Pharmaceutical Biotechnology

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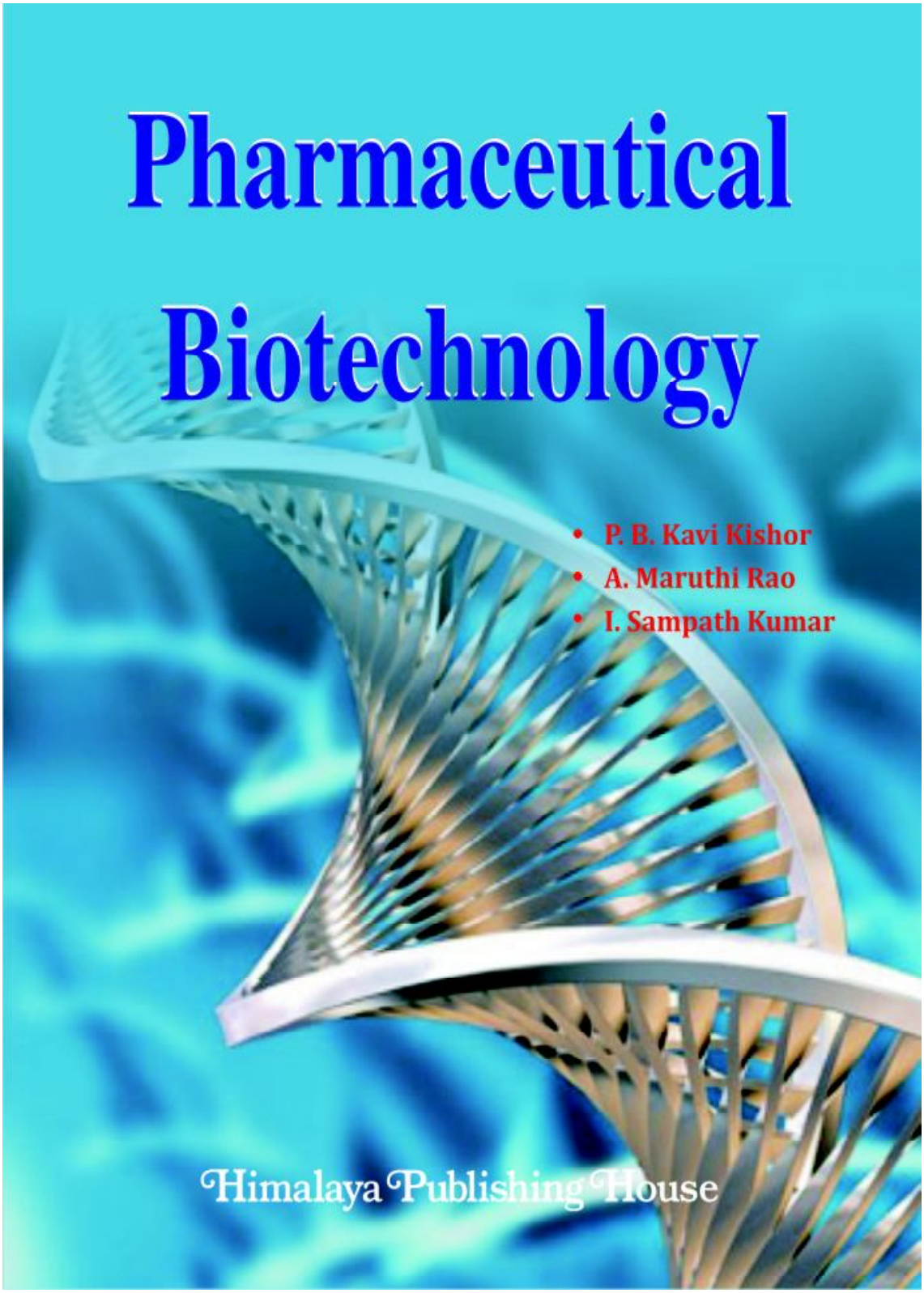

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Preface

The text book on "Pharmacuetical Biotechnology" has been written keeping in view of the B.Pharmacy (IV Year) syllabus of Jawaharlal Nehru Technological University, Hyderabad, though it can cater to the needs of other students and academicians in Biology as such. However, this is not a comprehensive book on "Pharmaceutical Biology". We hope that the students scholars and faculty members alike would appreciate our little endeavour and encourage this.

Our special thanks are due to Prof. K. Shiva Shankar, Registrar, Telangana University for his constant encouragement. We express our gratitude to Prof. M. Yadagiri, Principal, University College, Telangana University for his encouragement. We would like to thank Ms.T. Deepti, Department of Pharmacology, SRM University, Chennai for her significant contribution at every stage in the preparation of this book and providing much current literature. We also thank our student Mr. M. Ebenezer for his help at various stages of the preparation of this book and in designing the cover page.

We would like to thank Mr. Krishna Poojari, Himalaya Publishing House, Hyderabad branch for supporting us and bringing out the book timely with a nice get-up.

P.B. Kavi Kishor

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I. Sampath Kumar

Contents

CHAPTER 1

Fermentation Technology

Isolation and Selection of Industrially important Microbes :

A suitable microorganism is a critical requisite for any fermentation process. A wide variety of microorganisms have the property of producing some specific compounds in the medium. Such microbes are isolated from the pool of microbes found in a culture. The most suitable organism is sought by screening (identifying) from a population or creating specific strains of microorganisms that will yield high quantities of the desired product by genetic manipulations. The microorganisms of industrial importance are generally, bacteria, actinomycetes, fungi and algae. These organisms occur virtually everywhere, ex: in air, water and soil, on the surfaces of the plants and animals, and in plant and animal tissues. But, the good sources for the isolation of microorganisms are soils, lakes and river muds. Many different microorganisms can be isolated by using specialised enrichment techniques, Eg: soil treatment (UV irradiation, air drying or heating at 70-120^{0}C, filteration or continuous percolation, washings from root system, treatment with detergents (or) alcohols, pre incubation with toxic agents), selective inhibitors (antimetabolites, antibiotics, etc.,), nutritional variations (specific C and N sources), variations in pH, temperature, aeration etc.

The right microorganism which produces a large amount of novel product is selected and screened. The selected strain should have relatively stable characterstics and the ability to grow rapidly and vigourously and economically important strain should be genetically stable, but amenable to genetic modification. The strain should essentially be non-pathogenic, non-producer of any unwanted by-products or toxins. An ideal producer (strain) should be amenable to long-term conservation and the risk of contamination should be minimal under the optimum conditions.

Screening of Industrially important Microbes :

Screening is defined as the use of highly selective procedures to allow the detection and isolation of microorganisms producing the desired metabolite from among a large microbial population.

Primary Screening :

Primary screening allows the detection and isolation of microorganisms that posses potentionally interesting industrial applications. Primary screening is followed by secondary screening which can be qualitative or quantitative in its approach. Secondary screening is conducted on agar plates, in flasks or small fermenters containing liquid media, or as a combination of these approaches. The qualitative approach, tells us the spectrum or range of microorganisms which are sensitive to a newly discovered antibiotic. The quantitative approach tells us the yeilds of antibiotic which can be expected when the microorganism is grown in various media.

Secondary Screening :

Secondary screening should yeild the types of information which are needed in order to evaluate the true potential of a microorganism for industrial usage. It should determine whether the microorganisms are actually producing new chemical compounds not previously described. Secondary screening should reveal whether there are pH, aeration, or other critical requirements associated with particular microorganisms, both for the growth of microorganism and for the formation of chemical products. Secondary screening should reveal whether a product resulting from the microbial fermentation occurs in the cultured broth in more than one chemical form, and whether it is an optically or biologically active material. It should also reveal whether microorganisms are able to chemically alter or even destroy their own fermentation products.

Table 1.1 : Some selected valuable products from microbial sources representing potential targets for directed screening.

Product	***Activity***	***Source (Producing Microorganism)***
Avermectin	Antihelmintic	*Streptomyces avermitilis*
Bestatin	Antitumour	*Streptomyces olivoreticuli*
1, 3-Diphenethylurea	Antidepressant	*Streptomycs sp.*
Detoxin	Detoxicant	*S. caespitosus*
Herbicidin	Herbicide	*S. sagamonensis*
Cyclosporin	Immunosuppressor	*Trichoderma polysporum*
Piercidin	Insecticide	*S. mobaraensis*
Tetranactin	Miticide	*S. aureus*
Gibberellic acid	Plant growth regulator	*Gibberella fujikuroi*
Slaframine	Salivation inducer	*Rhizoctoniz leguminicola*
Azalomycin	Antiprozoal	*S. hygroscopicus*
Aabomycin A	Antiviral	*S. hygroscopicus*

Table 1.2 : Fermentation based commercial products :

Microorganism	Final product
Industrial 'oxychemicals' *(alcohols & solvents)*	
Saccharomyces cerevisiae	Ethanol
Kluyveromyces fragillis	Ethanol
Clostridium acetobutylicum	Acetone, isopropanol & butanol
Saccharomyces sp.	Glycerol
Acetobacter sp.	Sorbitol
Bacillus sp.	Propylene glycol
Organic acids	
Aspergillus niger	Citric acid
Lactobacills delbrueckii	Lactic acid
Bacillus sp.	Acrylic acid
Acetobacter sp.	Acetic acid
Propionibacterium shermanii	Propionic acid
Rhizopus sp.	Fumaric acid
Enzymes	
Aspergillus niger / A. oryzae	Glucoamylase
Bacillus subtilis	Amylase / neutral protease
Trichoderma reesei	Cellulase
Saccharmyces cerevisiae	Invertase
S. lipolytica	Lipase
Aspergillus spp. / Rhizopus oryzae	Pectinases
Saccharomyces lactis / Rhizopus oryzae	Lactase
Bacillus licheniformis	Alkaline protease
Bacillus coagulans	Glucose isomerase
Amino acids	
Corynebacterium glutamicum	L-Lysine
Brevibacterium spp.	Glutamic acid
Vitamins	
Ashbya gossypii	Riboflavin
Pseudomonas denitrificans	Vitamin B12
Propionibacterium shermanii	Vitamin B12
Polysaccharides	
Leuconostoc mesenterodies	Dextran
Xanthomonas campestris	Xanthan gum

Microorganism	Final product
Bioinsecticides	
Bacillus thuringiensis	Bt-toxin (anti-insect larval compd.)
Bacillus popillae	Control of mosquitoes
Food supplements	
Methanogenic bacteria	Single cell protein (SCP)
Spirulina sp. / Fusarium sp.	SCP
Rhizopus oryzae	Single cell oil (SCO)
Pharmaceutical (Antibiotics)	
Penicillium chrysogenum	Penicillin and it relatives
Cephalosporium acremonium	Cephalosporins
Streptomyces spp.	Streptomycin, Neomycins, Tetracyclines, Amphoterecin-B, Kanamycins, Polyxins, Actidione
Bacillus brevis	Gramicidin - S
Bacillus polymyxa	Polymixin - B

Strain Improvement :

Strain improvement or strain development (or) strain evaluation are the techniques and approaches used to genetically modify strains of industrial importance to increase the production of desired product. The utility of strain improvement arises because of the existence of rate-limiting steps within all metabolic pathways. The conventional method of strain improvement is to induce mutations by using mutagens (chemical mutagens or UV light) and screening the mutants for products. The other method is protoplast fusion in which two strains with two independent desirable characteristics could be fused to get a hybrid with both the characteristics, say better growth from one and better product yield from another strain.

The modern approach to strain improvement is largely based on molecular techniques and recombinant DNA technology. In this approach two things can be achieved, (i) Over-production of a gene product by molecular techniques, (ii) insertion of new genes into a known good strain to further improve it.

(i) Mutation : Mutants can be generated by using either chemical or physical treatments in order to modify the genome of the target organism. The agent responsible for mutation is known as mutagen. Different mutagens are presumed to have different mechanism of action, such as genetic attraction by base transitions or by frame shifts. During a long-term strain improvement program, it is advisable to change mutagens periodically to take advantage of these different mechanism of action. A small population of these cells could be the one which produces large amount of bioactive components of interest. Major mutations bring about marked changes in the biochemical characters and are useful in strain improvement. For example, the original strain of *Streptomyces griseus* produced small amounts of streptomycin and large amounts of mannosido streptomycin,

which has low antibiotic activity. A major mutant isolated from this strain produced less amount of manosido streptomycin and much larger quantities of streptomycin. Similarly, 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.

Usually, mutations are rare, about one per 10^7 to 10^{11} cells, therefore, it is necessary to have a very sensitive detections system. The best example for direct detection of mutants is replica plating technique which is used to detect auxotrophic mutants. An auxotrophic mutant has a defect in one of its biosynthetic pathways so that it requires a specific biochemical for normal growth and development. Resistance selection technique is one of the important techniques employed in mutant selection. Resistance selection technique can be used together with virutally any environmental parameter, resistance to bacteriophages, antibiotics or temperature is most commonly used. The mutant selection approaches are used when the biosynthetic pathways for the concerned product is known, as are the precursors and the regulatory mechanisms.

(ii) Recombination : Recombination has been applied for the production of vaious industrial strains. Recombination occurs when ever new gene arrangements are formed through exchange, elimination or insertion of DNA. The utilization and practical applicability for strain improvement by utilizing this technique is quite less in context with improvement of industrial strains due to the basic reason of complexity of process and non-availability of genetic literature of industrial strains. Sexual reproduction by means of conjugation in some bacteria and actinomycetes leads to the formation of, usually, partial diploids in which crossing over produces recombinant genotypes. In parasexual cycle, the nuclear fusion and gene segregation could take place outside the sexual organs of fungus, *Aspergillus nidulans*, *Pencillium chrysogenum*, etc. Here, heterokaryon formation takes place by nuclear fusion of genetically indifferent nuclei.

(iii) Protoplast fusion : A protoplast is defined as the cell which is devoid of the cell wall. Protoplasts of bacteria, actinomycetes and fungi are isolated by subjecting the cells to the action of wall-degrading enzymes in isotonic solutions. This technique can offer a means of combining favorable traits from two lineages or parental cultures. The protoplasts require an osmoticum for stability and fusion is induced by PEG (polyethlene glycol) treatment. Protoplast fusion between non-producing strains of *Streptomyces griseus* and *Streptomyces tenjimariensis*, has yielded a strain that produces indolizomycin, a new indolizine antibiotic.

(iv) Recombinant DNA Technology : Recombinant DNA technology has been used to achieve the objectives like production of recombinant proteins by transfering the genes of commercial values into bacteria and modification of the organism's metabolic pattern for obtaining new, modified or more quantity of metabolites. This process of modifying the metabolic pattern is known as metabolic engineering.

Table 1.3 : Recombinat DNA technology mediated changes in the production of various metabolites.

Approach	Features	Examples / Remarks
Recombinant DNA Technology	Genes from other organisms transferred into micro-organisms	New genes transferred; entirely new products, modified products, enhanced product yields, etc.
1. Recombinant proteins	Proteins encoded by the transgenes are the products of interest.	
2. Metabolic engineering	Metabolites generated by the transgene encoded enzymes are the products of interest.	Existing metabolic pathways modified, extended, made more efficient or new pathways introduced.
(i) Product modification	The new enzyme modifies the product of existing biosynthetic pathway.	Conversion of cephalosporin C into 7-aminocephalosporanic acid by D amino biosynthetic pathway. acid oxidase (*in A. chrysogenum*)
(ii) New substrate utilization	Inacessible substrate converted into acessible form	Beer fermentation by yeast: cyclodextrins convered into glucose, which is utilized by yeast
(iii) Completely new metabolite	All the genes of a new pathway transferred	E. *coli*; transfer of two genes for polyhydroxybutyrate synthesis from *Alcaligenes eutrophus*
(iv) Enhanced metabolite production	Amplification of the gene/ enzyme whose activity is rate limiting	Gene *cef* G of *C. acremonium* catalysing the conversion of penicillin N; increased cyclosporin yield
(v) Enhanced growth	Enhanced substrate utilization	*E. coli* glutamate dehydrogenase into *M. methylotrophus*; carbon conversion increased from 4% to 7%

Bioreactors (Fermentors)

A bioreactor is basically a large vessel, generally made of thick stainless steel body for the culture of microorganisms. A fermentor usually refers to the containment system for the cultivation of prokaryotic cells (bacteria), while in a bioreactor grows the eukaryotic cells (Mammalian, insect, plant). Industrial fermentors are designed to provide the best possible growth and biosynthesis conditions for industrially important microbial cultures, and to allow ease of manipulation for all operations associated with the use of the fermentors. The main function of a fermentor is to provide a controlled environment for growth of a microorganism, or a defined mixture of microorganism, to obtain a desired product. An ideal fermentor should have the provisions and control over various operations like temperature, pH, adequate aeration and agitation, less evaporation loss, low power consumption, proper sampling facilities, use of cheapest material, operated aseptically for a long period throughout the

operation, vessel of similar geometry (small and larger vessels), vessel with minimal use of labour during operation, harvesting, cleaning, suitable for variety of reactions etc. Modern fermentors are usually integrated with computers for efficient process monitoring, data acquisition etc.

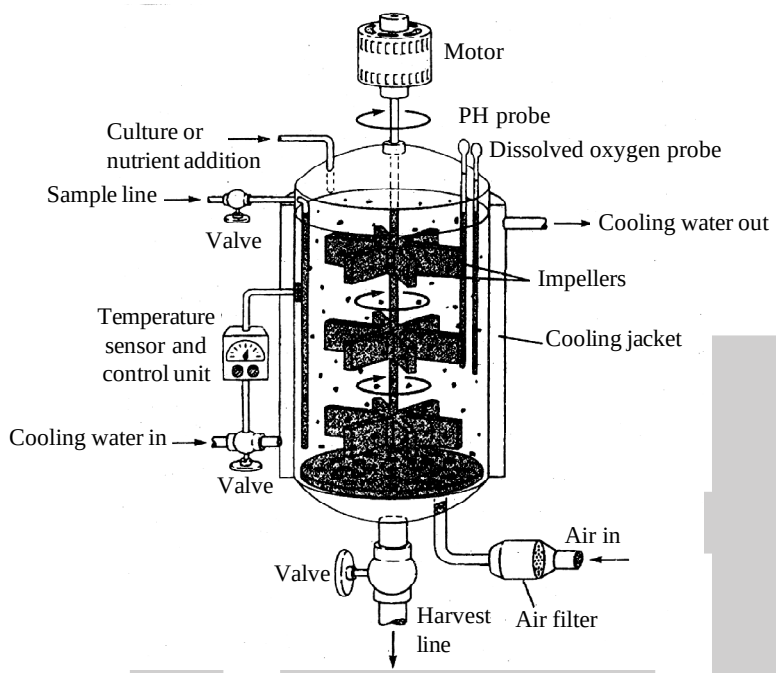

Fig. 1.1: A typical bioreactor

Essential features of a bioreactor

(i) The vessel should be strong enough to hold large volumes of culture broth and pressure that could be generated at times due to gas production. It should be completely free from leakages, otherwise long-term operations will be difficult and contaminations will occur.

(ii) Adequate aeration and agitation of the fermenting broth should be provided for the microbial metabolism to proceed at the optimum level.

(iii) It should have a system for monitoring and regulating temperature, pH.

(iv) Allow monitoring and / or control of dissolved oxygen.

(v) The vessel should have internal vertical plates called baffles to prevent vortexing and the vessel should be connected to inlet pipes to receive culture medium and inoculum of the microorganism.

(vi) It should have facility for sampling and should require a minimum of labour in maintenance, cleaning, operating and harvesting operations.

(vii) It should be constructed using the cheapest materials and should be adequate service provisions for individual plants.

(viii) The size and the internal volume of the bioreactor is designed by bio-engineers with proper knowledge of fluid dynamics, and the microbiologists should maintain the sterility of the entire system.

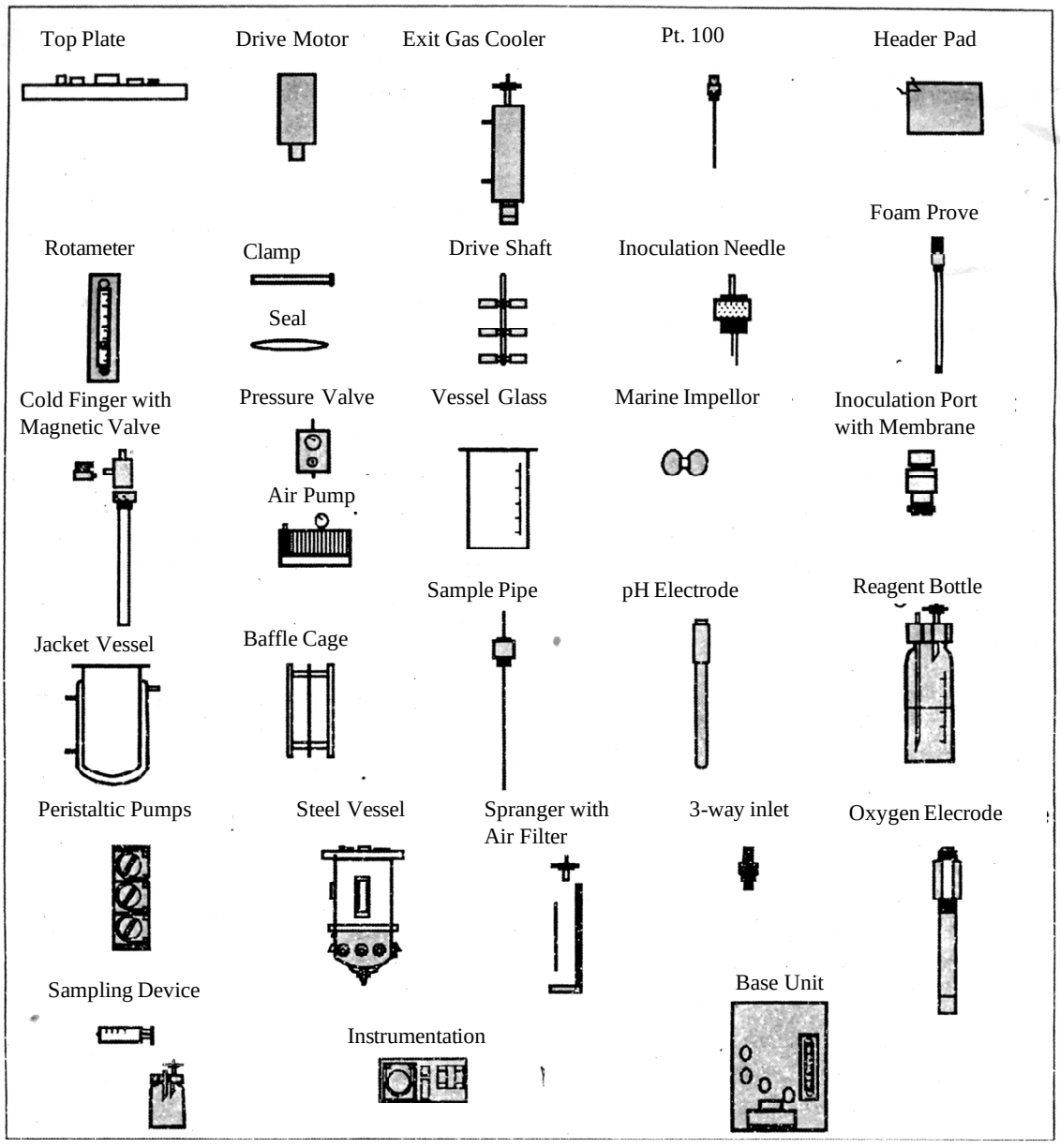

Fig. 1.2 : Major components of fermentor

Types of Fermentors (Bioreactors) :

The most widely used bioreactors in industries are

1. Stirred tank bioreactor
2. Tower bioreactors
3. Air lift bioreactors
4. Packed - bed bioreactors
5. Fluidized bed bioreactors
6. Photo bioreactors

1. **Stirred tank bioreactor (STRS):** It is an upright cylindrical vessel with motor driven central shaft that supports one or more agitators (impellars) so as to maintain the homogeneity. The number of impellars is variable and depends on the size of the bioreactor i.e; height to diameter ratio, referred to as aspect ratio. Both the ends of the vessel are closed with hemi-spherical basins.

   These bioreactors are most versatile and are used in a range of sizes from one litre laboratory unit to production scale vessels of typically 100 ton capacity. Continuous-flow culture systems, a type of stirred tank reactors, are either of chemostat or turbidostat type. A continuous flow culture provides a continuous source of cells, and are suitable for product generation.

   At the lateral side of the fermentor, there is an opening through which culture medium is being pumped into the fermentor. Another opening acts as the outlet for harvesting the products is present at the base of the fermentor. The volume of the vessel is about 30 to 50% larger than the required culture volume leaving a head space-allowing disengagement of the liquid droplets from the exhaust gas and room for foaming. A heating coil is used to raise the temperature inside the vessel. Sterile air is pumed into the fermenter through a pipeline found just below the stirrer.

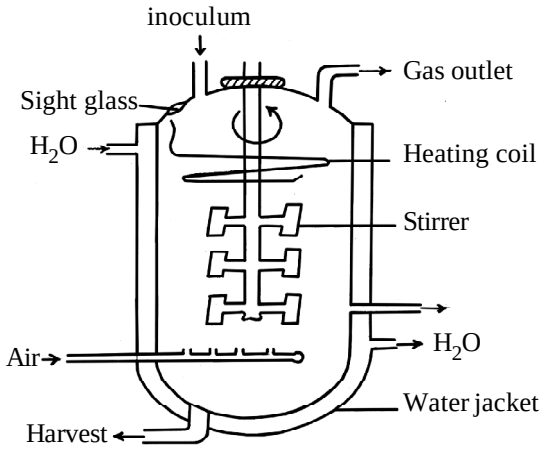

Fig. 1.3 : Stirred tank reactor in a schematic view

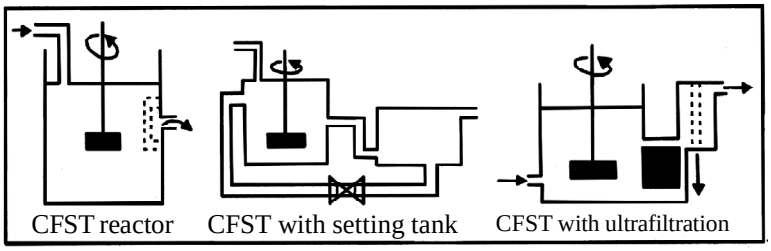

Fig. 1.4 : Types of continuous flow stirred reactors

2. **Tower bioreactors :** These are huge bioreactors mainly designed for the brewing industry. In these, the cells tend to aggregate and settle at the bottom of the vessel inspite of the upflow of the fluid.

   A high hydrostatic pressure generated at the bottom of the reactor increases the solubility of oxygen in the medium. Tower fermentors are of different types based on their design. They are Bubble-up fermentors, Bubble column tower fermentors, Multistage tower fermenters, Vertical-tower Beer fermentors.

   The high density of cells may create anaerobic condition which is advantageous to alcohol fermentation. Tower bioreactor has high aeration capacities without having moving parts. Bubble column tower fermentors are used for citric acid and tetracycline production and for a range of other fermentations based on mycelial fungi. Vertical-tower beer fermentors are designed to maximise yeast biomass yields and for beer production. Multistage tower fermenters are used for continuous culture of *E. coli*, *S. cerevisiae* and activated sludge.

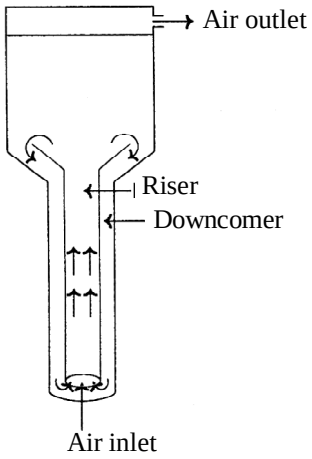

Fig. 1.5 : Tower reactor

3. **Air lift bioreactors :** In airlift bioreactors, the medium is circulated around two nested columns within a long tubular tower. The rate of airflow of the reactor depends on the volumetric mass transfer co-efficient in the reactor system. The incoming air forces the medium up the inner column, or riser, and it then descends down the outer column, or down comer tube. The riser tube may be placed within the down comer tube or it may be externally located and connected to the latter.

Air lift bioreactors are of two types. They are (i) Internal-loop airlift bioreactor, (ii) External-loop airlift bioreactor. Internal-loop airlift bioreactor has a single container with a central draft tube that creates interior liquid circulation channels. External-loop airlift bioreactor possesses an external loop so that the liquid circulates through separate independent channels. An internal heat exchanger coil is located at the bottom loop connecting the riser and downflow tubes; it maintains the temperature at 30^0C. Airlift bioreactors are commonly used for aerobic bioprocessing technology. These bioreactors are mainly employed in the production of single cell proteins, methanol production and waste water treatment.

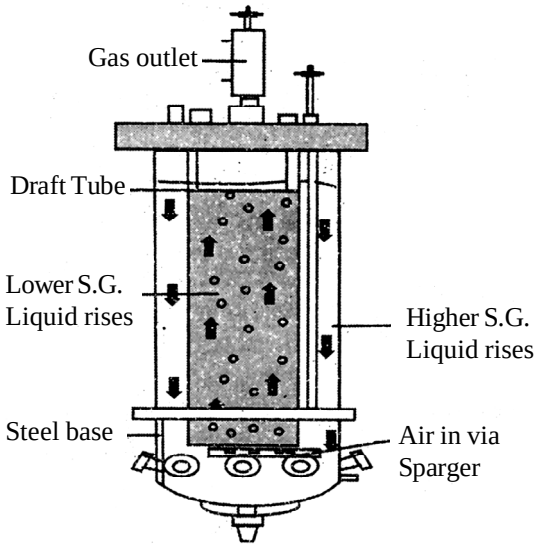

Fig. 1.6 : A Typical air lift bioreactor

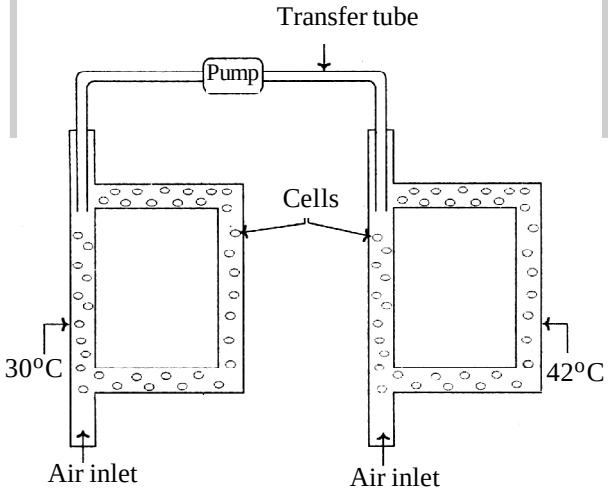

Fig. 1.7 : Two stage air lift bioreactor

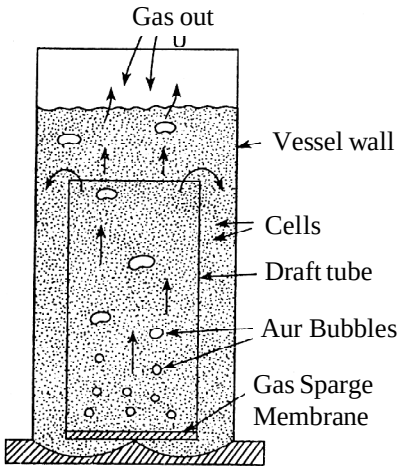

Fig. 1.8 : Airlift fermenter with internal loop

4. **Packed - bed bioreactors :** A packed-bed bioreactor is a vessel filled about 2/3rd with solid particles. These fermentors are usually installed with a forced aeration device. A column which is filled with a solid matrix, traps microorganisms within it. The solid matrix may be gels absorbed with the biocatalyst (enzyme/cells), or more rigid particles, e.g. compressible polymeric particles or particles of silica. The temperature adjustment between the top and bottom of the substrate depends on the thickness of the bed and the aeration rate. A nutrient broth is continuously poured over the immobilized biocatalyst. The products obtained in the bioreactor are released into the fluid and removed. The concentration of the nutrients can be increased by increasing the flowrate of the nutrient broth. The depth of the packed bed is limited by several factors like pH gradient formation, nutrient concentration and oxygen requirement.

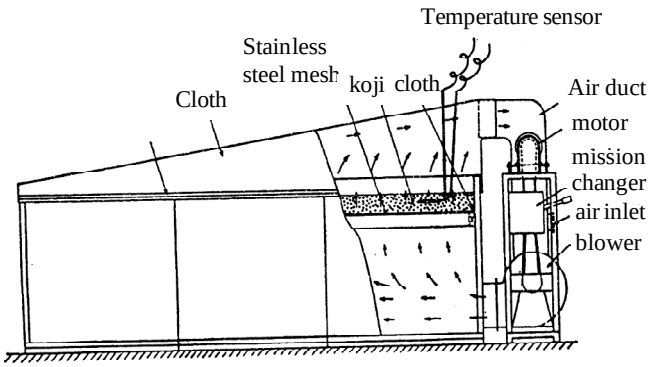

Fig. 1.9 : A packed-bed fermentor with forced aeration

5. **Fluidized bed bioreactors :** In the fluidized bed bioreactor, the solid substrate is fluidzed by upward flow. In this type of bioreactor, the solids are retained in the rector while the liquid flows out. A fluidized bed has a biological film developed on particles which are suspended in an upward flow of liquid in which they are then free to circulate. Fluidized beds are designed using a biocatalyst such as immobilised enzyme or cells adsorbed to particles. The support particles can be solids such as sand or glass beads or porous such as plastic or stainless steel mesh. The solid particles carrying the biocatalyst are suspended in the liquid substrate. For an efficient operation of fluidized beds, gas is sparged to create a suitable gas-liquid-solid fluid bed. The upflowing stream of nutrient (substrate) is used to fluidise the solid particles which get dispersed in the liquid. The top of the reactor is kept broad and the bottom narrow so that the particles concentrate more on the lower narrow region. These bioreactors are mostly used in conjuction with immobilised cells or enzyme system and are operated continuously.

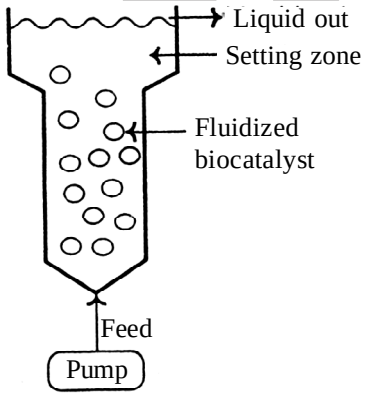

Fig. 1.10 : Fluidized bed bioreactor

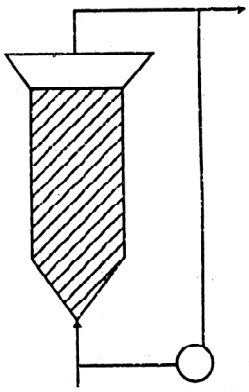

Fig. 1.11: Fluidized bed recycle reactor

6. **Photo bioreactors :** The photosynthetic organisms (Cyanobacteria, Microalgae) require light for the production of important products (e.g β-Carotene, single cell protein and astaxanthin) and bioreactors designed for them are called photobioreactors. In addition to light, the cells require CO_2 which can be provided by dissolving bicarbonate. These bioreactors comprise an array of transparent tubes (glass or clear plastic tubes), that are placed horizontally or vertically. The array of tubes or flat pannels constitute light receiving system. The culture is circulated through the light receivings system solar receivers by centrifugal pumps or airlift pumps. The light penetration depends on the density of the biomass and the temperature is maintained at 22-37^{0}C.

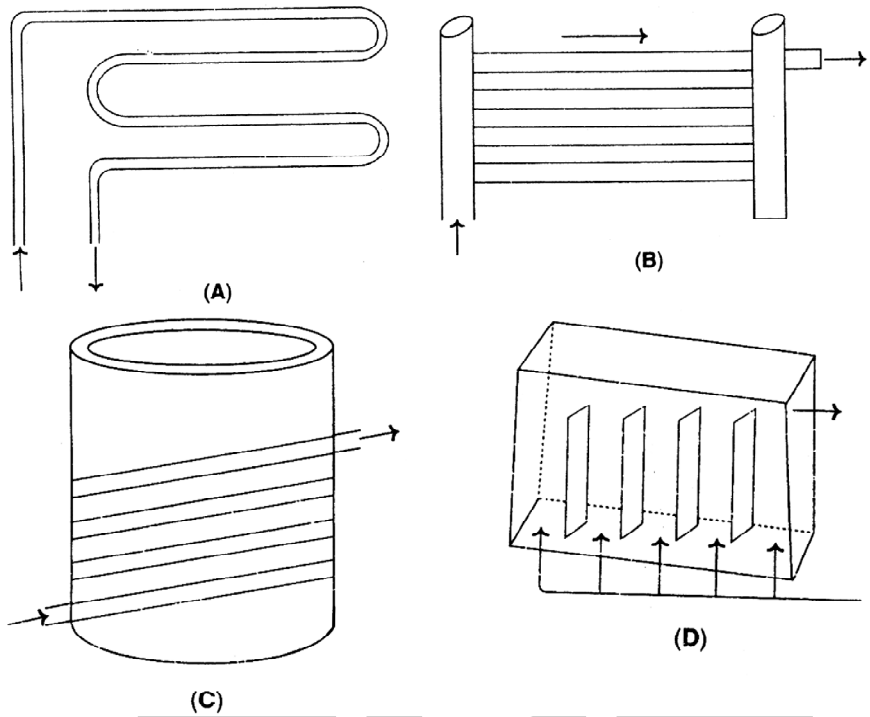

Fig. 1.12 : Types of photobioreactors (A) Continuous run tubular loop (B) Multiple parallel tube (C) Helical wounder tubular loop (D) Flat panel configuration.

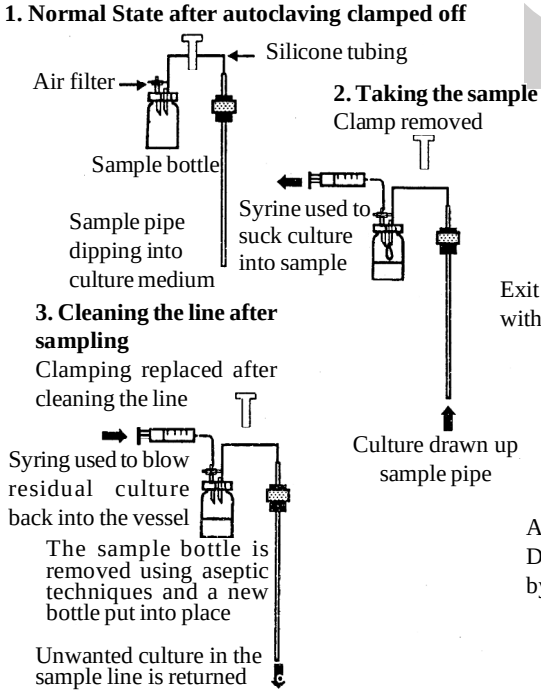

Fig. 1.13 : Sampling

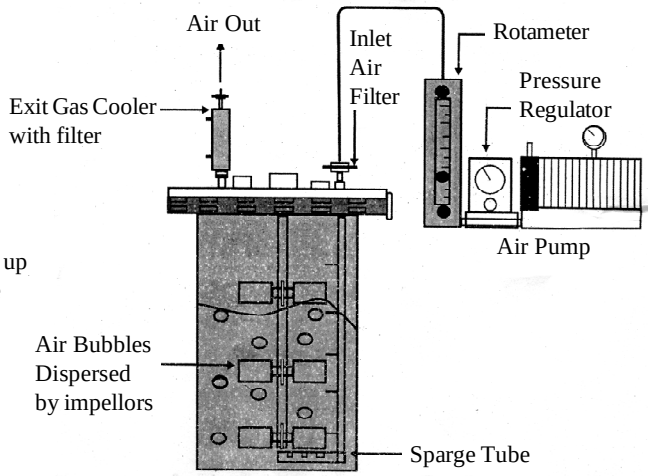

Fig. 1.14 : Gas supply system

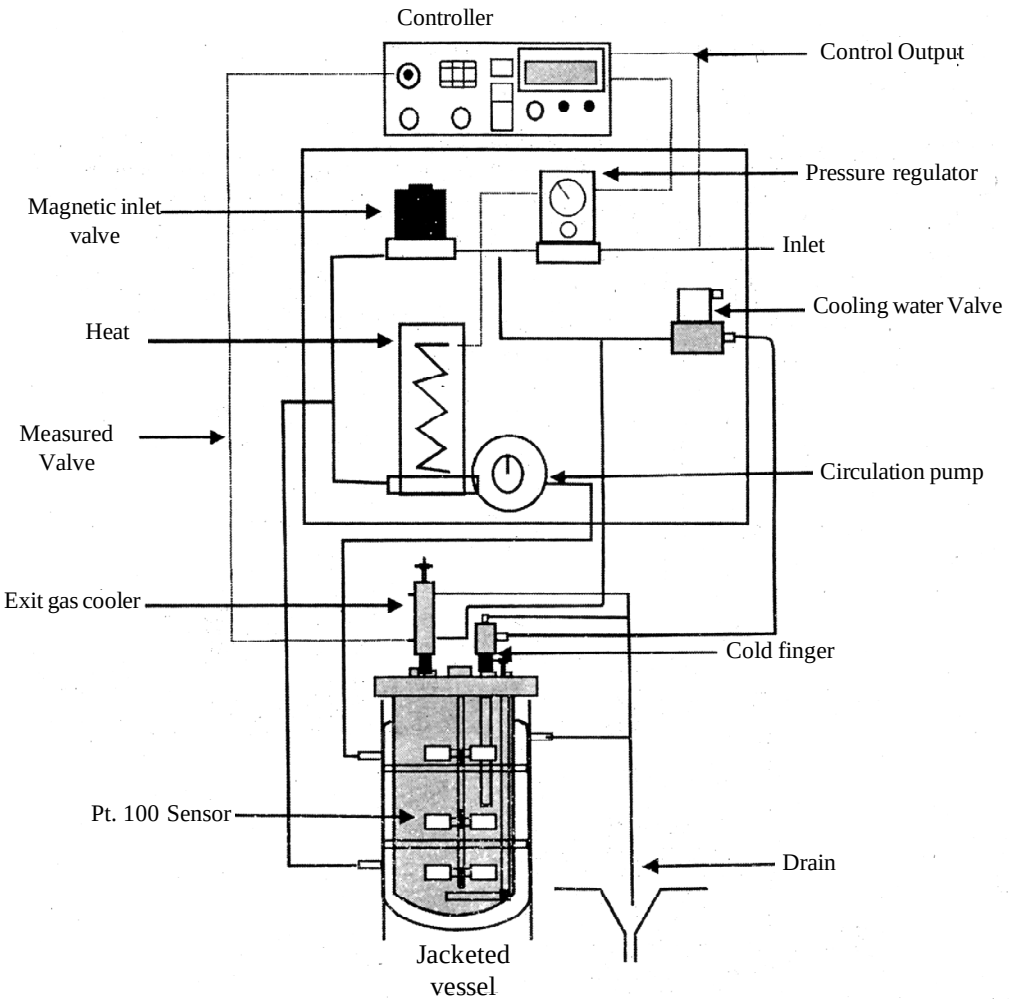

Fig. 1.15 : Temperature control system using water circulation.

Design and operation of bioreactor :

Industrial fermentors are designed to provide the best possible growth and biosynthesis for industrially important microbial cultures, and to allow ease of manipulation for all operations associated with the use of fermentors. These vessels must be strong enough to withstand the pressure of large volume of agitating medium. Since, most industrial fermentations utilise pure cultures, the fermentors must make some provision for the control of or prevention of the growth of contaminating microorganisms. Conventional bioreactors are cylindrical vessels with domed top and bottom. The reaction vessel, surrounded by a jacket, is provided with a sparger. The agitator shaft is connected to a motor at the bottom. The reaction vessel has side ports for pH, temperature and dissolved oxygen sensors.

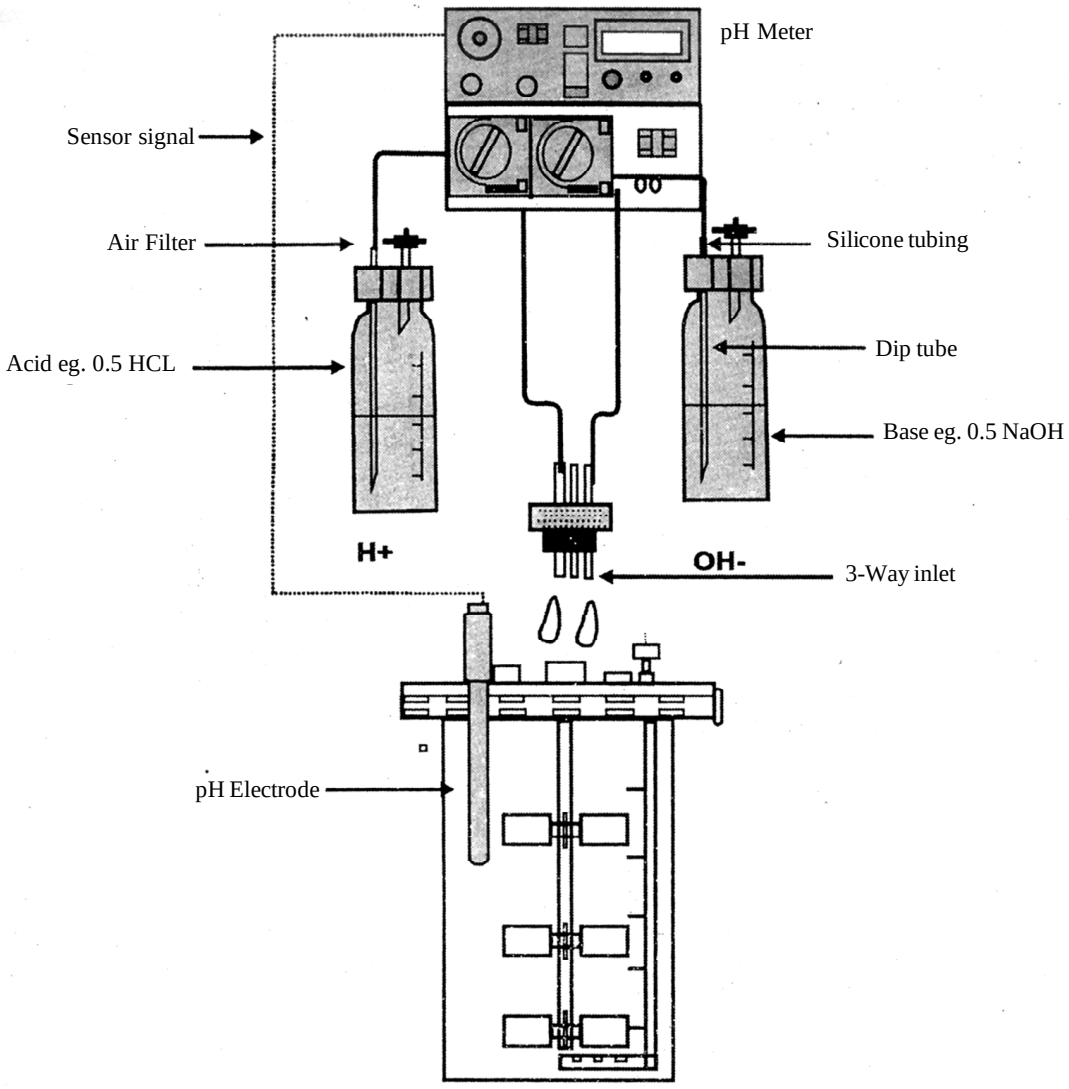

Fig. 1.16 : pH measurement and control

The bioreactor is usually designed to work at high temperature, high pressure. There must be provision for inoculation and sampling, as well as for charging and discharging the vessel. In order to maintain aseptic operation the mechanical integrity of the plant should be maintained. Entry points to the aseptic region of the plant such as mechanical seals for agitators and pump shafts, valve closers, probe insertions, sample codes and joints are loci of contamination risks and should be as few in number as possible. A good aseptic fermentor should not have direct connections between sterile and non-sterile parts of a system and

should minimise flange connections. All other connections to the aseptic region of the plant should be steam sealed, including the glands of the valves used to control liquid flow in the pipe and steam flow in the pipe. If the industrial fermentations are aerobic, the fermentor should have mechanical stirrers to mix the solution, baffles to increase turbulence and ensure adequate mixing, and forced aeration to provide needed oxygen. The fermentor should have a provision for the intermittent addition of antifoam agent. The fermentor is designed in such a way that it should avoid stagnant regions, dead spaces, pockets, pipe branches and crevices which cannot only collect stagnant liquid and microorganisms but also difficult to sterilize effectively.

Suitable grade of stainless steel is employed in the construction of a fermentor : stainless steel is the most reliable than the ordinary mild steel due to its high mechanical strength as well as corrosion resistance. Diaphragm valves are needed to regulate fluid flow in the fermentor or a piston valve for aseptic conditions with a steam sealed gland is used. Porous spargers made up of sintered glass, ceramics or a metal are used to introduce air into the fermentor. Antifoaming agents like alcohols (sterile and octile decanol); esters, fatty acids and their derivatives, silicones, sulphonates and miscellaneons compounds like oxaline and polypropylene glycol are used to prevent or to decrease the foaming during fermentation. Integration of computers in the fermentation systems is based on the computer's capacity for process monitoring, data storage and error detection. The use of computers in fermentation technology has produced a remarkable impact in fermentation work in recent years.

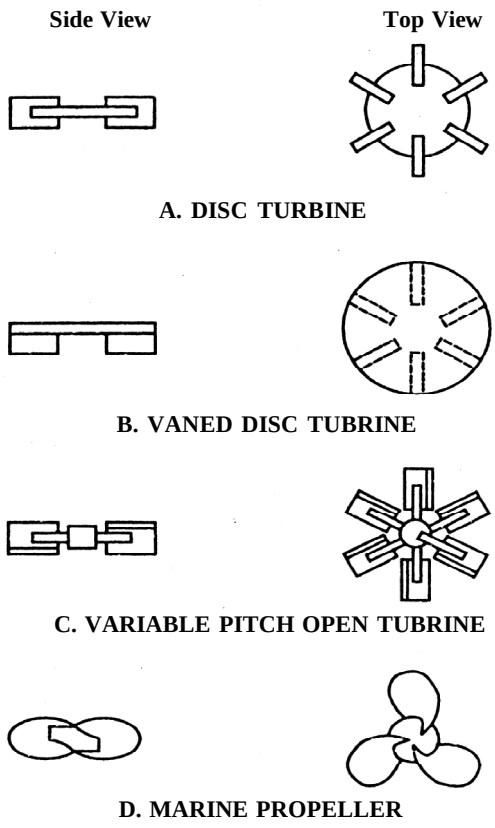

Fig. 1.17 : Different types of agitators. : A. Disc turbine; B. vaned disc; C. open turbine, variable pitch; and D. marine propeller agitators.

Table 1.4: Typical fermenter control ranges

S.No	Control	Range
1.	Temperature	8^0C above coolant to 60^0C $\pm$ 1^0C
2.	Agitator speed	0 - 1000 rpm
3.	Stability	> 98%
4.	pH range	2-12 $\pm$ 0.1
5.	Pressure	2000 mbar
6.	CO_2 range	0-100%
7.	Air flow	0-6 liters/minute

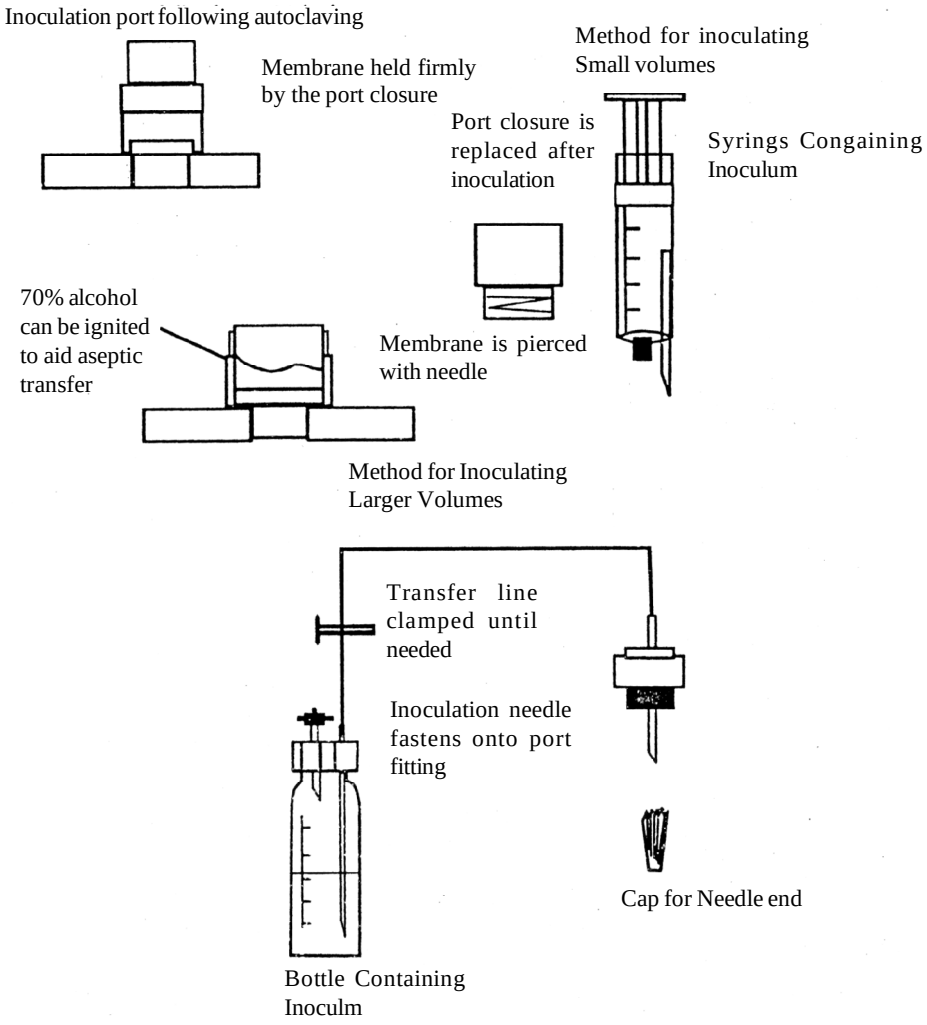

Fig. 1.18 : Inoculation process of a fermentor vessel.

During operation of a bioreactor, the sterilization (maintaing aseptic conditions) procedures are followed strictly for successful fermentation process. Sterilization can be *in situ* sterilization and continuous heat sterlization. In in situ sterilization, the whole system is heated to about 120^0C and held at this temperature for about 20 minutes. In continuous heat sterilization, the medium is rapidly heated to 140^0C for a short period, by injecting the pressurised steam. Later, inoculation of the specific industrial microorganism into the growth medium is done. The size of the incolum is genereally 1-10% of the total volume of the medium. During the fermentation process, samples are regularly drawn from the bioreactor and checked for contamination (if any). Adequate aeration and agitation of the fermenting broth should be provided for the microbial metabolism to proceed at the optimum level. Impellars are used for agitation, and they rotate through a motor. Spargers are structures used for aeration. Aeration may be air-lift system of aeration or stirred system of aeration. Bioreactors meant for growing mycelial fungi may require different kinds of spargers with holes of larger diameter about 8 to 10 times the diameter specified for the unicelled organism. Impellars (agitators) distribute the air bubbles released from the spargers uniformly in the culture vessel medium. The number of impellars in a vessel depends on the vessel volume, usually 2 to 3 impellars may be connected to a single shaft at a difference of about 1.2 times the impellar diameter. Control systems are essential to maintain optimal growth environment in the reaction vessel for maximum product formation. It is necessary to maintain the pH of the broth at the optimum level (pH control). This can be done by adding alkali or acid, after testing a sample at intervals. Temperature control is absolutely essential for a good fermentation process. Rapidly growing microorganisms can generate a large amount of heat that must be dissipated to prevent inactivation of enzymes. Cooling coils are often employed in fermentors to regulate temperature and to maximize the rate of product accumlation. Some thermophilic organisms may require high temperature, and in such cases, it may be necessary to pass steam to raise the temperature using internal coils. Oxygen is sparingly soluble in water and it should be 0.0084 g/L at 25^0C (dissolved oxygen). During the fermentation process, because of aeration and agitation, foam is formed. The antifoams are added in small quantities only, other wise they will reduce oxygen transfer within the broth and may affect product formation. After completion of the fermentation process, the bioreactor is harvested and is prepared for the next round of fermentation after cleaning.

Types of Fermentations

1. **Solid substrate (solide state) fermentation (SSF) :** Solid-state fermentation (SSF) is a microbial process in which a solid material is used as the substrate or the inert support of microorganisms growing on it. Although SSF was developed for the manufacturing of traditional foods and alcoholic beverages, its application has been extended to the pharmaceutical and biochemical industries. The most commonly used solid substrates for SSF are cereal grains, wheat bran, saw dust, wood shavings and several other plant and animal materials. Fungi and actinomycetes are the best suited for SSF, because of their larger biomass and reach by means of hyphae. SSF techniques are also being used for the production of high-value products such as enzymes and toxins. Most enzymes from fungi are now being cheaply produced through SSF. More recently, this approach has been used for the production of extracellular enzymes, certain valuable chemicals, fungal toxins, and fungal spores (used for biotransformation).

   In solid substrate fermentation, the microbial distribution occurs on the solid surface, and microbial growth and product formation also occur mainly on the surface. The moisture content required for SSF is normally low, depending on the physical or chemical characteristics of the substrate. Heat derived from the metabolism and growth of the microorganism raises the temperature of the solid substrate bed and causes the loss of moisture. The air supply and temperature of the solid substrate bed is controlled by forced aeration, in which the large surface area of the solid substrate promotes heat transfer and gas exchange of oxygen and carbondioxide. The bioreactors used in SSF are tray fermentors without any agitation, drum fermentors with continuous or staggered slow agitation, and column fermentors with forced aeration. The tray type fermentors consists of wooden, metallic (aluminium, iron), or plastic trays with perforated bottom. The trays are sterlized and filled with a layer of substrate mixed with inoculum. The trays are stacked one above the other to a convenient height. A humid atmosphere is created inside the chamber, and the temperature is controlled by cool or warm air. After fermentation process is completed, the trays are removed and the fermented mash is pooled for down stream processing for product recovery.

   The column fermentor is a glass or plastic column, with a jacket for water circulation and usually aerated through forced air and it is more expensive.

Table 1.5: Examples of solid-state fermentations on natural substrates

Product	Microorganisms	Materials
Enzymes		
α-Amylase	*Aspergillus oryzae, Rhizopus* sp., *Bacillus licheniformis, Bacillus* sp.	Wheat bran, cassava
Glucoamylase	*Aspergillus niger, Aspergillus* sp., *Rhizopus* sp.	Cassava, wheat bran, corn
Cellulase	*Trichoderma reesei,* A. niger	Wheat bran, wheat straw
	Penicillium sp., Thermoascus auranriacus	Beet pulp, cellulosic biomass
Xylanase	*Aspergillus fumigantS, Thermoascus lanuginos*	Wheat bran, jute fiber + wheat germ
Pectinase	*Talaromyces flavus,* A. *niger,* A. *carbanerius*	Fruit pomace, wheat bran, coffee pulp
Glucose oxidase	*Penicillium notatum, Penicillium* sp.	
β-Galactosidase	*Kluyveromyces laccis*	Whey + corn or wheat bran
Protease	*Penicillium caseicolum, Martierella renispora,* A. *oryzae,* A. *niger*	Wheat bran, dried skim milk
Rennin	*Mucor pusillus, Mucor miehei*	Wheat bran
Metabolites		
Ethanol	*Saccharomyces cerevisiae*	Fruit pomace, sweet sorghum, beet, com, carob pods
Citric acid	A. *niger*	Sugarcane bagasse, fruit pomace, wheat bran
Lactic acid	*Lactobacillus* sp., *Rhizopus oryae*	Sweet sorghum, sugarcane bagasse + glucose
Gibberellic acid	*Gibberella fujikuroi*	Wheat bran
Red pigment	*Manascus anka*	Rice, bread flake
Antibiotics		
Penicillin	*Penicillium chrysogenum*	Sugarcane bagasse
Tetracycline	*Streptomyces viridifaciens*	Sweet potato residue
Cephalosporins	*Cephalosporium acremonium*	Barley
Iturin, surfactin	*Bacillus subeilis*	Soybean curd residue
Foods		
Natto	*Bacillus natto*	Soybean
Tempeh	*Rhizopus oligosparus*	Soybean
Tape	*Amylomyces rouxii, Rhizobium chinensis*	Rice, cassava, maize
Ontjom	*Neurospora sitophila*	Peanut meal
Cheese	*Penicillium roqueforti*	Milk curd
Bread dough	*Saccharomyces cereliisiae, Lactobacillus*	Wheat powder
Koji	*sanfrancisco*	
Sake, shochu	A. *oryzae,* A. *kawachii*	Rice, barley
Soy sauce	*Aspergillus sojae*	Soybean, wheat
Miso	A. *oryzae*	Soybean, rice
Shao-hsing wine	*Rhizopus* sp., *Mucor* sp. (A. *oryzae*)	Wheat (rice)
Kao-liang liquor	*Rhizopus* sp., *Mucor* sp.	Sorghum
Ragi	*Rhizopus* sp., *Saccharamycopsis* sp.	Rice
Single-cell proteins	Many yeasts and molds	Starchy or cellulosic biomass
Compost	White-rot fungi (mixed culture)	Cellulosic biomass

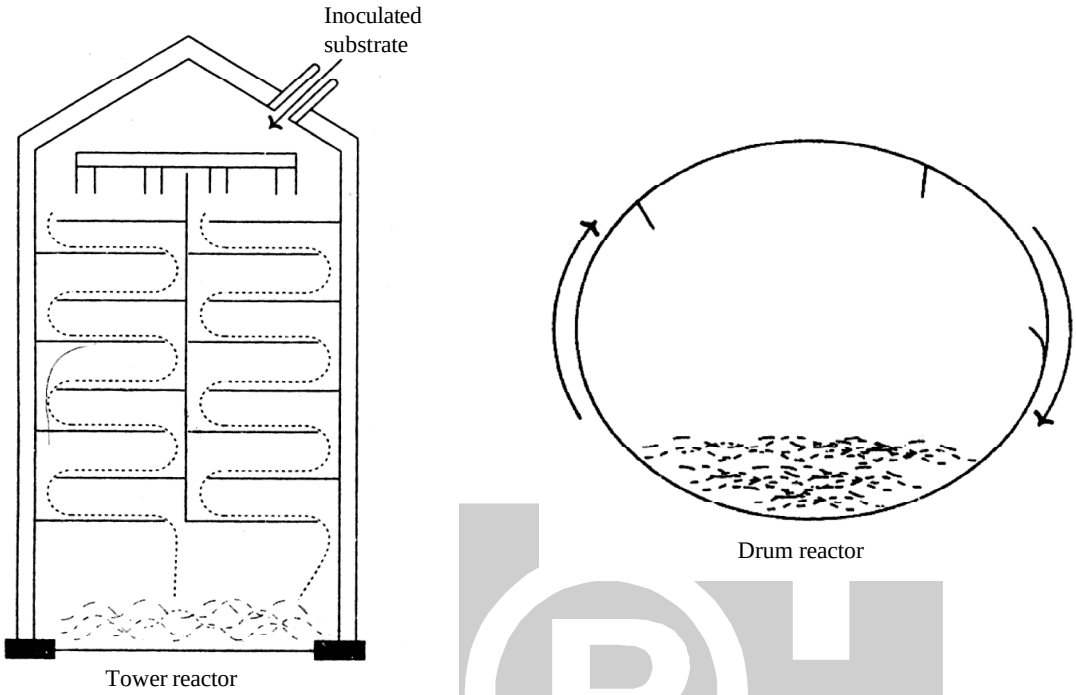

Fig. 1.19 : Bioreactors used for solid-state fermentations

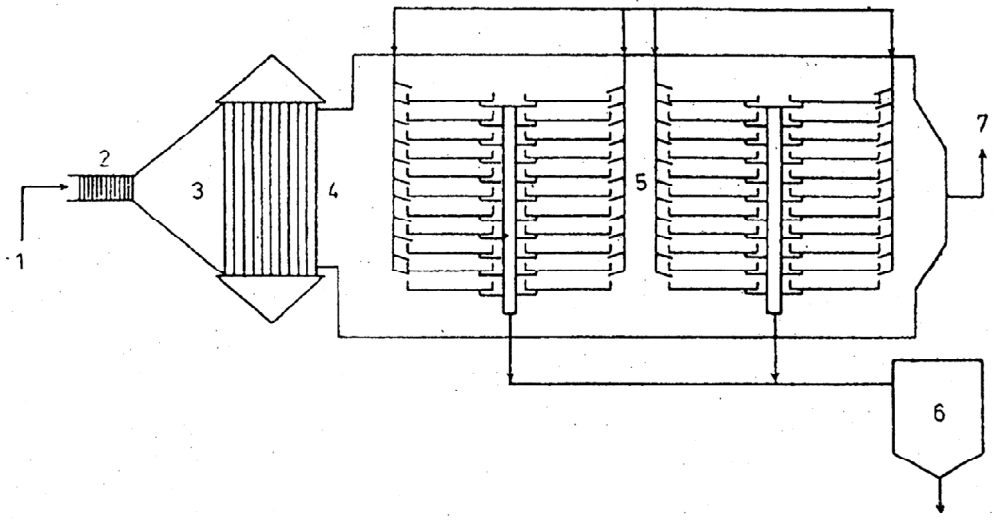

Fig. 1.20 : Solid state fermentation : A set up for surface culture in trays.
1. Air inlet, 2. air filter, 3. air moistener, 4. air heater,
5. fermentation chamber with stacked trays, 6. culture collectror, 7. air outlet.

2. Submerged fermentation

(i) Batch culture (or) Batch fermentation : The growth of the fermentation organism occurs in a closed system in a batch culture. A microbe grows in the medium until the nutrients are exhausted or toxic metabolites secreted by it reach to inhibitory level. Important nutrients given to the microorganisms are being utilized for the reproduction and the accumulation of the metabolic products. This results in continually changing conditions in the culture in which the composition of the cell wall and the concentrations of a number of cytoplasmic components vary as growth of the microorganisms proceed. The incubation is carried out under optimal physiological conditions (pH, temperature O_2 supply etc.). After inoculation, the culture enters lag phase, during which there is increase in the size of the cells and not in their number. It is the period of adaptation to new conditions or prior to active growth. The length of the lag phase is variable and is mostly determined by the new set of physiological conditions, and the phase at which the microorganisms were existing when inoculated.

The period of maximum growth rate is known as log or exponential phase. The time taken for the population to double in size is referred as "doubling time", or mean generation time, which is the average time taken by a single cell to divide into two daughter cells. The two parameters that is the doubling time and mean generation time are identical under favourable conditions, but if some daughter cells are non-viable the mean generation time is less than the observed doubling time. The growth rate of the microbes also decreases due to the nutrient limitation because of the depletion in the substrate coupled with accumulation of toxic metabolites. This phase is known as stationary phase. The biomass remain almost constant during this phase. Later the cells enter death phase in which cessation of metabolic activity and depletion of energy reserves take place.

(ii) Fed-batch culture : This process is mainly employed in mammalian and insect cell cultures. More recently, advances have been made in high density fed-batch cultures by using sophisticated media designs and feeding schemes beyond the addition of carbon and energy substrates. Generally in fed-batch culture, the culture is subsequently fed with fresh nutrient medium without removing the growing microbial culture and allows one to supplement the medium with such nutrients that are depleted or that may be needed for the terminal stages of the culture.

(iii) Continuous culture (or) Continuous fermentation : Continuous culture provides well-defined cultivation conditions for genetic, biochemical and physiological characterization of cells and allows independent variation of growth parameters, enabling reliable kinetic studies of cell growth and metabolism for process optimization. Continuous cultures have been used for production of some primary metabolites (e.g, ethanol and organic acids), in waste water treatment, and fermented foods and for some reactions catalyzed by enzymes. It is also used for the production of monoclonal antibodies and recombinant proteins by animal cell cultures.

In continuous fermentations, fresh medium is added either continuously or intermittently to the fermentation vessel, accompanied by a corresponding continuous or intermittent withdrawl of a portion of the medium for recovery of cells or fermentation products. In continuous cultures, the growth of the bacterial population can be maintained in steady state over a long period of time. Here the rate of supply of nutrients in the form of raw material and removal of products/cells should be volumetrically the same i.e; volume added is equal to volume removed. It means that volume of the medium always remains constant. The different types of bioreactors employed in continuous fermentation include chemostat bioreactors, turbidostat bioreacters and plug flow bioreactors.

Chemostat :

A chemostat maintains the nutrient feed and harvest culture withdrawl rates at constant values, but always less than that which allows a maximum growth rate. A chemostat is defined as a continuous culture system in which the feed rate is set externally and cell growth is limited by a selected nutrient.

Tubidostat :

In tubidostat, the total cell population is held constant by employing a device that measures the culture turbidity so as to regulate both the nutrient feed rate to the fermentor and the culture withdrawl rate from the fermentor. In plug flow bioreactors the culture solution flows through a tubular reaction vessel without back mixing.

Equipment in continuous culture:

The equipment set up and design of a continuous culture includes the following parts. A reaction vessel, i.e a bioreactor (at center) equipped with elements for aeration, mixing of liquid (medium) and solid (cells or insoluble substrate) phases, sampling and control of temperature, pH, dissolved oxygen, foaming, and reaction volume, components may be mixed together before being fed into the reactor by a metering device (e.g. a pump) or may have separate connecting lines to the reactor. There should be vessels for withdrawl of broth and collection.

Table 1.6: Representative Organisms Grown in Continuous Culture

Organisms	Genera
Actinomycetes	*Streptomyces*
Algae	*Chorella*
	Euglena
	Scenedesmus
Bacteria	*Aerobacter*
	Azatobacter
	Bacillus
	Brucella
	Clostridium
	Sallmonella
Fungi	*Ophiostoma*
	Penicillium
Protozoa	*Tetrahymena*
Yeast	*Aaccharomyces*
	Torula
Mammalian Cells	Embryo rabbit kidney

Source. W.D. Maxon. 1960. Adv. App. Microbiol, 2, 335-349

Table 1.7 : Representative chemical products from continuous fermentation

Growth	Not Growth-Associated
Acetic acid	Acetone
Butanediol	Butanol
Ethanol	Glycogen
Gluconic acid	Subtilin
Hydrogen sulfide	Chloramphenicol
Lactic acid	Penicillin
	Streptomycin
	Vitamin B_{12}

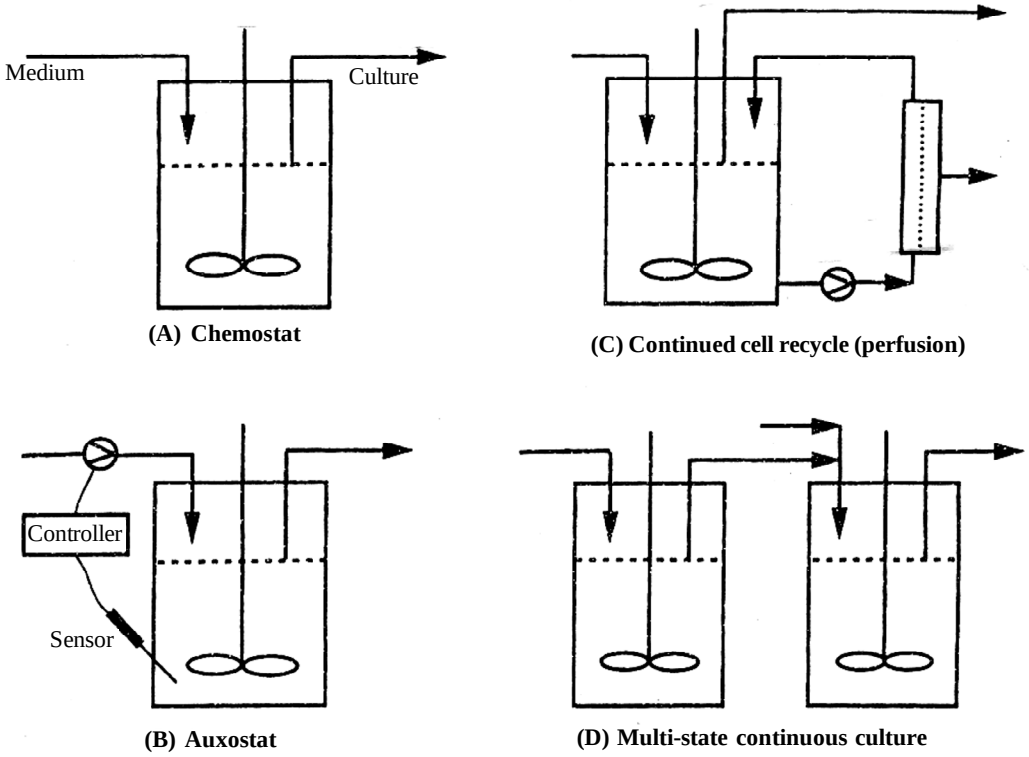

Fig. 1.21 : Schematic diagram of four types of continuous culture.
(A) Chemostat. (B) Auxostat. (C) Continuous culture with cell recycle. (D) Multistate continuous culture.

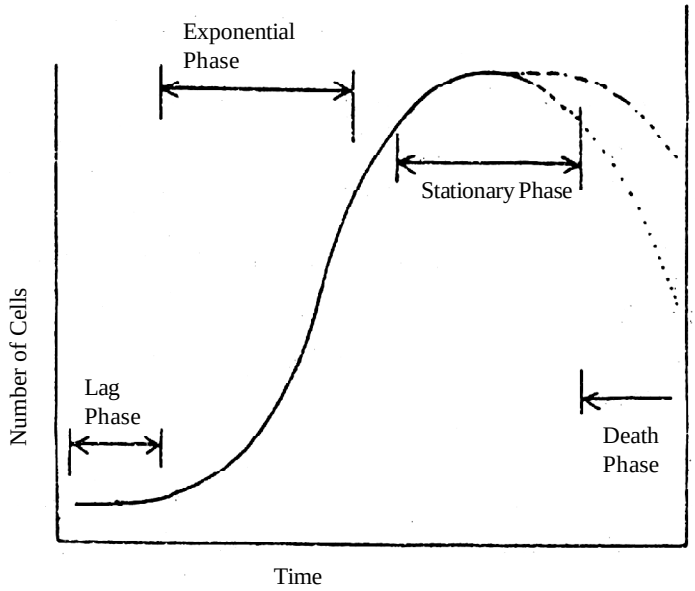

Fig. 1.22 : Growth curve showing lag, log, stationary and death phases.

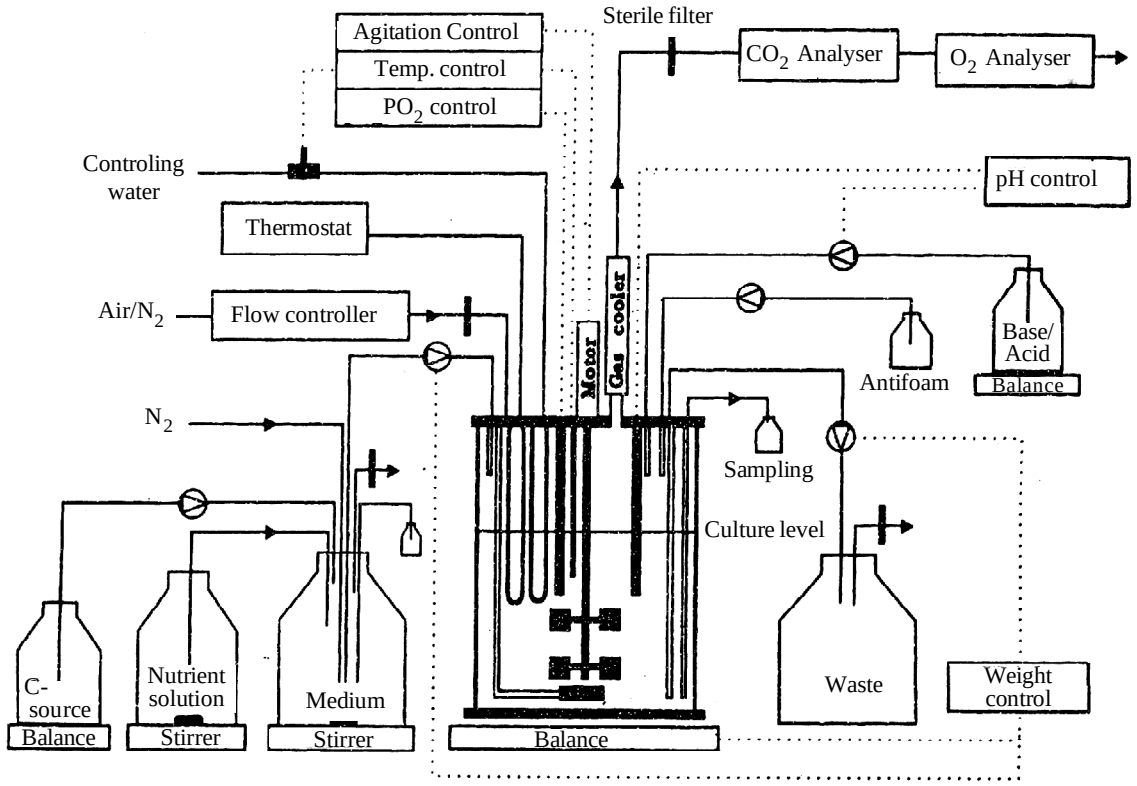

Fig. 1.23 : A typical setup of a bench-scale continuous-culture system. The feeding rate and the culture volume are controlled by a weight control unit.

3. Anaerobic Fermentation

One of the largest areas of industrial microbiology is anaerobic digestion of wastes, and anaerobes that are having an increasing role in detoxicification of hazardous wastes and pollutants. Anaerobic microorganisms do not utilise molecular oxygen in biosynthesis and are not capable of using oxygen as a terminal electron acceptor. Instead, they use diverse array of organic and inorganic electron donors and acceptors in their energy metabolism. In anaerobic fermentations, the production of biomass is less than aerobic organisms. This is due to their characteristic energy metabolism and consequent formation of small quantities of ATP. With less biomass production, more carbon can be converted to end-products and the product yeild is higher. In case of acetogens and other gas utilising bacteria, oxygen free sterile CO_2 or other gases are bubbled through the medium. The culture vessel (fermentor) required for acetogens is 400 lts size and 3 kg cells could be harvested in each run.

Anaerobes can use a variety of substrates including polysaccharides, molasses, sugars and other complex substrates which may be obtained from agricultural waste streams and reduce the overall cost of the fermentation process. The fermentation usually produces high

yeilds of CH_4, organic acids or other compounds by fermenting complex organic substrates such as polysaccharides or proteins. Many anaerobes grow at high temperatures at which oxygen is poorly soluble and growth under these conditions can contribute to efficient product recovery. Anaerobic organisms of industrial importance includes a wide range of genera in the domains of bacteria and archaea. The genus *Clostridium* is important medically and for the production of metabolites including enzymes and solvents. Toxigenic *Clostridia* are employed in production of human and animal vaccines and for human theraupatics. Lactic acid bacteria (*Lactococcus, Lactobacillus, Streptococcus, Propionebacterium* and *Leuconostoc*) are important in food fermentations and also in the production of organic acids. Sulphate reducing bacteria (*Desulphovibrio, Desulphobacter* and *Desulphotomaculum*) are used in biotransformation of organic substrates including hazardous compounds and toxic wastes. These bacteria are industrially important in oil and gas industry; in corrosion of iron, steel and concrete; and in sewage treatment. Anaerobic Thermophiles (*Clostridium, Thermoanaerobacter, Thermoanaerobium* and *Dictyoglomus*) offer advantages for biotechnological processes including high growth rates, enhanced recovery of volatile end products, and processes resistant to the negative effects of contamination. Some sulphate reducing thermophiles like thermodesulfotobacterium, desulphovibrio and desulfurella; thermophilic methanogens including methanobacterium, methanopyres and methanothermus have applications in industrial processes. Anoxygenic phototrophic bacteria are employed for industrial production of vitamins, single cell proteins, enzymes and ubiquinones and in waste treatment.

The domain archaea includes methanogens and thermophiles which are important in anaerobic fermentations. Methanogens use carbondioxide or methyl groups as electron acceptors and form methane as their end product. Hyperthermophilic methanogens (*Methanococcus, Methanothermus*) have maximum growth at 91-110^{0}C. Heterotrophic anaerobic thermophiles (order thermoproteales, thermococcales) are dependent upon organic carbon and nitrogen as electron donors for growth. They grow at a temperature of 67-110^{0}C. Sulphate reducers (genus *Archaeologus*) are capable of oxidising carbon substrates or hydrogen (H_2) and maximum growth is obtained at a temperature of 97^{0}C and is employed in transformations of industrial sulphur residues and in petroleum microbiology.

4. Aerobic Fermentation

If the growth of the fermentation microorganism is to occur aerobically, then provision must be made for rapid incorporation of sterile air into the medium in such a manner that the oxygen of this air is dissolved in the medium and, therefore, readily available to the microorganism, and the carbondioxide resulting from microbial metabolism is largely flushed from the medium. Usually, the sterile air is supplied to the fermentor. The fermentors mostly employed in aerobic fermentations include stirred tank type and air lift type. The fermentor should provide aseptic means for the withdrawal of culture samples during the fermentation as well as for the introduction of inoculum at the initiation of the fermentation.

Table 1.8 : Selected anaerobic industrial processes

Product or process	Representative organism(s)
Degradative and bioremediation process	
Biopolymer degradation	*Clostridum* spp. Thermophiles
Hydrolases from extremophiles	Various
Salt-resistant enzymes	Various
Bioremediation of toxic wastes	Consortia
Aromatic metabolism	Various
Benzene, benzoate degradation	*Rhodoseudomonas* spp.
Degradation of chlorinated ethanes	Methanogens
Dehalogenation of wastes	Various, consortia
Degradation of nitroaromatics	Various
Bioremediation of metals	Sulfate, reducers, others
Biodegradation of agricultural chemicals	Various
Metal corrosion	Sulfate reducers, various
Wastewater treatment	Consortio
Solvent, acid, and fuel production	
Ethanol	*Saccharomyces* spp. *Clostridium* spp. *Themoanaerobacter*, others
Methane	*Methanobacterium* spp. *Methanococcus* spp. *Thermophilic methanogens*
Acetic acid	*Clostridium thermoaceticum*
Acetone and butanol	*Clostridium acetobutylicum*
Conversion of coal-derived synthesis gases to fuels	Various
Selective oxidation of crude oil	Various
Conversion of waste sulfides	Extremophiles
Biotransformations and novel enzymes	
Redox enzymes	Various
DNA polymerases	*Thermus* spp., others
β-Lactam precursor	*Clostridium tetanomorphum*
Enzymes for increased rumen efficiency	Various
Therapeutics and vaccines	
Vaccine production	*Clostridium* spp.
Botulinum toxin as therapeutic	*Clostridium botulinum*
Tumor targeting	*Clostridium* spp.

Optimization of Fermentation Process

The optimization of fermentation process is based upon utilization of metabolic and enzymatic activities of various microorganisms so as to transform organic compounds. The study of kinetics of cell growth has put forth some of the applicability so as to obtain highest end product. The objectives of statistically designed optimization study are to confirm previous effects and interactions, to estimate specific quadratic effects, to determine optimal settings for the critical factors. The use of central composite designs in fermentation has been widely reported and includes optimization for ethanol, citric acid, streptomycin, cellulose, antifungal agents, proteins and agents and microbial growth. Mathematical optimization is performed by most statistical software packages and involves finding the critical value.

Optimization in bioreactor can be done by a simple approach and is used to continuously improve an existing fermentation process. The procedure begins by selecting two or more critical fermentation process. Three bioreactors are needed to study two factors. One bioreactor is run under control conditions, while the other two are set-up to form an equilateral triangle in the two dimensional design space. The results from these three fermentations establish a direction for improvement of the process response. Other optimization designs include mixture designs which is used in pharmaceutical formulation and have been applied to medium composition in fermentation. D-optimal designs are computer generated designs and are useful in fermentation when certain trials cannot be run due to physical or operational constraints. In order to design an optimum fermentor following considerations are required:

(i) Inoculum should be active, (ii) large volumes of inoculum should be used, (iii) inoculum medium should correspond as closely as possible to the fermentor.

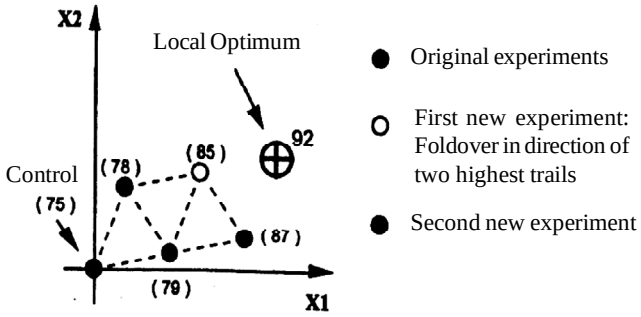

Fig 1.24 : Simplex method for optimizing two factors in a fementation.

Downstream Processing : (DSP)

The efficiency and successful working of a bioreactor depends on adequate upstream operations, and the recovery of the product requires a series of careful and meticulous steps collectively referred to as downstream processing. These include purification, economic analysis, legal and regulatory affairs, and production on larger scales. The products of fermentation may be whole cells (biomass), enzymes, amino acids, organic acids, solvents, antibiotics, therapeutic proteins, vaccines, gums etc., depending on the end product, different separation principles are required.

A representative sample of some DSP operations may include filteration, centrifugation,

flotation, disruption (separation operation); solubilization, extraction, thermal processing, membrane filteration, precipitation (concentration operation); crystallization, chromatography (purification operations); and drying, packing and storage (marketing operations). Downstream processes require more knowledge in chemistry, biology, chemical engineering molecular engineering etc., then only, the extraction and purification of the product from the spent medium will be possible. The cost of downstram processing is often more than 50% of the manufacturing cost, and there is product loss at each step of DSP.

The various steps involved in down-stream processing are as follows :

1. Solid-liquid separation.
2. Disintegration of cells (release of intracellular products)
3. Extraction
4. Concentration
5. Purification
6. Formulation

Fig 1.25 : Steps involved in down stream processing (DSP)

1. Solid-Liquid Seperation

The first step in DSP is the separation of solid, usually cells, from the liquid medium.

(i) Centrifugation : It is used to separate bacteria and is based on the principle of density differences between the particles to be separated and the medium. In continuous flow industrial centrifuges, there is a continuous feeding of the slurry and collection of clarified fluid, while the solids deposited can be removed intermittently. The commonly used centrifuges are disc centrfuge, scroll centrifuge, tabular bowl centrifuge and multichamber centrifuge. Centrifugation difficulties arise due to small differences in the densities of the particles and the medium. The disadvantages of the centrifugation are equipment cost, power consumption, temperature etc.

(ii) Flocculation and Flotation : The formation of large aggregates (floccules) of cells to settle down for easy removal is known as flocculation. Since, sedimentation rate of a particle increases with size, flocculated cells can be recovered by centrifugation. Flotation is mainly used in alcoholic beverages industry. Gas bubbles can be created by sparging, release of overpressure or electrolysis; and adhere to cells or flocs, making them float at the surface of the liquid from where they are skimmed off. Collector substances like long chain fatty acid amines facilitates stable foam formation during flotation process.

(iii) Filteration : Separation of biomass and culture filterate is usually carried out by filteration. Filteration uses pressure created by over pressure or vacuum, and its rate depends on filter area, fluid viscosity and the resistance generated by filter-cake, which increases with time.

Table 1.9 : Types of Filteration Processes

Type	Sizes of particles separated	Compound or particles separated
1. Microfilltration	0.1-10 μm	Cells or cell fractions, virsues.
2. Ultrafiltration	0.001-0.1 μm	Compounds with molecular weights greater than 100 (e.g. enzymes).
3. Reverse osmosis (hyperfiltration)	0.0001-0.001μm	Compounds with molecular weights less than 1000 (e.g. lactoses).

(a) Cake filteration : In this method, the solid particles are retained as a cake in the filter medium. The filters are made up of sintered metal, glass wool, cloth, fibres, ceramics, cellulose etc.

(b) Rotary vaccum drum filters : These are useful in separating mycelia of filamentous fungi. Rotary vacuum drum filters have proved to be quite satisfactory for continuous or semi-continuous separation of cells from whole broth. The filter is in the form of drum and a portion of the drum rotates through the medium which is partially immersed in a tank of broth.

(c) Ultrafilteration : It is a pressure driven membrane separation process for dissolved and suspended materials. Two types of products can be processed by ultrafilteration, namely, (a) dissolved macromolecules and (b) cellular products. Substances with molecular weights ranging from 500-1,000,000 can be separated by ultrafilteration, eg: substances like peptides, proteins, vaccines, viruses etc. Ultrafilteration is mainly used for (a) purification of culture medium prior to bioconversion (or) fermentation, (b) removal of cell debris after lysis, (c) harvesting of cells after fermentation and (d) concentration of product. Ultrafilteration is mainly employed in pharmaceutical, chemical and food processing industries.

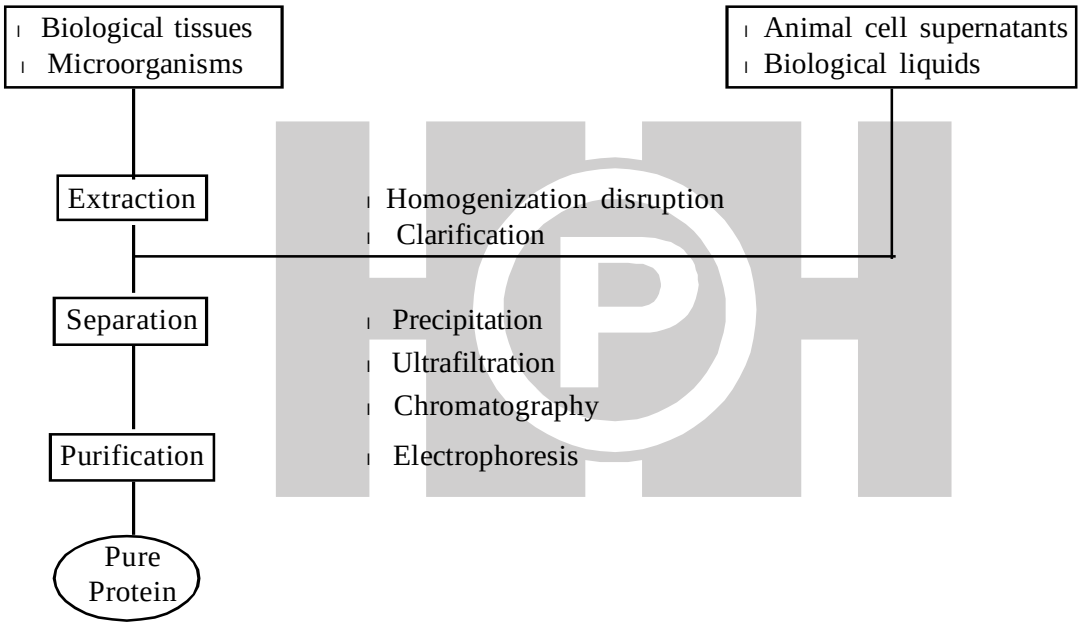

Fig. 1.26 : Principal steps in protein DSP

(d) Membrane filteration : Millipore membrane filters are available in different pore sizes. However, the membranes are prone to choking and blocking. Membranes with asymetric pores (asypore filters) are more efficient. Membranes and membrane processes have received much attention in recent times as a potent tool in modern biotechnology for down stream processing of bioreactor constituents, sterilization of feed streams, or immobilization of enzymes. Mass-scale separation can be achieved physically at ambient temperature without any chemical change is one of the advantages of membranes. In cross flow membrane filteration, the broth is pumped in a crosswise fashion across the membrane. Factors influencing the efficiency of the cross flow technique include permeate flux, voltage and degree of turbulence.

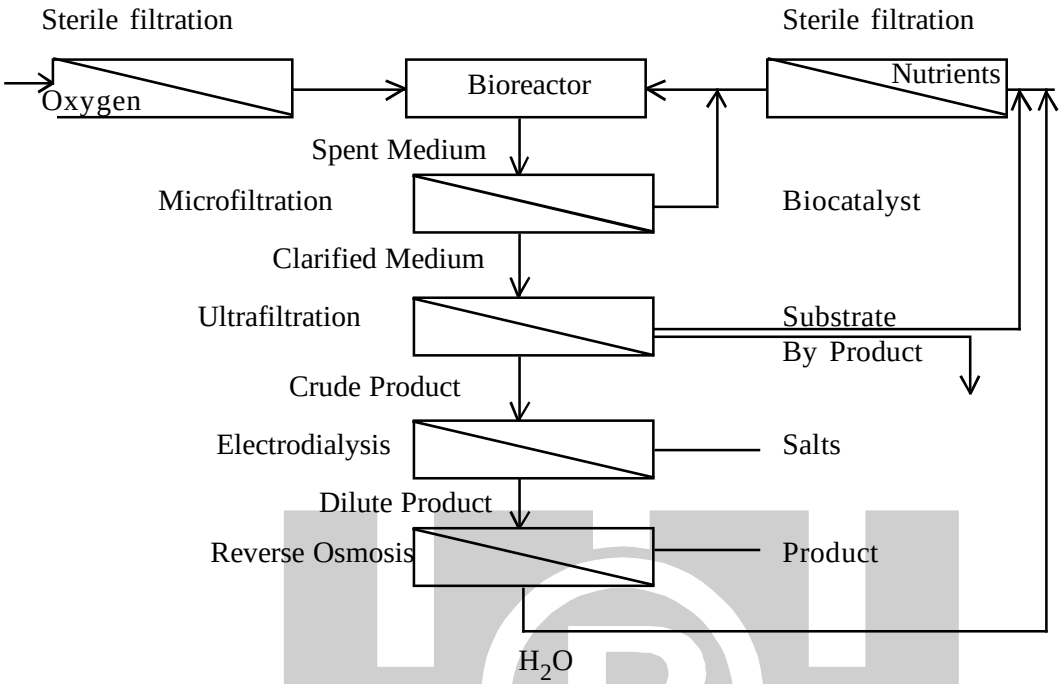

Fig. 1.27 : Uses of membrane process in DSP of biorecator products

2. Disintegration of cells (release of intracelluar products) :

If the desired product is intracellular, the cell biomass can be disrupted so that the product should be released. Disruption of microbial cells is usually difficult due to their small size, strong cell wall and high osmotic pressure inside the cells. There are three methods of cell disruption. They are enzymatic, chemical and physical methods.

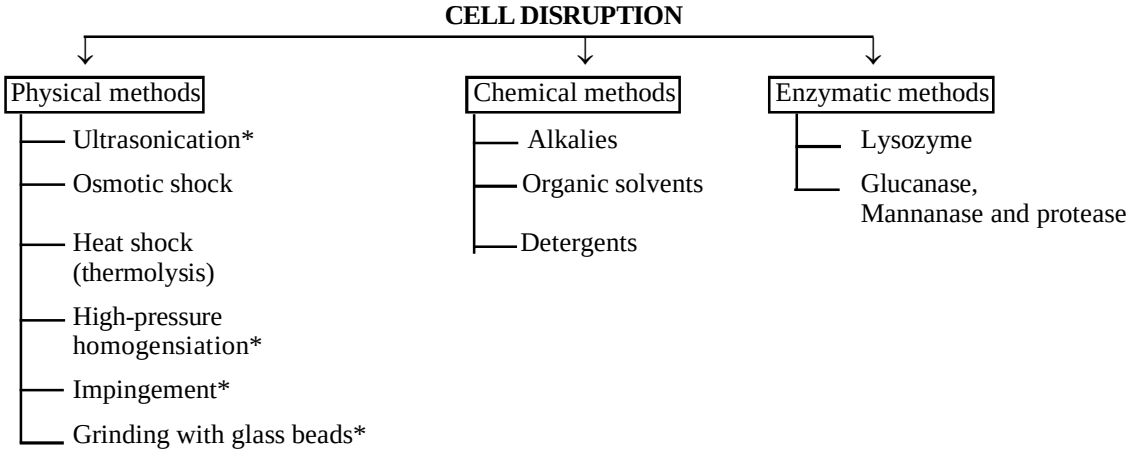

Fig. 1.28 : Methods of cell disruption to release intracellular products

(i) Enzymatic Methods : Cell disruption by enzymatic methods has certain advantages ie., lysis of cells occurs under mild conditions in a selective manner. Enzymatic methods

include the use of egg white lysozyme to lyse gram negative bacteria. Lysozyme is commercially available and it hydrolyses β-1,4-glycosidic bonds of the mucopeptide in bacterial cell walls. Lysozyme plus EDTA is used for the disruption of *Pseudomonas fluorescens* cells to release acylamidase. As the cell wall gets digested by the lysozyme, the osmotic effects break the periplasmic membrane to release the intracellular contents.

(ii) Chemical Methods : Alkali treatment has been used for the extraction of some bacterial proteins. Alkali lysis is used at pH-11 for 20 minutes, using sodium or ptoassium hydroxide for the release of L-asparagine from *Erwinia chrysanthemi*. Organic solvents like methanol, ethanol, isopropanol, butanol can be used in cell disruption. The use of detergent is another chemical method of cell lysis. The detergents may be ionic (sodium dodecyl sulphate), anionic (sodium cholate), cationic (acetyl trimethyl ammonium bromide) or non-ionic (Triton-X-100, Tween 80). The ionic detergents are more reactive than non-ionic detergents, and can cause denaturation of many proteins, Ex : triton-X-100 is used to release cholesterol oxidase from *Nocardia* species, sodium cholate is used for the release of pullulanase (pullulan 6-glucon hydrogenase), a membrane bound enzyme from *Klebsiella pneumoniae*.

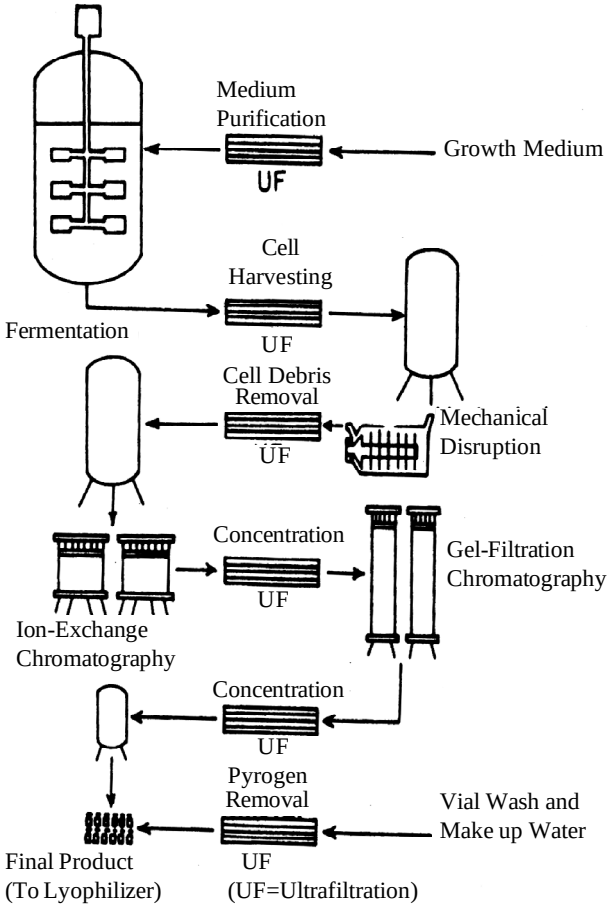

Fig. 1.29 : An illustration of recovery process for intracellular product of fermentation

(iii) Physical Methods : Mechanical methods include sonication, grinding, glass bead mills, the french press, and the Manton-Gaulin homogeniser. Each of these methods destroy proteins as well as cells and requires optimization of the release of active protein. Periplasmic proteins can be selectively liberated by osmotic shock, which ruptures only the outer membrane by sudden transfer from hyper to hypertonic medium. Gram negative bacteria such as *Salmonella typhimurium* and *E.coli* are easily lysed by osmotic shock. The method (osmotic shock) involves washing the cells in a buffer, suspending them in 20% sucrose and resuspending them in distilled water at 4^0C. Grinding the cells by mixing with glass beads are subjected to a very high speed in a reaction vessel. Dynomill is an apparatus used for the release of proteins from cells by abrasion. It is a chamber containing glass beads and a number of fixed and rotating impeller discs. A dynomill can process 5 kg of bacteria per hour. The french press is a standard tool for lysis of some tonnes of grams of wet cell paste by forcing cells through a small orifies at high pressure. It is necessary to precool the cell suspension before homogenization.

3. Extraction :

It is a compound recovery process from a mixture. The purification of microbial metabolite requires a wide range of techniques since these compounds have very diverse chemical structures. They range from extremely water-soluble compounds to very solvent-extractable substances that have all types of ionic character and a wide range of molecular weights. The most difficult compounds to purify are the very water-soluble, neutral, or amphoteric substances. To recover solvent extractable metabolites, a typical extraction can be accomplished by mixing two volumes of the fermentation filterates with one volume of ethyl acetate, separating the organic layer and repeating the process. If the metabolite is an acid, the extraction will in general be more efficient if carried out under acidic conditions, and the opposite effect is observed for basic metabolites. Another approach to solvent-extractable and some near-solvent-extractable metabolites is to adsorb the metabolite from the fermentation filterate with a reverse-phase resin such as Amberlite XAD-2, Diaion HP-20, or Diaion HP-21. The aqueous two phase separation method involves precipitation with polyethylene glycol (PEG) and dextran, PEG and ammonium sulphate, followed by centrifugation. This method is mainly employed for extraction of compounds with high molecular weight.

4. Concentration :

After extraction, both intracellular and extracellular proteins are usually concentrated from the cell-free broth. The commonly used techniques for concentrating biological products are ultrafilteration or adsorption, evaporation and membrane filteration, using ion-exchange resins. Ultrafilteration can be carried out at small scale in centrifugally driven cells, or at larger scale in stirred cells or tangential flow units. Evaporation is generally used in cases of solvent extraction. The evaporators 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 different types of evaporators include continuous flow evaporators, centrifugal forced filan evaporators.

Membrane filteration is mainly used for the separation of biomolecules and particles, and for the concentration of fluids. The different processes involved in membrane filteration are namely hyperfilteration, ultrafilteration, microfilteration and reverse osmosis. Ion exchange resins can firmly attach to the ionizable groups (anions or cations) which ionize under a suitable environment. These may be solids or liquids. Examples of solid ion exchangers are polyamine, cellulose, dextran etc., liquid ion exchangers dissolve only in non-aqueous solvent carriers.

5. Purification :

It is the final step in the recovery of the products. Chromatography is widely used in the purification of proteins. In Industrial downstream processing, selection of the matrix for chromatography is important as the process involves large scale purification. The matrix chosen should be porous, rigid, stable, inert and reusable. The commonly used matrices and their trade names are : (i) Cross linked dextran (Sephadex), (ii) Cross linked polyacrylamide (biogen-P), (iii) Agarose (Sepharose, ultragel-A), (iv) Cross linked agarose (Sepharose-CL, Superose), (v) Porous silica-silicaceous particles coated with agarose (Spherosil), (vi) Kieselguhr (Macrosorb), (vii) rigid organic polymers (Monobeads), (viii) Polysternedivinyl benzene (Poros), and (ix) cellulose (Ultragel-A, Whatman TM, Cellufine, cellex). Most forms of chromatography involve selective adsorption of proteins on the surface of porous particles, throguh interactions that can be broadly classified as ion exchange, hydrophobic and affinity. Chromatography operations are also classified as low-pressure, high-pressure (HPLC), medium-pressure (including Pharamacia's popular FPLC), depending on the pressure used to force liquid through the packed bed of adsorbent particles. Apparatus used for chromatography includes a column into which the particles are packed (glass, plastic or steel), a pump to drive liquid flow, some method for introducing the sample into the flow before the column (a switch wall or manual pipetting), and a fraction collector that deposits the emerging, separted proteins into different vessels.

Table 1.10 : Types of Chromatography

Feature	Low-pressure	Medium-pressure	High-pressure (HPLC)
Particles size	40-150 μm	10-75 μm	2-15 μm
Flow Driver	Gravity, peristaltic	Piston or syringe	Positive displacement
Run time	40-1,000 min	15-60 min	0.5-30 min
Apparatus cost	Low	Medium high	High
Resolving power	Lowest	Intermediate	Highest
Particulate tolerance	Low	Very low	Lowest

Table 1.11 : Chromatography techniques and principles

Chromatography	Principle
Gel-filtration (Size exclusion)	Size and shape
Ion-exchange	Net charge
Chromatofocussing	Net charge
Affinity	Biological affinity and molecular recognition
Hydrophobic interaction	Polarity (hydrophobicity of molecules)
Immobilized metal-ion affinity	Metal ion binding

Ion exchange chromatography is the most common high resolution method for separation of proteins. In this type of chromatography, cellulose ion exchangers with various charged groups are used. Matrices bearing negative charges (carboxylates and sulphonates eg. CM, SP) are called cation exchangers and are used at low pH. Adsorbents with fixed positive charges (from immobilized amines, eg. DEAE, QMA) are called anion exchangers and are employed at pH above the isoelectric point of the protein to be adsorbed. Ion exchange adsorption depends strongly on the ionic strength, equal to half the sum of the concentratrion of all ions present, each multiplied by the square of the ions charge. Affinity chromatography depends on the interaction of a protein with an immobilized ligand. A ligand can be a substrate for the particulate protein (enzyme), substrate analogue, inhibitor or antibody. Hydrophobic interaction chromatography (HIC) exploites the presence of exposed hydrophobic groups on the surfaces of proteins, which can interact with immobilized non-polar moieties such as short alkyl chains and phenyl rings. Most proteins can be purified using agarose substituted with phenyl or octyl groups. HIC is widely used in industrial processes because the cost and capacity of HIC matrices are attractive, as is the method, and tolerance of high salt concentrations, which can allow immediate processing of proteins.

Table 1.12 : Types of absorptive chromatography

Feature	Ion-exchange	Hydrophobic	Metal chelate	Biospecific
Adsorbent	Carboxyl, amine, Sulfonate	Propyl, butyl, phenyl	Chelator, loaded with Ni^{2+}, Zn^{2+}	Antibody, cofactor, receptor
Selectivity	Moderate-high	Moderate-high	Moderate-high	High-very high
Capacity	High	High	Moderate-High	Moderate-High
Matrix cost	Low	Low	Moderate	High
Eluction	High slat, pH	Low salt	pH, imidazole	pH, chaotrope
Initial salt	Low	High	Indifferent	Often indifferent

6. Formulation :

For antibiotics and acids like citric acid, formulation can be done by crystallization by adding salts. Crystallization may be initiated by changing pH or temperature at constant salt concentration. The formulation of low molecular weight products (solvents, organic acids) can be achieved by concentrating them with removal of most of the water. In order to increase the shelf-life of proteins, stabilizers like sugars, salts, polymers are added. Proteins may be formulated in the form of solutions, suspension or dry powder. Drying is an essential component of product formulation. Drying makes the product suitable for handling and storage. In freeze drying, very low pressure is maintained to promote sublimation and the energy needed for sublimation is provided by heated plates and radiation onto the surface. The wide range of products such as diagnostic food stuffs, pharmaceuticals are freeze dried.

Effluent treatment (Biological waste treatment)

The type of microorganisms and their quantity will vary depending on the nature of the waste water. Most industrial processes produce waste waters that contain varying amounts of salts and organic matter. Thus, the waste waters associated with the industrial fermentation industries contain spent media, waste waters and waters accumulating in various steps of product recovery. The manufacturing industries in general releases substances which are biodegradable. Often these wastes can be utilized and recycled by microorganisms and plants. Agriculture and Dairy industries produce crop residues and manures, which are biodegradable. The waste waters resulting from industrial fermentation processes contain water-soluble, colloidal, and suspended wastes. If a plant or animal pathogen has been employed in the fermentation, the fermentation wastes may require-sterilization before undergoing waste treatment. The spent media or media residues also may require preliminary filtration before further treatment to remove the larger solids and masses of microbial cells. Generally, the bacteria present in the sewage include coliforms, faecal streptococci, anaerobic spore forming bacilli, proteus group etc. A fermentation company, depending on its size and on the type of waste waters that it produces, either may process its own waste waters or arrange with the local muncipal sewerage for treatment at the local sewage treatment plant. The natural microbial flora of the waste waters include heterotrophic bacteria, to certain extent, actinomycetes and fungi. Protozoa also are very active in organic matter decomposition. Anaerobic bacteria are helpful in the anaerobic-digestion of organic matters.

Table 1.13 : Illustration of different types of waste and pollutants generated by human activities.

Human activity	Wastes	Pollutants		
		Air	Surface water	Soil/ground water
Manufacturing				
1. Chemical industry	Chemical refuse (solid/liquid)	Voltile organic compounds, SO_2*, NO_2*	Organic and inorganic compounds, heavy metals, cynides	Organic and inorganic compounds, heavy metals, cyanides
2. Oil refinery	Waste water	Volatile hydrocarbons, SO_2 NO_2, aerosols, etc.	Oil, acids, heavy metals, phenolics, etc.	Oil, acids, phenolics, etc.
3. Fertilizer industry	Contaminated gypsum	SO_2, NO_2, NH_3	Inorganic chemicals	Inogranic chemicals
Energy Production	Fly ash, heavy metals, radioactive waste, etc.	CO, CO_2,SO_2, NO_2 aerosols, water vapour	Heat, coal residues, fly ash, etc.	Flyash, coal residues
1. Agriculture	Crop residues pesticides, plastics	Pesticides,	NO_3^-, PO_4^{4-}	Pesticides, NO_3^- PO_4^{3-}
2. Dairy	Animal refuse, feed residues	NH_3^-, CH_4	NO_3^-, PO_4^{4-}(from manures	NO_3^-, PO_4^{4-}(from manure), feed residues
Transport	Scrapped vehicles used up oil, greese etc.	CO_2, CO, SO_2, NO_2 volatile hydrocarbons, aerosols, lead	Oil products	Oil products
House-building and Domestic				
1. House-building	Building and demolition refuse: wood, metals, asbestos, fly ash, etc.	CO_2, CO, SO_2, NO_2 heavy metals, volatile organic compounds, etc.	NO_3^-, PO_4^{4-},O_2^- binding compounds	Metals, wood, fly ash, etc.
2. Domestic	Sewage, garbage, scrapped utensils, appliances, etc.	CO_2, CO, SO_2, NO_2 C.F.C.**m CH_4	Percolation water, NO_3^-, PO_4^{4-}	Solid refuse, percolation water

*SO_2 and NO_2, different oxides of S and N, respectively

Basically, sewage treatment (or) waste water treatment is of two types.

(I) Small scale sewage treatment (septic tank)

(II) Large scale sewage treatment (For towns and cities)

(I) Small scale sewage treatment :

The septic tank : Treatment and disposable of sewage from single-dwelling units can be accomplished by anaerobic digestion of solids. Septic tanks are anaerobic digestion chambers used for bringing about the decomposition of simple or complex organic materials to simple organic molecules and fermentation gases. Septic tank is a metallic or concrete tank which is kept below the ground level somewhere near the homes. It is a relatively small closed tank vented for the escape of fermentation gases and it finds little present-day industrial use. The organic materials accumulated in septic tank is decomposed by anaerboic bacteria releasing into water several by-products such as sugars, alcohols, organic acids, amino acids, glycerol and gases. The undigested organic matter is called 'sludge' and is removed at certain intervals by pumping process other -wise, it will block the tank and pipes.

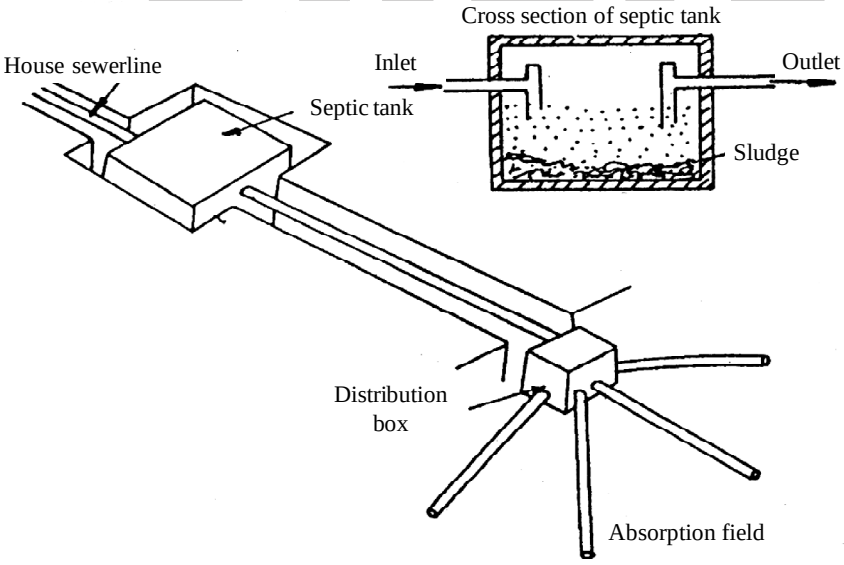

Fig. 1.30 : Illustration of septic tank.

(II) Large scale sewage treatment : Sewage treatment on a large scale of population of towns and cities is known as large scale treatment. This sewage is treated by municipal plants. The overall treatment process is divided into three steps.

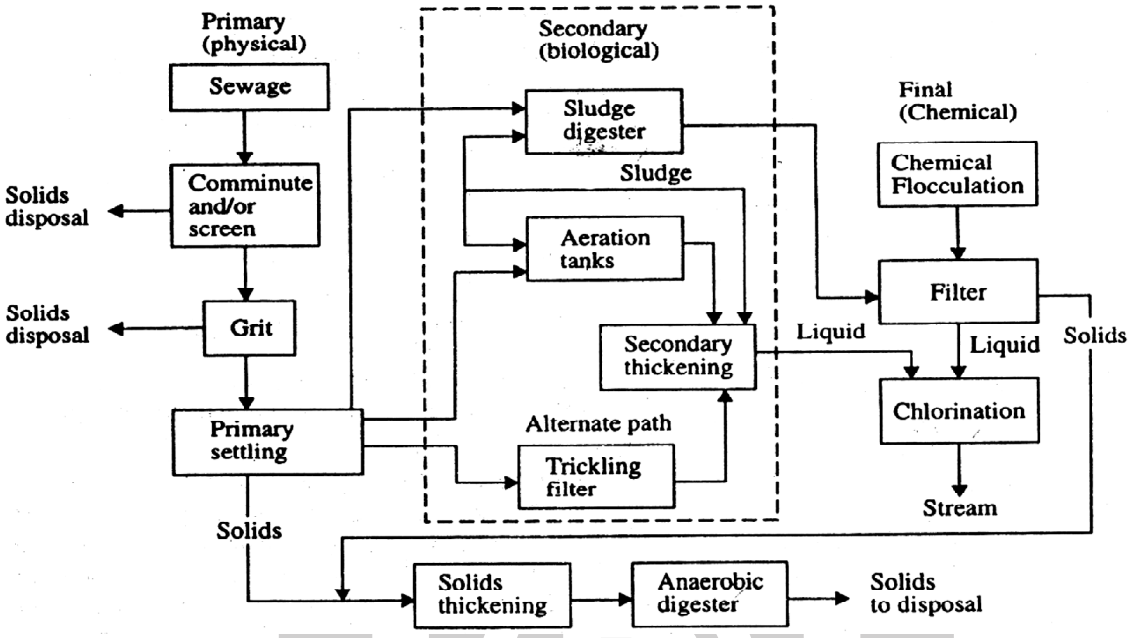

Fig. 1.31 : Illustration of different stages of sewage treatment.

1. Primary treatment
2. Secondary treatment
3. Tertiary treatment

1. **Primary treatment :** It is to remove the settleable solids present in the sewage. The solids are removed by screening or grinding. The solid material (sludge) is removed and kept in land fill/composting for anaerobic digestion. Sedimentation tanks are used for primary treatment and are subjected to alluminium sulfate so that the organic materials and microorganisms can be trapped in sedimentation process of water purification. Sedimentation is influenced by factors like size, shape and specific gravity of the particles. The sedimentation tanks are constructed based on the flow of sewage, shape and the purpose and position and operation. By chemical precipitation about 60-80% of the suspended particles can be removed. The most commonly used coagulants in sewage treatment are alum (aluminium sulphate), iron salts (ferric sulfate, ferrous sulfate, ferric chloride), lime and soda ash (sodium carbonate), sodium silicate and sodium aluminate.
2. **Secondary treatment (Biological) :** It is carried to absorb and oxidise organic constituents of waste water thus reducing BOD. This process involves the oxidation of organic material in the waste water by microbial activity. This process involves the use of microorganisms like bacteria, algae, fungi, protozoa, rotifers, nematodes that

decompose the unstable organic matter to stable inorganic form. The microbial activity may be aerobic or anaerobic. The methods employed are

(i) Filteration by trickling filters

(ii) Activated sludge process

(iii) Use of oxidation ponds

(iv) Anaerobic digestors

(i) Tricking Filters : A tricking filter consists of a large bed of stone pieces, slag or synthetic material with drains made at the bottom of the tank. The sewage is made to slowly trickle over this pile of stones, by a rotating arm or by sprinkelers. Due to the spraying process, sewage is saturated by oxygen. Due to aerobic condition, microbial flora will develop in the bed (bacteria, fungi, protozoa and algae). This microbial growth is generally referred to as zoogleal film or biological film and as the sewage trickles through it, microorganisms metabolize organic constitutents. This film has a thickness of 0.1 to 2 mm and is rich in microorganisms. Few weeks are required for the microbial film to develop in the trickling filter. The biological film is rich in the bacteria-*Beggiatoa alba, Sphaerotilus natons, Achromobacter* spp., species of *Pseudomonas* and algae-chlorella, *Utotrix* and *Stigeochlonium* and some fungi and yeast. The upper region of the tricking filter is favourable for growth of algae and if too much algae are allowed to grow, other microorganisms may be suppressed and efficiency of the filter may be reduced.

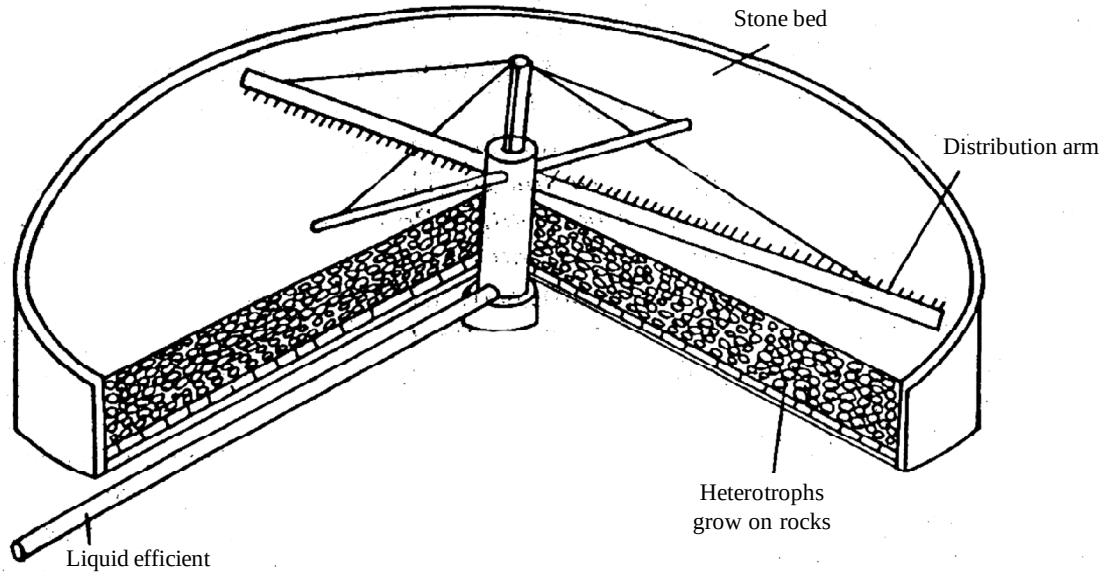

Fig 1.32: The Trickling Filter

(ii) Activated sludge process : In this method, the waste water is subjected to vigorous aeration which results in the formation of flock. The sewage is passed onto an aeration tank from primary settling tank. Sewage is aerated by mechanical stirring. The fine colloidal particles along with microorganisms form aggregates known as floculues. The flocks are permitted to settle down secondary settling tank. Activated sludge contains actively metabolizing bacteria, yeast, molds and protozoa. Activated sludge process reduces the BOD of effluent to 10-15% as compared to raw sewage. The advantages of activated sludge process are significant reduction in BOD and suspended solids, low cost, requirement of little land, reduction in intestinal pathogens. Some of the bacteria that are present in activated sludge flocks are Pseudomonas, *E.coli*, *Achromobacter*, *Enterobacter*, *Zooglea*, *Arthrobacter*, *Mycobaterium*, *Corynebacterium* and large filamentous bacteria, fungi and protozoa.

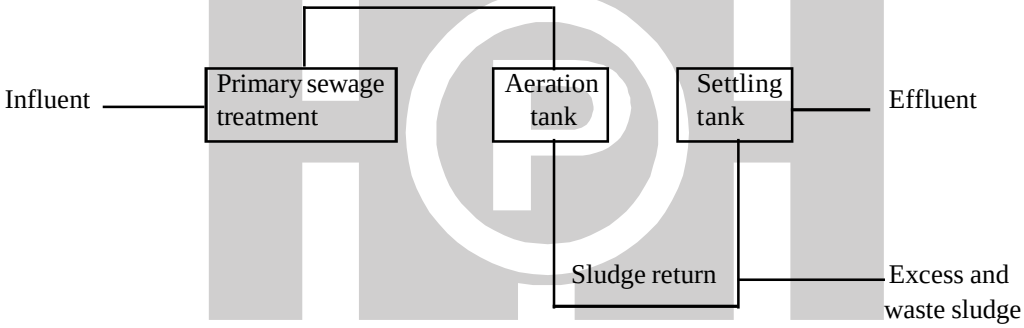

Fig. 1.33 : Flow chart of activated sludge process

(iii) Oxidation ponds : It is used for secondary treatment in rural areas or industrial sectors. The oxidation ponds (aerated ponds, stabilization ponds) are shallow ponds 2-4 feet in depth, designed to allow algal growth. The organic materials are degraded by heterotrophic bacteria into simpler forms, they in turn support the growth of algae. *Chlorella pyrenoidosa*, *C.ellipsoides*, *Scendesmus acutus*, *S. quadricauda* are some of the algae growing in stabilization ponds.

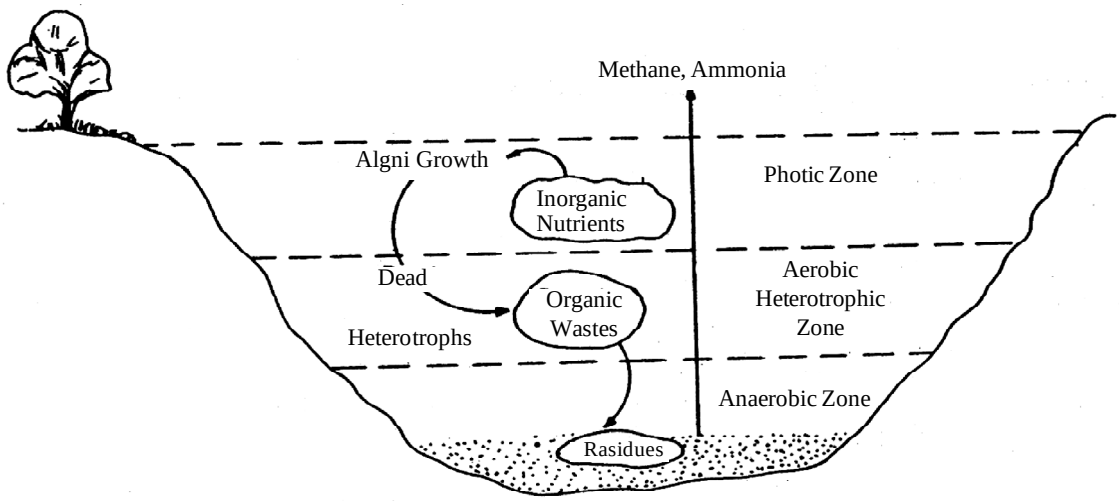

Fig. 1.34 : Oxidation ponds

(iv) Anaerobic digestors : The sludge which accumulates in aerobic sewage treatment are treated in anaerobic digestors through the process of anaerobic digestion. Anaerobic digestion is mostly useful for the stabilization of concentrated sludges and the process is carried out in an airtight reactor. The microorganisms degrade sludge into soluble substances and gaseous products. Sludge is introduced into the digestor continuously or intermittently. These digestors are provided with provisions for mechanical mixing, heating, gas collection, sludge addition and removal of final stabilized sludge. Anaerobic digestion involves following steps :

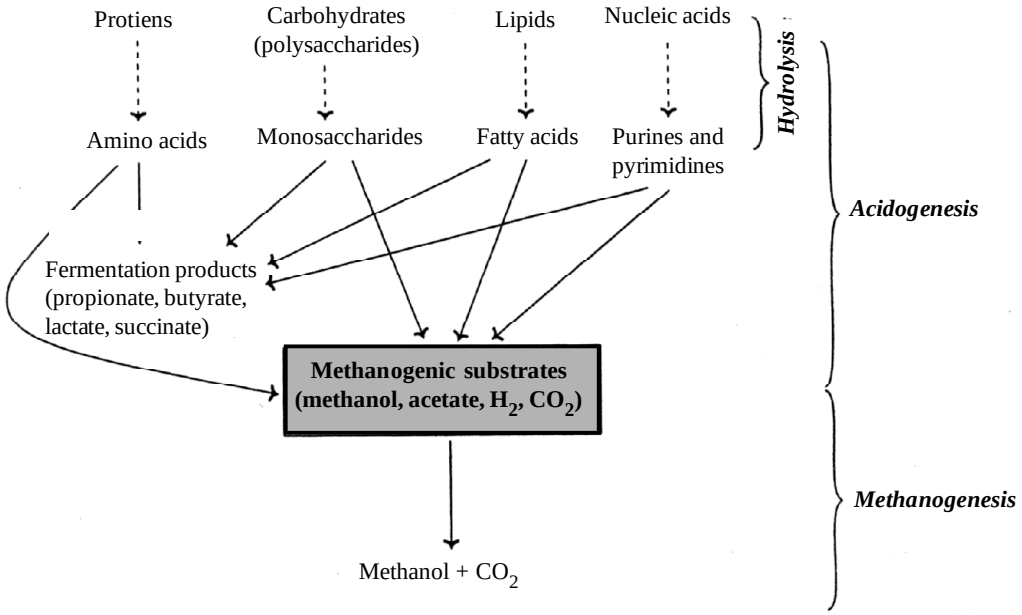

Fig 1.35 : Steps in anaerobic digestion

(a) Fermentation : The formation of organic acids from organic polymers is due to their fermentation of sludge components by bacteria (*Clostridum, Eubacterium, Lactobacillus, Peptostreptococus* etc.,) in the digestor. The organic acids produced are butyrate, propionate, succinate, lactate, acetate along with ethanol and hydrogen, CO_2 etc.

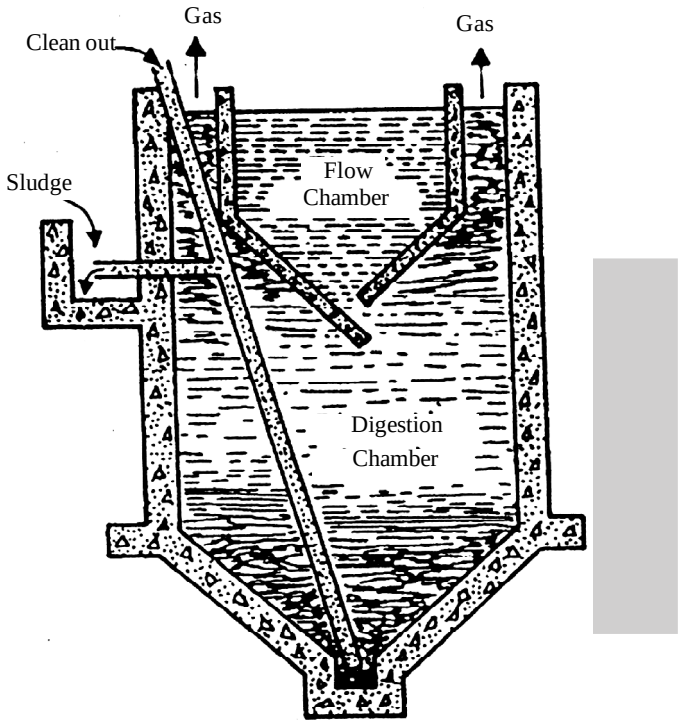

Fig. 1.36 : Anaerobic sludge digestor.

(b) Acidogenesis : The organic acids produced during fermentation are converted to acidic products (acetate) by several acidogenic bacteria viz., *Syntrophomonas, Syntrophobacter* and *Acidobacterium.*

(c) Methanogenesis : This is the last stage and involves the utilization of products produced during acidogenesis as substrates by methanogenic bacteria and produces methane and carbondioxide. Methane gas is highly insoluble and is released from the digestor represents the stabilization of sewage or sludge. The products obtained after anaerobic digestion are a mixture of gases (70% CH_4, 30% CO_2), microbial biomass and non-biodegradable compounds like heavy metals, polychlorinated biphenyl etc.

3. Tertiary treatment

It is needed to remove non-biodegradable organic material, heavy metals and minerals. Tertiary teatment involves the processes like removal of solids, biological nitrogen, biologic phosphorous and killing of microorganisms (disinifection). The solids can be removed by granualar medium filteration and microscreening methods. The nitrogen is removed in the form of nitrate by converting to nitrogen gas (dentirification). Acinetobacter species are employed in the removal of phosphorous by phosphate stripper and process system. Disinfection kills the microorganisms and this process is necessary from the point of view of public health as most of the effluents are discharged to rivers and other water bodies. Physical agents like heat and light are used as disinfectants, and the chemicals like bromine, ozone, iodine, phenols, alcohols, alkalies, acids are also used as disinfectants. If disinfection is not done, the effluents may affect water supply systems downstream by harming irrigation and fish harvesting. Chlorination (chlorine) is the usually employed method of disinfection.

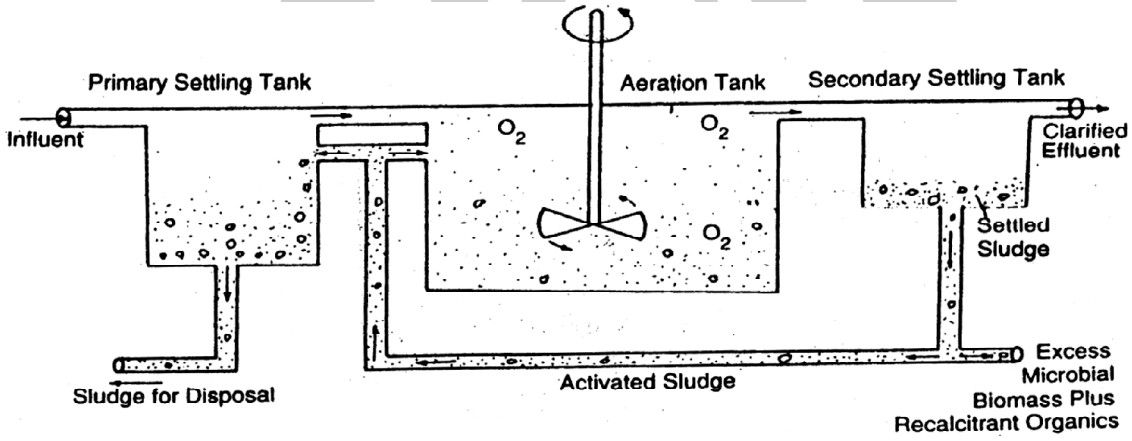

Fig. 1.37 : Activated sludge process

Questions

(I) Essay type questions

1. Write an essay on strain improvement of Microorganisms.
2. Give a brief account of different types of bioreactors.
3. Write an essay on effluent treatment.

(II) Short answer questions.

(i) Design and operation of Bioreactor

(ii) Screening of industrially important microbes.

(iii) Anaerobic digesters

(iv) Downstream processing

(v) Batch fermentation.