# INDUSTRIAL MICROBIOLOGY MCB 406

Course Lecturer
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**Industrial Microbiology**

Scope of Industrial Microbiology. Microorganisms of industrial importance. Fermentation processes including culture methods – solid and submerged. Strain selection and development, media formulation and economics. Optimization of the fermentation process including fermentation design, fermenters and operations. Primary and secondary metabolites. Microbiological quality control of foods, drugs and cosmetics. Use of enzymes in the industries

## INDUSTRIAL MICROBIOLOGY

### 1.0 Scope of Industrial Microbiology

### Industrial Microorganisms and Their Products

The major organisms used in industrial microbiology are fungi (yeasts and molds) and certain prokaryotes, in particular species of the genus Streptomyces. Industrial microorganisms can be thought of as metabolic specialists, capable of synthesizing one or more products in high yield. Industrial microbiologists often use classical genetic methods to select high-yielding microbial variants, with the goal being to increase the yield of the product to the point that an economically feasible process is possible. Thus the behaviour of the actual production strain may be far removed from that of the original wild-type strain.

*The use of microbes to obtain a product or service of economic value constitutes industrial microbiology*. *Any process mediated by or involving microorganisms in which a product of economic value is obtained is called fermentation*. The terms industrial microbiology and fermentation are virtually synonymous in their scope, objectives and activities. The microbial product may be

- microbial cells (living or dead), microbial biomass, and components of microbial cells, • microbial metabolites,
- intracellular or extracellular enzymes or
- chemicals produced by the microbes utilizing the medium constituents or the provided substrate
- and/or modified compound that has been microbiologically transformed • Recombinant products through the DNA recombinant technology.

The services generated by microorganisms range from the

- degradation of organic wastes,
- detoxification of industrial wastes and toxic compounds,
- the degradation of petroleum to manage oil spills, etc.

- Industrial microbiology also encompasses activities like production of biocontrol agents, inoculants used as biofertilizers, biofuel: etc.

The activities in industrial microbiology begin with the isolation of microorganisms from nature, their screening for product formation, improvement of product yields, maintenance of cultures, mass culture using bioreactors, and usually end with the recovery of products and their purification.

**Microbial Products of Potential Importance -**

| Product / Activity | Examples |
|---|---|
| **Products** | |
| 1. Amino acids | L-glutamic acid, L-lysine |
| 2. Antibiotics | Streptomycin, penicillin, tetracyclines, polymyxin |
| 3. Beverages | Wine, beer, distilled beverages |
| 4. Biodegradable plastic | β-polyhydroxybutyrate |
| 5. Enzymes | Amylase, proteases, pectinases, invertase, cellulase |
| 6. Flavouring agents | Monosodium glutamate, nucleotides |
| 7. Foods | Cheese, pickles, yoghurt, bread, vinegar |
| 8. Gases | $CO_2$, $H_2$, $CH_4$ |
| 9. Organic acids | Lactic, citric, acetic, butyric, fumaric |
| 10. Organic solvents | Acetone, ethanol, butanol, amyl alcohol |
| 11. Others | Glycerol, fats, steroids, gibberellins |
| 11 a. Vitamins | B12, riboflavin, A |
| 12. Recombinant proteins | Insulin, interferon, subunit vaccines |
| 13. Substrates | A wide range of compounds used for chemical syntheses of valuable products. |
| **Cells/Biomass** | |
| 14. Biomass | Food and feed yeast, other organisms used as single cell protein (SCP) |
| 15. Cells | Biofertilizers, biocontrol agents, bacterial insecticides, mycorrhyzae |
| 16. Vaccines | A variety of viral and bacterial vaccines |
| **Activities** | |
| Biotransformation | Steroids, antibiotics D-sorbitol |
| Degradation | Disposal of biological and industrial wastes, detoxification of toxic compounds, petroleum |
| Solubilization/accumulation | Improved recovery of oil and metals, discovery of new oil reserves, removal of toxic metals |

**Properties of a Useful Industrial Microorganism**

A microorganism used in an industrial process must have other features besides just being able to produce the substance of interest in high yield.

1. First and foremost, the organism must be capable of growth and product formation in large-scale culture.
2. It should produce spores (if fungi or yeast) or some other reproductive cell form so that it can be easily inoculated into the large vessels used to grow the producing organism on an industrial scale.
3. It must also grow rapidly and produce the desired product in a relatively short period of time.
4. It must also be able to grow in a liquid culture medium obtainable in bulk quantities at a low price. Many industrial microbiological processes use waste carbon from other industries as major or supplemental ingredients for large-scale culture media. These include corn steep liquor (a product of the corn wet-milling industry that is rich in nitrogen and growth factors) and whey (a waste liquid of the dairy industry containing lactose and minerals).
5. An industrial microorganism should not be pathogenic, especially to humans or

economically important animals or plants. Because of the high cell densities in industrial microbial processes and the virtual impossibility of avoiding contamination of the environment outside the growth vessel, a pathogen would present potentially disastrous problems.

6. Finally, an industrial microorganism should be amenable to genetic manipulation because increased yields are often obtained by means of mutation and classical genetic selection techniques. A genetically stable and easily engineered microorganism is thus a clear advantage for an industrial process.

**Problems often associated with Industrial Microbial Processes**

1. Finding the ***least expensive medium*** in which to grow the microbe so as to **maximize** yield and profits.
   - Often this is a **waste product** from another industrial process, such as corn steep liquor, sugar processing wastes or whey.
2. Maintaining strain purity and developing better strains for **improving the yield**. o A single mutation may decrease the yield by a significant percentage or result in undesirable substances being produced. The industrial research laboratories constantly seek better strains for the production of their product.
3. Preventing contamination by other microbes and by viruses (phage) that live on the microbe involved.
   - The media must be sterilized prior to being inoculated with the desired organism and purity must be maintained throughout the production process. A small quantity of a contaminant may produce an enzyme that can destroy the product in thousands of gallons of medium. For many microbes, viruses present a constant danger as a single virus can infect and destroy the desired microbe in an entire tank. The sterilization of large containers and huge quantities of media represent both an engineering and microbial challenge.
4. Developing rapid and efficient methods for purification of the desired produce in a stable form that is safe to use.
   - The products of many fermentations are often unstable in the IMPURE FORM or subject to unwanted modifications if they are not purified quickly. The final growth mixture may contain dangerous substances from which the desired product must be separated. As every step in the purification results in a loss of the product, the search for more efficient purification procedures is never ending.
5. Always striving to improve yield by modifying the strain, nutrients or environmental conditions.
   - As product yields are **sensitive** to subtle modifications in the nutrient and the environmental conditions, these are constantly monitored. For example, the pH, oxygen content, nitrogen/phosphorous ratio etc. may be adjusted during the production process.
6. Safe and inexpensive disposal of the massive quantities of **waste products** remaining after the product is formed. The waste products of these large fermentations present major waste disposal problems as they are rich in organic matter that are highly polluting if released untreated into the environment. However, the cost of treatment cuts into the profit margin and increases the cost of the product.

**Microbes History**

- Microbes have been employed for product generation, e.g., wines, bread, etc., since thousands of years, but these activities were purely art.
    - The science of industrial microbiology is only about 150 years old.
    - The first observations of microorganisms by Anthony Leeuwenhoek were published in 1677.
    - The experiments of Spallanzani in 1799 and of Schwan in 1837 not only disproved the idea of spontaneous generation of microorganisms, but also provided a means of sterilization of liquids (by heat) and air (by heat), respectively.
    - Schwan's findings also suggested that alcoholic fermentation was due to a fungus or mold, i.e., yeast, and inoculation resulted in quicker fermentation.
    - But microbiology is widely considered to have begun in 1857 when Luis Pasteur reported his studies on lactic acid fermentation, including the microscopic features of the microorganisms and a suitable medium for the process; the scientific basis of industrial microbiology began with this paper.
    - In 1860, Pasteur reported the first synthetic medium for microorganisms, and used it to study alcohol fermentation.
    - In 1861, Luis Pasteur showed that growth and physiology of yeast (and hence the accumulation of fermentation product, alcohol) differs depending on the presence or absence of $CO_2$. This phenomenon is known as Pasteur effect and is applicable to other microorganisms as well.
    - In 1878, Lister described the dilution technique for obtaining the first pure microbial culture of lactic acid bacterium.
    - A simpler and more effective technique for obtaining pure cultures from isolated separate colonies developed on solidified medium was described by Robert Koch in 1881; this technique is widely followed even today
    - In 1876, Cohn showed that bacterial spores have a high level of heat resistance and developed the technique of 'intermittent sterilization' for their inactivation. - In 1897, Buchner demonstrated alcohol fermentation by cell-free yeast juice; he suggested that a proteinaceous enzyme was responsible for fermentation. - Wildiers demonstrated in 1901 that yeast required growth factors (vitamins) for growth, especially at low inoculum levels; vitamins are used in fermentation even today. - In 1929, Alexander Fleming accidentally discovered penicillin produced by *Penicillium* growing as contaminant in a Petri plate of *Staphylococcus*. Fleming developed the technique for assay of antibacterial activity of penicillin using bacteria and showed its low toxicity to man and animals. This was followed by an intensive search for antibiotics during the Second World War leading to the discovery of streptomycin, chloramphenicol, tetracyclines, etc. Later developments have resulted in the use of metabolically blocked mutants of microorganisms, which accumulate large amounts of metabolic intermediates.

## 2.0 STRAIN SELECTION AND DEVELOPMENT

**Isolation and screening of microorganisms**

**Isolation of Microorganisms**

The first step in developing a producer strain is the isolation of concerned microorganisms from their natural habitats. Alternatively, microorganisms can be obtained as pure cultures from organisation, which maintain culture collections, e.g., **American Type Culture Collection** (**ATCC**) Rockville, Maryland, U.S.A.; **Commonwealth Mycological Institute** (CMI), Kew, Surrey, England; **Fermentation Research Institute** (FERM), Tokyo, Japan; U.S.S.R. **Research Institute for Antibiotics** (RIA), Moscow, U.S.S.R., etc.

The *microorganisms of industrial importance* are, generally, bacteria, actinomycetes, fungi and algae. These organisms occur virtually everywhere, e.g., in air, water, soil, surfaces of plants and animals, and plant and animals tissues. But most common sources of industrial microorganisms are soils, lake and river mud. Often the ecological habitat from which a desired microorganism is more likely to be isolated will depend on the characteristics of the product desired from it, and of process development. For example, if the objective is to isolate a source of enzymes, which can withstand high temperatures, the obvious place to look will be hot water springs. A variety of complex isolation procedures have been developed, but no single method can reveal all the microorganisms present in a sample. Many different microorganisms can be isolated by using specialized enrichment techniques, e.g., soil treatment (UV irradiation, air drying or heating at 120°C, filtration or continuous percolation, washings from root systems, treatment with detergents or alcohols, pre-inoculation with toxic agents), selective inhibitors (antimetabolites, antibiotics, etc.), nutritional (specific C and N sources), variations in pH, temperature, aeration, etc. The enrichment techniques are designed for selective multiplication of only some of the microorganisms present in a sample. These approaches however take a long time (20-40 days), and require considerable labour and money. The main isolation methods used routinely for isolation from soil samples are: sponging (soil directly), dilution, gradient plate, aerosol dilution, flotation, and differential centrifugation. Often these methods are used in conjunction with an enrichment technique.

**Screening of Microorganisms for New Products**

The next step after isolation of microorganisms is their screening.

A set of highly selective procedures, which allows the detection and isolation of microorganisms producing the desired metabolite, constitutes primary screening. Ideally, primary screening should be *rapid, inexpensive, predictive, specific* but effective for a broad range of compounds and applicable on a large scale. Primary screening is time consuming and labour intensive since a large number of isolates have to be screened to identify a few potential ones. However this is possibly the most critical step since it eliminates the large bulk of unwanted useless isolates, which are either non producers or producers of known compounds. Computer based databases play an important role by instantaneously providing detailed information about the already known microbial antibiotic compounds. Rapid and effective screening techniques have been devised for a variety of microbial products, which utilize either a property of the product or that of its biosynthetic pathway for detection of desirable isolates. Some of the screening techniques are relatively simple, e.g., for extracellular enzymes and enzyme inhibitors. However for most microbial products of high value, the screening is usually complex and tedious, and often may involve two or more steps, e.g., for antimicrobials. In some cases, it may be desirable to concentrate on a group of organisms expected to yield new products. For example, the search for new antibiotics now focusses on rare Actinomycetes, i.e., Actinomycetes other than those belonging to the genus

Streptomyces. Suitably designed specialized screening techniques may be used to detect compounds having various pharmacological activities other than antibiotics.

**Inoculum Development**

- The preparation of a population of microorganisms from a dormant stock culture to an active state of growth that is suitable for inoculation in the final production stage is called inoculum development. As a first step in inoculum development, inoculum is taken from a working stock culture to initiate growth in a suitable liquid medium. Bacterial vegetative cells and spores are suspended, usually, in sterile tap water, which is then added to the broth. In case of non-sporulating fungi and actinomycetes the hyphae are fragmented and then transferred to the broth. Inoculum development is generally done using flask cultures; flasks of 50 ml to 12 litres may be used and their number can be increased as per need. Where needed, small fermenters may be used. Inoculum development is usually done in a stepwise sequence to increase the volume to the desired level. At each step, inoculum is used at 0.5-5% of the medium volume; this allows a 20-200-fold increase in inoculum volume at each step. Typically, the inoculum used for production stage is about 5% of the medium volume.

## 3.0 Media Formulation and Economics

**Culture Media**

Inoculum preparation media are quite different from production media. These media are designed for rapid microbial growth, and little or no product accumulation will normally occur. Many production processes depend on inducible enzymes. In all such cases, the appropriate inducers must be included either in all the stages or at least in the final stages of inoculum development. This will ensure the presence of the concerned inducible enzymes at high levels for the production to start immediately after inoculation.

**Contamination**

- The inoculum used for production tanks must be contamination free. But the risk of contamination is always present during inoculum development. Therefore, every effort must be made to detect as well as prevent contamination.

**Sterilization**

- Sterilization is the process of inactivating or removing all living organisms from a substance or surface. In concept, it is regarded as absolute in all living cells must be inactivated / removed, usually in a single step at the given time. But in practice, the success of sterilization procedures is only a probability. Therefore, the probability of a cell escaping inactivation/filtration does exist although it is usually very small. When a closed system is sterilized once, it remains so indefinitely since it has no openings for the entry of microorganisms. But most fermentation vessels are open systems; such systems are initially sterilized and must be kept sterilized by ensuring the removal of living cells at their entry points, e.g., the cotton plug of a culture flask. **Common Contaminants**

- The most common contaminants of different industrial processes are considerably different. Some examples are given below

1. In canning industry, *Clostridium butylicum* is the chief concern. This obligate anaerobe can grow in sealed cans, and produce heat resistant spores and a deadly toxin. However, it is not a problem for catsup (too acidic), jam and jellies (too high sugar concentration) and milk (stored at low temperature).
2. Organisms like lactobacillus are a problem in production of wine.

3. In antibiotic industry, potential contaminants are many, e.g., molds, yeast, and many bacteria, including Bacillus.
4.The most dreaded contaminants of fermentation industry are phages. The only effective protection against phages is to develop resistant strains.

**Sterilization Procedures**

- Sterilization involves either inactivation or removal of living organisms. This may be achieved by

(i) heating,
(ii)irradiation.
(iii) Chemicals or
(iv) filtration; these are briefly discussed below.

**Heating.**

- It is the most commonly used and the least expensive sterilizing agent. - Dry heat is used in ovens and is suitable for sterilization of solids, which can withstand the high temperatures needed for sterilization, e.g., laboratory glassware, talc, etc. Steam, i.e., moist or wet heat, is used for sterilization of media and fermenter vessels. An autoclave uses steam for sterilization (at 121°C and 15p.s.i.). the period of time at this temperature pressure depending on medium volume, e.g., 12-15 min for 200 ml. 17-22 min for 500 ml, 20-25 min for 1 L and 30-35 min for 2 L.
- But sterilization of oils will require a few hours, and concentrated media (10-20% solid) must be agitated for effective sterilization. Autoclaves can also be used to sterilize laboratory vessels, small volumes of media and even small fermenters. Large fermenters are sterilized by either a direct injection of steam or by indirect heating by passing steam through heat exchange coils or a jacket. The steam should always be saturated. Media sterilization may be achieved in a continuous flow sterilization system either by direct steam injection or by indirect steam heating, and then filled in a sterile fermenter. Alternatively, the medium may be filled in the fermenter and steam sterilized with the latter. Heat killing in most part is due to protein inactivation. In general, moist heat is far superior to dry heat.
- Bacterial spores are the most heat resistant, e.g., spores of thermophilic bacteria can survive steam at 30p.s.i. at 134°C for 1-10 min and dry heat at 180°C for up to 15 min.

**Radiation.**

High energy X-rays are used for sterilization of a variety of lab ware and of food. In general, vegetative cells are much more susceptible than bacterial spores (Clostridium spores can resist nearly 0.5 M rad). But *Deinococcus radiodurans* vegetative cells can survive 6 M rad.

Viruses are usually similar to bacterial spores but some viruses, e.g., encephalitis virus require up to 4.5 M rad. In practice, 2.5 M rad is used for sterilizing pharmaceutical and medical products. X-rays cause inactivation by inducing single and double strand DNA breaks, and by producing free radicals and peroxides, to which -SH enzymes are particularly susceptible.

**Chemicals**

. The chemicals used for sterilization cause inactivation by oxidation or alkylation; these are formaldehyde, $H_2O_2$, ethylene oxide, propylene oxide etc. $H_2O_2$ (10-25% w/v) is being increasingly used in the sterilization of milk and of containers for food products. It is a powerful oxidizing agent, kills both vegetative cells and spores and is very safe. Ethylene oxide is used for sterilizing equipment, which are likely to be damaged by heat, and is very effective, but highly toxic and violently explosive if mixed with air.

**Filtration.**
Aerobic fermentation requires a very high rate of air supply often amounting to 1 vol of air (equal to medium volume) every minute. Air contains both fungal spores and bacteria, which are ordinarily removed by filtration using either a depth filter or a screen filter. Depth filters are made from fibrous or powdered materials pressed or bonded together in a relatively thick layer; the materials used are fiberglass, cotton, mineral wool, cellulose fibers, etc. in form of mats, wads or cylinders. Modern depth filters are cylinders of bonded borosilicate microfibers. Depth filters allow higher filtration rates and efficiencies than screen filters, but are not suitable for filtration of moist air. Screen filters are membranes of cellulose esters or other polymers with pores of 0.45 µm or smaller (bacterial contaminants are 0.5 µm or larger). Usually, a microfibers profiler is used with such filters to remove gross contamination. All filters themselves must be sterilized before they can be used to sterilize the air. Filters are also used to sterilize the effluent gasesfrom fermenters, especially in case of pathogenic microorganisms.

## 4.0 Strain Selection and Development

**Strain Improvement**
- After an organism producing a valuable product is identified, it becomes necessary to increase the product yield from fermentation to minimise production costs. Product yields can be increased by
(i) developing a suitable medium for fermentation,
(ii) refining the fermentation process and
(iii) improving the productivity of the strain

Generally, major improvements arise from the last approach; therefore, all fermentation enterprises place a considerable emphasis on this activity. The techniques and approaches used to genetically modify strains, to increase the production of the desired product are called strain improvement or strain development. Strain improvement is based on the following three approaches: (i) mutant selection, (ii) recombination, and (iii) recombinant DNA technology.

**(i) Mutant Selection**
Large scale mutant selection programmes begin when favourable reports of clinical trials are obtained. In the early stages, selection of spontaneous mutants may be helpful, but induced mutations are the most common sources of improvements. Many mutations bring about marked changes in a biochemical character of practical interest; these are called major mutations. Some major mutations can be useful in strain improvement. For example, a mutant strain (S-604) of *Streptomyces aureofaciens* produces 6-demethyl tetracycline in place of tetracycline; this demethylated form of tetracycline is the major commercial form of tetracycline. In contrast, most improvements in biochemical production have been due to the stepwise accumulation of so called minor genes. These genes lead to small increases (or decreases) in the antibiotic or other biochemical production, and selection may be expected to result in a10-15% increase in yield. The selected strains are usually subjected to successive cycles of mutagenesis and selection; after several cycles, a large increase is yield is likely to be obtained. Mutants of *Penicillium chrysogenum* were selected for increased penicillin production; each cycle of selection was preceded by Mutagen (chemical) treatment and

resulted in only small changes in penicillin yield. The two types are associated with different strains of *Agrobacterium. tumefaciens*. There is also a related disease ("hairy root disease") in which infected tissues proliferate in root tissue and produce an opine. This disease is associated with the bacterium *A. rhizogenes*. Formation of opines explains the ecological significance of tumor formation. Each strain of Agrobacterium synthesizes enzymes (permease, dehydrogenase) that allow it to metabolize the specific type of opine formed by the tumor it induces. Thus by stimulating the plant to form opines, the bacteria insure themselves a supply of nutrients specifically designed for them. After several (about dozen) cycles of selection, a strain (E 15-1) was obtained that yielded 55% more penicillin than the original strain (Fleming strain).

**Selective Isolation of Mutants**

A majority of desirable mutants, especially the 'minor gene' mutants, showing increased production are isolated by screening a large number of clones surviving the mutagen treatment; this is called secondary screening. But this approach requires a large amount of work. Therefore, efforts have increasingly focussed on developing techniques for the isolation of particular classes of mutants, which are likely to be overproducers.

1. Isolation of auxotrophic mutants is the basis for commercial amino acid production in Japan from the bacterium *Corynebacterium glutamicus*. For example, phe-mutants of *C glutamicus* accumulate tyrosine.

2. Many analogue-resistant mutants have feed-back insensitive enzymes of the biosynthetic pathway the analogue of whose product was used for selection of such cells. Such mutants tend to overproduce the end product of the concerned pathway.

3. Sometimes revertants from nonproducing mutants of a strain are high producers, e.g., one such reversion mutant of *Streptomyces viridifaciens* showed over 6-fold increase in chlortetracycline production over the original strain from which the nonproducing mutant was obtained.

4. Reversion mutants of appropriate auxotrophs may often be high producers. 5. In some cases, selection for resistance to the antibiotic produced by the organism itself may lead to increased yields.

6. Sometimes, mutants with altered cell membrane permeability show high production of some metabolites.

7. Mutants have been selected to produce altered metabolites, especially in case of aminogycoside antibiotics. For example, *Pseudomonas aureofaciens* produces the antibiotic pyrrolnitrin; a mutant of this fungus yields 4'- fluoropyrrolnitrin. Mutant selection has been the most successful approach for strain improvement, but major advances are being made in the exploitation of other strategies, i.e., recombination and recombinant DNA technology.

**Different Approaches in Utilization of Mutation and Genetic Recombination for Strain Improvement -**

| Approach | Chief feature | Example/Remark |
|---|---|---|
| **A. Mutant Selection : Types** | | The main approach to strain improvement; produces new alleles of existing genes |
| 1. Spontaneous Mutations | Occur without any treatment with a mutagen | Used in, the initial stages of strain improvement; also for maintenance of improved strains |
| 2. Induced Mutations | Induced by chemical (mainly) or physical mutagens | Mutagenesis followed by selection; several cycles employed |
| 3. Major Mutations | Affect the pattern of metabolite production | Production of 6-demethyl tetracycline in place of tetracycline by S. aureofaciens |
| 4. Minor Mutations | Affect the rate metabolite production | Small gains in each cycle of selection; substantial improvement after several cycles |
| **B. Mutant Selection :Strategies** | | |
| 1. Auxotrophic mutants | Defective biosynthesis of a biochemical | Enhanced production of an amino acid, e.g., phe mutants accumulate tyrosine |
| 2. Analogue resistant mutants | Feedback insensitive enzymes | Overproduction of metabolites, e.g., amino acids by C. glutamicus |
| 3. Revertants of nonproducing mutants | | Some mutants are high producers, e.g., chlortetracycline by S. viridifaciens |
| 4. Revertants of auxotrophic mutants | | Some are high produces, e.g. chlortetracycline by S. viridifaciens |
| 5. Resistance to the antibiotic produced by the organism itself | | Increased production, e.g., chlortetracycline by S. aureofaciens |
| **C. Recombination** | | Produces new combinations of existing alleles |
| 1. Sexual reproduction | Conjugation; fusion of gametes | Some bacteria and Actinomycetes; fungi and yeast |
| 2. Heterokaryosis | Nuclear fusion followed by mitotic recombination and mitotic reduction | Fungi |
| 3. Protoplast fusion | Protoplasts produced by lytic enzymes fusion by PEG, recombinant recovery | Bacteria, Actinomycetes, fungi; quite; successful |

## 5.0 Optimization of Fermentation Process

**Bioreactors -**

*A bioreactor is a device in which a substrate of low value is utilized by living cells or enzymes to generate a product of higher value*. Bioreactors are extensively used for food processing, fermentation, waste treatment, etc. On the basis of the agent used, bioreactors are grouped into two broad classes:

(i) those based on living cells and,

(ii) those employingenzymes.

But in terms of process requirements, they are of the following types:

(i) aerobic,

(ii) anaerobic,

(iii) solid state, and

(iv) immobilized cell bioreactors.

All bioreactors deal with heterogeneous systems having two or more phases, e.g., liquid, gas, solid. Therefore, optimal conditions for fermentation necessitate efficient transfer of mass, heat and momentum from one phase to the other. A bioreactor should provide for the following: (i) agitation (for mixing of cells and medium),

(ii) aeration (aerobic fermenters; for $O_2$ supply),

(iii) regulation of factors like temperature, pH, pressure, aeration, nutrient feeding, liquid level, etc.,

(iv) sterilization and maintenance of sterility, and

(v) withdrawal of cells/medium (for continuous fermenters). Modem fermenters are usually integrated with computers for efficient process monitoring, data acquisition, etc.

The size of fermenters ranges from 1-2 Litres laboratory fermenters to 500,000 litres or,

occasionally, even more; fermenters of up to 1.2 million litres have been used. Generally, 20-25% of fermenter volume is left unfilled with medium as "head space" to allow for splashing, foaming and aeration. The fermenter design varies greatly depending on the type of fermentation for which it is used.

**Immobilized Cell Bioreactors**

Bioreactors of this type are based on immobilized cells. Cell immobilization is advantageous when

(i) the enzymes of interest are intracellular,
(ii) extracted enzymes are unstable,
(iii) the cells do not have interfering enzymes or such enzymes are easily inactivated/removed, and (iv) the products are low molecular weight compounds released into the medium.

Under these conditions, immobilized cells offer the following advantages over enzyme immobilization:

(i) enzyme purification is not needed,
(ii) high activity of even unstable enzymes,
(iii) high operational stability,
(iv) lower cost and
(v) Possibility of application in multistep enzyme reactions.
(vi) In addition, immobilization permits continuous operation of bioreactor, which reduces the reactor volume and, consequently, pollution problems. Obviously, immobilized cells are used forsuch biotransformation of compounds, which require action of a single enzyme.

Cell immobilization may be achieved in one of the following ways:

1. Cells may be directly bound to water insoluble carriers, e.g., cellulose, dextran, ion exchange resins, porous glass, brick, sand, etc., by adsorption, ionic bonds or covalent bonds.
2. They can be cross linked to bi- or multi-functional reagents, e.g., glutaraldehyde, etc. 3. Polymer matrices may be used for entrapping cells; such matrices are polyacrylamide cell, κ-carrageenan (a polysaccharide isolated from a seaweed), calcium alginate (alginate is extracted from seaweed), poly glycol oligomers, etc. Out of these approaches, calcium alginate immobilization is the most commonly used since it can be used for even very sensitive cells. Cell immobilization has been used for commercial production of amino acids, organic acids, etc.

**Bioreactor Media**

The medium composition is as critical to product yields as high producing strains of microorganisms. The medium not only provides the nutrients needed for microbial growth but also for the metabolite production. The organisms vary greatly in their nutrient requirements from autotrophs, which produce all the biochemical required from simple inorganic nutrients deriving their energy from oxidation of some inorganic component of the medium to the difficult organisms like lactic acid bacteria, which require many organic compounds for their growth.

The various media for bioreactors may be grouped into two broad categories:

(i) **Synthetic** and (ii) **Complex**.

A synthetic or chemically defined medium is desirable for various studies, but product yields from such media are generally low. Foaming is not a problem with such media. The complex media contain undefined constituents like soybean meal, molasses, corn steep liquor, etc., and give much higher yields of metabolites. Carbon source can be simple, e.g., sugar, alcohol, etc., or complex carbohydrates, proteins, molasses, potatoes, sweet potatoes, etc. In many processes, precursors need to be provided, e.g., phenylacetic acid for penicillin G, inorganic cobalt for vit. $B_{12}$. Buffers are also added to prevent drastic changes in pH, and anti-foam would often be needed when complex media are used. For much fermentation, e.g., antibiotic production, medium suited for rapid cell growth is unsuitable for product formation. In such cases, specialized media for production have to be devised.

**Downstream Processing**

- *The various processes used for the actual recovery of useful products from a fermentation or any other industrial process is called downstream processing.* The cost of **downstream processing** (DSP) is often more than 50% of the manufacturing cost, and there is product loss at each step of DSP.

Therefore, the DSP should be efficient, involve asfew steps as possible (to avoid product loss), and be cost-effective. The various steps in DSPareasfollows:

(i) separation of particles,
(ii) disintegration of cells,
(iii) extraction,
(iv) concentration,
(v) purification and
(vi) drying.

**(i) Separation of Particles**

- The first step in DSP is the separation of solids, usually cells, from the liquid medium. This is generally achieved as follows.

**(a) Filtration**

. It is used for the separation of filamentous fungi and filamentous bacteria, e.g., streptomycetes, and often for yeast flocks. The various techniques of filtration employed are, *surface filtration, depth filtration, centrifugal filtration, cross flow filtration, and rotary drum vacuum filtration*.

**Centrifugation.**

It may be used to separate bacteria and usually protein precipitates. But difficulties arise due to small differences in the densities of the particles and the medium. In addition, equipment cost, power consumption, temperature, etc. are the other disadvantages. **Flocculation and Floatation**

Flocculation, i.e., sticking together of cells, can be induced by inorganic salts, mineral hydrocolloids are organic polyelectrolytes. Since sedimentation rate of a particle increases with size, flocculated cells can be recovered by centrifugation. In cases, where flocculation is not effective, very fine gas bubbles can be created by sparging, release of overpressure or electrolysis. The gas bubbles adsorb to and surround the cells, raising them to the surface of medium in form of foam (floatation); long chain fatty acids or amines promote stable foam

formation. The cells collected in the foam are readily recovered. Flocculation and floatation are used for the most efficient recovery of microbial biomass in some single cell protein production systems.

## 6.0 Fermentation Processes

### Fermentation

Inoculum is developed in several stages. The organisms are often multiplied in one or more seed tank stages. The medium used for these stages is rather rich to support good growth. The seed tank culture is finally inoculated into the production stage fermenter (25,000-100,000 1).The production medium is almost always complex and is devised to maximise yields of the enzyme. Appropriate measures must be employed to ensure contamination control. Biomass of the organism begins to increase early during fermentation, while enzyme activity shows significant increases about midway through the fermentation; the end of fermentation is signalled by cessation of enzyme synthesis.

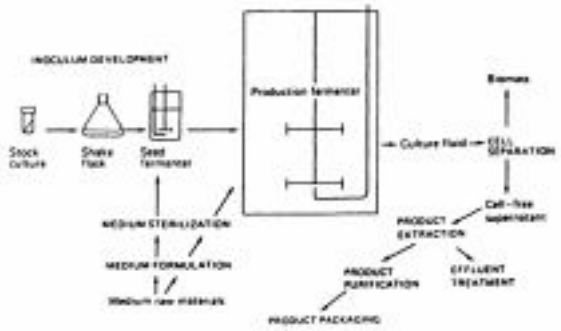



**Figure 3** *A generalized, schematic representation of a fermentation process* (Reproduced with permission from P. F. Stanbury, A. Whitaker and S. J. Hall, 'Principles of Fermentation Technology', Pergamon Press, Oxford, 1995)

### Isolation and Purification

Isolation and purification is done immediately after termination of fermentation in a manner that retains the enzyme activity. If the cells are to be used for immobilization, the biomass is isolated and treated to make it ready for use. The extracellular enzymes are recovered directly from broth, while enzymes localized within cells are isolated by rupturing the cells. Enzyme purification is based on various techniques whose efficacy and cost differ widely; the process used will mainly depend on the purity needed and the cost, which is acceptable.

### *Types of Fermentation Process*

The fermentation unit in industrial microbiology is similar to a chemical plant in the chemical industry. A fermentation process is a biological process and, therefore, has requirements of sterility and use of cellular enzymatic reactions instead of chemical reactions aided by inanimate catalysts, sometimes operating at elevated temperature and pressure. Industrial fermentation processes may be divided into two main types, with various combinations and modifications.

Some of the most important types of fermentation are as follows:

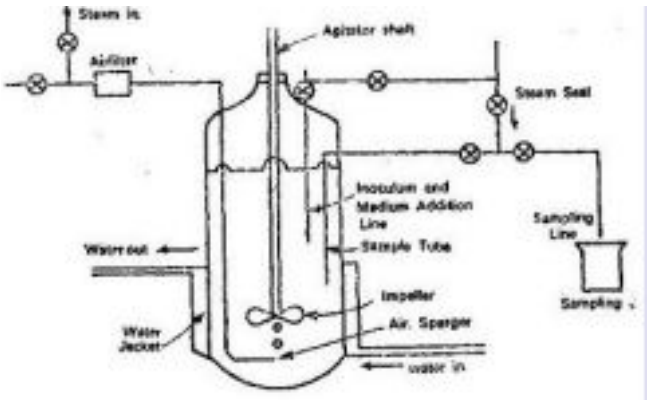
1. Solid State Fermentation
2. Submerged Fermentation
3. Anaerobic Fermentation
4. Aerobic Fermentation
5. Immobilized Cell Bioreactors
6. Immobilized Enzyme Bioreactors.

## 1. Sub-merged Fermentation (SLF)
## (Submerged Liquid Fermentation)

### (a) Batch Fermentation Process

Fermentation is derived from the latin word "*fervere*" which means to boil, a process typically manifested by the action of yeasts on malted grain during the production of alcoholic beverages. *Fermentation could be regarded as a metabolic process involved in the conversion of sugar to acids, gases and/or alcohol*. The growth of microorganisms generally results in metabolite production but specific metabolite requires optimal cultural conditions at a particular growth rate for save delivery. A closed loop system where yeasts or bacteria grow in a culture media and an additional material is not added such is called BATCH CULTURE. Growth in a batch culture undergo some series of steps. There is the initial phase that seems to record no growth, the lag phase, followed by the gradual increase that builds up into constant, maximum rate often referred to as the logarithmic phase or exponential phase.

A tank of fermenter is filled with the prepared mash of raw materials to be fermented. The temperature and pH for microbial fermentation is properly adjusted, and occasionally nutritive supplements are added to the prepared mash. The mash is steam sterilized in a pure culture process. The inoculum of a pure culture is added to the fermenter, from a separate pure culture vessel. Fermentation proceeds, and after the proper time the contents of the fermenter, are taken out for further processing. The fermenter is cleaned and the process is repeated. Thus each fermentation is a discontinuous process divided into batches




### (b) Continuous Fermentation Process

Growth of microorganisms during batch fermentation conforms to the characteristic growth curve, with a lag phase followed by a logarithmic phase. This, in turn, is terminated by progressive decrement in the rate of growth until the stationary phase is reached. This is because of limitation of one or more of the essential nutrients and /or production of inhibitory substances. In continuous fermentation, the substrate is added to the fermenter continuously at a fixed rate. This maintains the organisms in the logarithmic growth phase. The fermentation products are taken out continuously. The continuous fermentation is also referred to as chemostat.

### (c) Fed-Batch Culture:

When a batch culture is subsequently led with fresh nutrient medium without removing the growing microbial culture, it is called fed-batch culture. Fed-batch culture allows one to supplement the medium with such nutrients that are depleted or that may be needed for the terminal stages of the culture, e.g., production of secondary metabolites.

Therefore, the volume of a fed- batch culture increases with time. Fed-batch cultures achieve higher cell densities than batch cultures. It is used when high substrate concentration causes growth inhibition. It allows the substrate to be used at lower nontoxic levels, followed by subsequent feeding. It allows the maximum production of cellular metabolites by the culture.

**2. Solid State Fermentation (SSF)**

Solid-state fermentation (SSF) processes can be defined as "the growth of microorganisms (mainly fungi) on moist solid materials in the absence of free-flowing water".

Semi Solid OR Solid State Methods - In this, the culture medium is impregnated in a carrier such as bagasse, wheat bran, potato pulp, etc. and the organism is allowed to grow on this. This method allows greater surface area for growth. The production of the desirable substance and the recovery is generally easier and satisfactory. In the development of a fermentation process, the composition of the culture medium plays a major role and will determine to a very great extent the level of end product. For example, a culture medium containing sucrose enables better production of citric acid by *A. niger* than any other carbohydrate. The pH, temperature of incubation, aeration etc., are all important factors in fermentations and these have to be optimized for each type of fermentation. Emphasis is generally placed on the use of cheap raw materials so that the cost of production is low. In such fermentations, microbial growth and product formation occur at the surface of solid substrates. Examples of such fermentations are mushroom cultivation, mold ripened cheeses, starter cultures, etc. More recently, this approach has been used for the production of extracellular enzymes, certain valuable chemicals, fungal toxins, and fungal spores (used for biotransformation).Traditional substrates are several agricultural products, rice, wheat, maize, soybean, etc. The substrate provides a rich and complex source of nutrients, which may or may not need to be supplemented. Such substrates selectively support mycelial organisms, which can grow at high nutrient concentrations and produce a variety of extracellular enzymes, e.g., a large number of filamentous fungi, and a few bacteria (Actinomycetes and one strain of Bacillus).

According to the physical state, solid state fermentations are divided into two groups: (i) low moisture solids fermented without or with occasional/continuous agitation, and (ii) suspended solids fermented in packed columns, through which liquid is circulated. The fungi used for solid state fermentations are usually obligate aerobes. Solid state fermentations on large scale use stationary or rotary trays. Temperature and humidity controlled air is circulated through the stacked solids. Less frequently, rotary drum type fermenters have been used. Solid state fermentations offer certain unique advantages, but suffer from some important disadvantages. However, commercial application of this process for biochemical production is chiefly confined to Japan.

Solid State Fermentation, SSF

The following are some the advantages of SSF over submerge liquid fermentation (SLF): 1. Comparative studies between submerged liquid fermentation (SLF) and SSF claim higher yields than those obtained in the corresponding submerged cultures 2. The low availability of water reduces the possibilities of contamination by bacteria and yeast. This allows working in aseptic conditions in some cases.

3. Similar environment conditions to those of the natural habitats for fungi, which constitute the main group of microorganisms used in SSF.

4. Higher levels of aeration, especially adequate in those processes demanding an intensive oxidative metabolism.
5. The inoculation with spores (in those processes that involve fungi) facilitates its uniform dispersion through the medium.
6. Culture media are often quite simple. The substrate usually provides all the nutrients necessary for growth.
7. Simple design reactors, with few spatial requirements can be used due to the concentrated nature of the substrates.
8. Low energetic requirements (in some cases autoclaving or vapour treatment, mechanical agitation and aeration are not necessary).
9. Small volumes of polluting effluents. Fewer requirements of dissolvents are necessary for product extraction due to their high concentration.
10. The low moisture availability may favour the production of specific compounds that may not be produced or may be poorly produced in SLF.
11. In some cases, the products obtained have slightly different properties (e.g. more Thermo-tolerance) when produced in SSF in comparison to SLF.
12. Due to the concentrated nature of the substrate, smaller reactors in SSF with respect to SLF can be used to hold the same amounts of substrate

**In the same way, SSF has some disadvantages when compared with the submerged-liquid cultures:**

1. Only microorganisms that can grow at low moisture levels can be used. 2. Usually the substrates require pre-treatment (size reduction by grinding, rasping or chopping, homogenisation, physical, chemical or enzymatic hydrolysis, cooking or vapour treatment).
3. Biomass determination is very difficult.
4. The solid nature of the substrate causes problems in the monitoring of the process parameters (pH, moisture content, and substrate, oxygen and biomass concentration). 5. Agitation may be very difficult. For this reason static conditions are preferred. 6. Frequently needs high inoculum volumes.
7. Many important basic scientific and engineering aspects are poorly characterized. Information about the design and operation of reactors on a large scale is scarce. 8. Possibility of contamination by undesirable fungi.
9. The removal of metabolic heat generated during growth may be very difficult. 10. Extracts containing products obtained by leaching of fermented solids are often viscous of nature.
11. Mass transfer limited to diffusion.
12. In some SSF, aeration can be difficult due to the high solids concentration. 13. Spores have longer lag times due to the need for germination.
14. Cultivation times are longer than in SLF

**3. Anaerobic Fermentation**

Basically a fermenter designed to operate under microacrophilic or anaerobic conditions will be the same as that designed to operate under aerobic conditions, except that arrangements for intense agitation and aeration are unnecessary Many anaerobic fermentations do, however, require mild aeration for the initial growth phase, and sufficient agitation for mixing and maintenance of temperature. In anaerobic fermentation, a provision for aeration is usually not needed. But in some cases, aeration may be needed initially for inoculum build up. In most cases, a mixing device is also unnecessary; while in some cases initial mixing of the inoculum is necessary. Once the fermentation begins, the gas produced in the process generates sufficient mixing. The air present in the headspace of the fermentor should be replaced by $CO_2$, $H_2$, $N_2$ or a suitable mixture of these; this is particularly important for obligate anaerobes like Clostridium. The fermentation usually liberates $CO_2$ and $H_2$, which are collected and used, e.g., $CO_2$ for making dry ice and methanol, and for bubbling into freshly inoculated fermenters. In case of acetogens and other gas utilizing bacteria, $O_2$ free sterile $CO_2$ or other gases are bubbled through the medium. Acetogens have been cultured in 400 Litre-fermenters by bubbling sterile $CO_2$ and 3kg cells could be harvested in each run.
Recovery of products from anaerobic fermenters does not require anaerobic conditions. But many enzymes of such organisms are highly $O_2$, sensitive. Therefore, when recovery of such enzymes is the objective, cells must be harvested under strictly anaerobic conditions.

**4. Aerobic Fermentation**

A number of industrial processes, although called 'fermentations', are carried on by microorganisms under aerobic conditions. In older aerobic processesit was necessary to furnish a large surface area by exposing fermentation media to air. In modern fermentation processes aerobic conditions are maintained in a closed fermenter with submerged cultures. The contents of the fermenter are agitated with an impeller and aerated by forcing sterilized air The main feature of aerobic fermentation is the provision for adequate aeration; in some cases, the amount of air needed per hour is about 60-times the medium volume. Therefore, bioreactors used for aerobic fermentation have a provision for adequate supply of sterile air, which is generally sparged into the medium. In addition, these fermenters may have a mechanism for stirring and mixing of the medium and cells. Aerobic fermenters may be either of the (i) stirred tank type in which mechanical motor driven stirrers are provided or (ii) of air lift type in which no mechanical stirrers are used and the agitation is achieved by the air bubbles generated by the air supply. Generally, these bioreactors are of closed or batch type, but continuous flow reactors are also used, such reactors provide a continuous source of cells and are also suitable for product generation when the product is released into the medium

**5. Surface Culture Method**

In this method the organism is allowed to grow on the surface of a liquid medium without agitation. After an appropriate incubation period the culture filtrate is separated from the cell mass and is processed to recover the desirable product. Sometimes the biomass may be reused. Examples of such fermentations are the alcohol production, the beer production and citric acid production. This method is generally time consuming and needs large, area or space.

**6. Submerged Culture Method**

In this process, the organism is grown in a liquid medium which is vigorously aerated and agitated in large tanks called fermentors. The fermentor could be either an open tank or a closed tank and may be a batch type or a continuous type and are generally made of non-corrosive type of metal or glasslined or of wood. In batch fermentation, the organism is

grown in a known amount of culture medium for a defined period of time and then the cell mass is separated from the liquid before further processing while in the continuous culture, the culture medium is withdrawn depending on the rate of product formation and the inflow of fresh medium. Most fermentation industries today use the submerged process for the production of microbial products.