TABLE 21.6 A selected list of important immobilized enzymes and their industrial applications

Immobilized enzyme	*Application(s)*
Aminoacylase	Production of L-amino acids from D, L-acyl amino acids
Glucose isomerase	Production of high fructose syrup from glucose (or starch)
Amylase	Production of glucose from starch
Invertase	Splitting of sucrose to glucose and fructose
β-Galactosidasp	Splitting of lactose to glucose and galactose
Penicillin acylase	Commercial production of semi-synthetic penicillins
Aspartase	Production of aspartic acid from fumaric acid
Fumarase	Synthesis of malic acid from fumaric acid
Histidine ammonia lyase	Production of urocanic acid from histidine
Ribonuclease	Synthesis of nucleotides from RNA
Nitrilase	Production of acrylamide from acrylonitrile

Production of L-Amino Acids:

L-Amino acids (and not D-amino acids) are very important for use in food and feed supplements and medical purposes. The chemical methods employed for their production result in a racemic mixture of D- and L-amino acids. They can be acylated to form D, L-acyl amino acids. The immobilized enzyme aminoacylase (frequently immobilized on DEAE sephadex) can selectively hydrolyse D, L-acyl amino acids to produce L-amino acids.

$$\text{D, L-Acyl amino acids} \xrightarrow{\text{Aminoacylase}} \text{L-Amino acids} + \text{D, L-Acyl amino acids}$$

The free L-amino acids can separated from the un-hydrolysed D-acyl amino acids. The latter can be recemized to D, L-acyl amino acids and recycled through the enzyme reactor containing immobilized aminoacylase. Huge quantities of L-methionine, L-phenylalanine L-tryptophan and L-valine are produced worldwide by this approach.

Production of High Fructose Syrup:

Fructose is the sweetest among the monosaccharide's, and has twice the sweetening strength of sucrose. Glucose is about 75% as sweet as sucrose. Therefore, glucose (the most abundant monosaccharide) cannot be a good substitute for sucrose for sweetening. Thus, there is a great demand for fructose which is very sweet, but has the same calorific value as that of glucose or sucrose.

High fructose syrup (HFS) contains approximately equivalent amounts of glucose and fructose. HFS is almost similar to sucrose from nutritional point of view. HFS is a good substitute for sugar in the preparation of soft drinks, processed foods and baking.

High fructose syrup can be produced from glucose by employing an immobilized enzyme glucose isomerase. The starch containing raw materials (wheat, potato, corn) are subjected to hydrolysis to produce glucose. Glucose isomerase then isomerizes glucose to fructose (Fig. 21.11). The product formed is HFS containing about 50% fructose. (Note: Some authors use the term high fructose corn syrup i.e. HFCS in place of HFS).

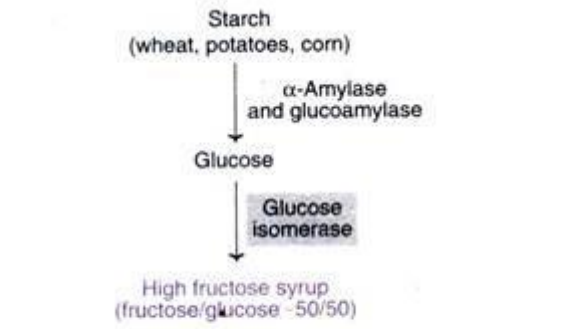

Fig. 21.11 : Production of high fructose syrup from starch (glucose isomerase is the immobilized enzyme).

Glucose isomerase:

This is an intracellular enzyme produced by a number of microorganisms. The species of Arthrobacter, Bacillus and Streptomyces are the preferred sources. Being an intracellular enzyme, the isolation of glucose isomerase without loss of biological activity requires special and costly techniques. Many a times, whole cells or partly broken cells are immobilized and used.

Immobilized Enzymes and Cells- Analytical Applications:
In Biochemical Analysis:

Immobilized enzymes (or cells) can be used for the development of precise and specific analytical techniques for the estimation of several biochemical compounds. The principle of analytical assay primarily involves the action of the immobilized enzyme on the substrate.

A decrease in the substrate concentration or an increase in the product level or an alteration in the cofactor concentration can be used for the assay. A selected list of examples of immobilized enzymes used in the assay of some substances is given in Table 21.7. Two types of detector systems are commonly employed.

Table 21.7 Selected examples of immobilized enzymes used in analytical biochemistry

Immobilized enzyme	*Substance assayed*
Glucose oxidase	Glucose
Urease	Urea
Cholesterol oxidase	Cholesterol
Lactate dehydrogenase	Lactate
Alcohol oxidase	Alcohol
Hexokinase	ATP
Galactose oxidase	Galactose
Penicillinase	Penicillin
Ascorbic acid oxidase	Ascorbic acid
L-Amino acid oxidase	L-Amino acids
Cephalosporinase	Cephalosporin
Monoamine oxidase	Monoamine

Thermistors are heat measuring devices which can record the heat generated in an enzyme catalysed reaction. Electrode devices are used for measuring potential differences in the reaction system. In the Fig. 21.12, an enzyme thermistor and an enzyme electrode, along with a specific urease electrode are depicted.

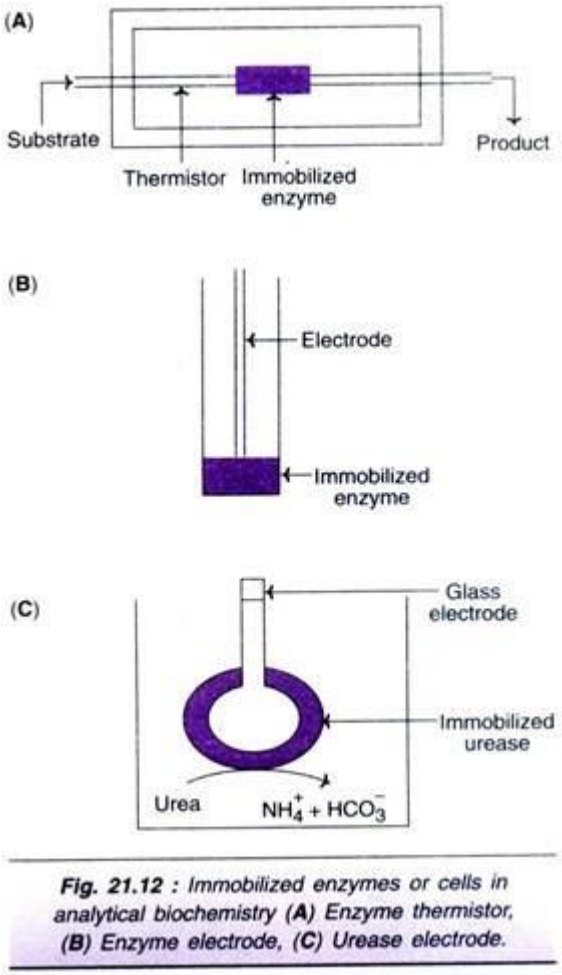

Fig. 21.12 : Immobilized enzymes or cells in analytical biochemistry (A) Enzyme thermistor, (B) Enzyme electrode, (C) Urease electrode.

In Affinity Chromatography and Purification:

Immobilized enzymes can be used in affinity chromatography. Based on the property of affinity, it is possible to purify several compounds e.g. antigens, antibodies, cofactors.

PENICILLIN GACYLASE

Penicillin Gacylase in one of the most important enzymes, it belonging to β-lactam antibiotics, first report on the enzyme penicillin acylase was in 1950 when they found in the mycelium of a Penicillium sp. The enzyme appeared to be a periplasmaticheterodimeric N-terminal serinehydrolase with a molecular mass of 86,183 Da, with a 23,817 Da (209 amino acids)α-subunit and a 62,366 Da (566 amino acids) β-subunit. This enzyme is capable of hydrolyzing penicillin G into phenyl acetic acid and 6-aminopenicillanic acid (6-APA) so this enzyme is the starting material for the manufacture of penicillin derivatives, which are the most widely used β-lactam antibiotics. Both natural and semi-synthetic penicillins contain 6-aminopenicillanic acid.