RA 1

Techniques in Microbial Culture

Bacteria and fungi may be cultured in liquid or solid media. These comprise a base of **agar** to which is added the nutrients required for microbial growth. Agar is a gelatinous colloidal extract of red algae, and can be used in solid or liquid form. It is used because of its two unique physical properties. Firstly, it melts at 100˚C and remains liquid until cooled to 40˚C, at which point it gels. Secondly, few microbes are capable of digesting agar so the medium is not used up during culture. The addition of microbes to an agar plate, or to liquid agar, is called **inoculation** and must be carried out under aseptic conditions. **Aseptic techniques** involve the **sterilisation** of equipment and culture media to prevent cross contamination by unwanted microbes. Sterilisation is a process by which all organisms and spores are destroyed, either by heat or by chemicals.

Conditions for the Culture of Bacteria and Fungi

Fungi

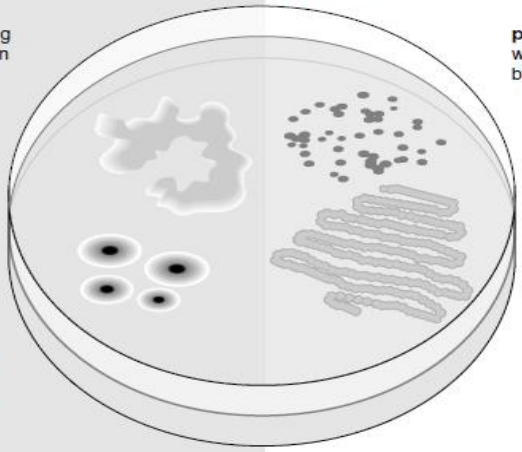
Temperature: Most fungi have an optimum temperature for growth of 25˚C, but most are adapted to survive between 5 and 35˚C.

pH: Fungi prefer a neutral (pH 7) growing environment, although most species can tolerate slightly acidic conditions.

Nutrients: Fungi require a source of carbon and nitrogen to produce protein. They also require trace elements such as potassium, phosphorus and magnesium. Growth factors can be added to increase the rate of fungal growth.

Water potential: Fungi are 85-90% water by mass. Water is constantly lost from the hyphae via evaporation and must be replaced through absorption from the media. To aid water uptake, media have a water potential that is less negative than that of the fungal tissue.

Gaseous environment: The majority of fungi are aerobic and very few species can tolerate anaerobic conditions. This is why fungi always grow on the surface of a culture medium, not inside it.



Bacteria

Temperature: Most bacteria cultured in the school laboratory are classified as **mesophiles**. Mesophiles prefer temperatures between 20 and 40˚C.

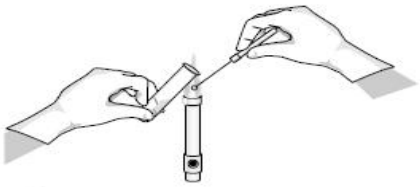
pH: Most bacteria grow optimally in media with a pH between 6 and 8. Very few bacteria can grow in acidic conditions.

Nutrients: Bacteria need a source of carbon, nitrogen and mineral salts as raw ingredients for cellular growth.

Water potential: All bacteria require water for growth. To prevent cell lysis or dehydration, the water potential of the medium must be such that net water fluxes into and out of the bacterial cell are minimised.

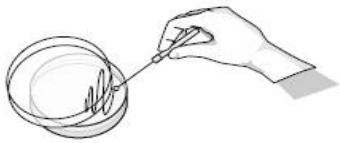
Gaseous environment: Aerobic bacteria will grow only in oxygenated environments, whereas obligate anaerobes (e.g. *Clostridium*) do not tolerate oxygen. Facultative anaerobes grow under aerobic conditions, but are able to metabolise anaerobically when oxygen is unavailable. All bacterial cultures benefit from a low concentration of carbon dioxide.

Inoculating Solid Media



1 Hold the inoculating loop in the flame until it glows red hot. Remove the lid from the culture broth and pass the neck of the bottle through the flame.

2 Dip the cool inoculating loop into the broth. Flame the neck of the bottle again and replace the lid.



3 Raise the lid of the plate just enough to allow the loop to streak the plate. Streak the surface of the media. Seal the plate with tape and incubate upside down.

RDA 2

Serial Dilution

The growth of microorganisms in culture can be measured in a number of ways. Some indirect methods measure culture dry weight or turbidity, both of which are often directly proportional to cell density. More commonly used are methods that directly or indirectly count the number of cells in a culture. Because microbial populations are often very large, most counting methods rely on counting a very small sample of the culture. A commonly used indirect method is serial dilution followed by plate counts (illustrated below).

Measuring Microbial Growth Using Serial Dilution

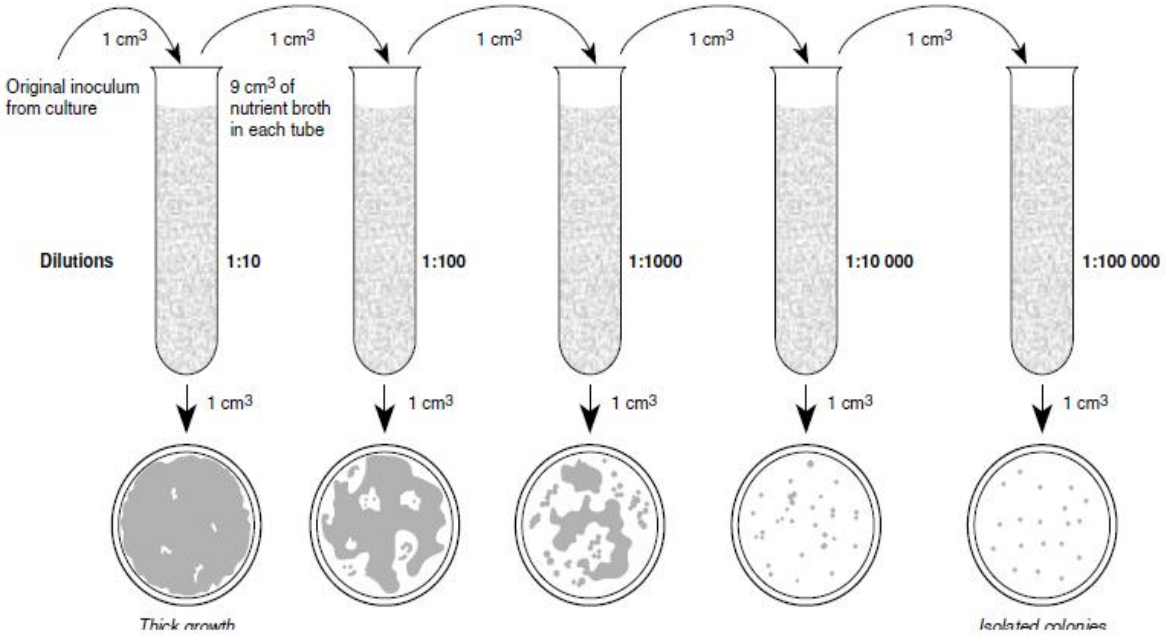
Serial dilution can be performed at different stages during the culture growth. By making a series of dilutions and then counting the colonies that arise after plating, the density of the original inoculum (starting culture) can be calculated. Colonies should be well separated and the number of colonies counted should ideally be neither too small nor too large (about 15-30 is good).

CALCULATION: No. of colonies on plate X reciprocal of sample dilution = no. of bacteria per cm^3.

EXAMPLE: *28 colonies on a plate of 1/1000 dilution, then the original culture contained:*

28 x 1000 = 28 x 10^3 cm^{-3} bacterial cells

Plate counts are widely used in microbiology. It is a useful technique because only the viable colonies are counted, but it requires some incubation time before colonies form. For quality control purposes in some food industries where the food product is perishable (e.g. milk processing) this time delay is unacceptable and direct methods (e.g., cell counts using oil immersion microscopy) are used.



FERMENTATION IN FOOD BIOTECHNOLOGY

Fermentation is an important part of our lives. The relevance of fermentation to day to day life is evident from the fact that food can be both spoiled and made by fermentation. Many of the foods used for human consumption are fermented foods. Fermentation is one of the oldest techniques used for food preservation. Muscle cells use fermentation to provide energy for a quick response. The oldest food biotechnological processes include the baking of yeast leavened breads, brewing of beer, sake and wine, and production of yogurt and cheese. Biotechnology can improve the baking process with improvements in cereal grains and starter culture

through recombinant DNA technology, use of enzymes as processing aids, and application of advanced batch and continuous fermentation technologies.

TYPES OF FERMENTATION

The fermentation process is mainly divided into two broad categories: **submerged fermentation and solid-state fermentation. The first** has been readily employed in industries for large scale **production of alcohol, organic acids, enzymes, antibiotics, vitamins, and amino acids. Solid-state fermentation** has been used for the **production of microbial metabolites from fungi**, but suffers from limitations of operation at large scales due to operational difficulties.

1- Submerged Fermentation

Submerged fermentation is the most popularly used technique for the production of a large number of products using a wide range of microorganisms. **The water activity of the medium is high (broth culture), making it prone to contamination if asepsis is not maintained.** Problems can be encountered at high substrate concentrations. Mass transfer from gas to liquid phase is usually a limiting factor, but due to better mixing, diffusion limitation of nutrients is not encountered in submerged fermentation. Bioreactors can be classified into three groups based on the type of biochemical process employed ;

1. Bioreactor with no agitation (mixed) and aeration (anaerobic processes, e.g., production of wine and beer)
2. Bioreactor with agitation and aeration (aerobic processes, e.g., production of citric acid and penicillin)
3. Bioreactor with aeration, but no agitation (aerobic solid state fermentation processes, e.g., production of food enzymes)

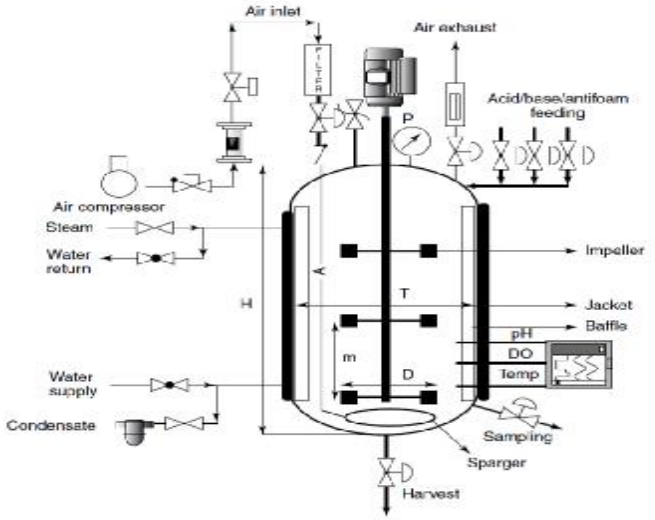


Figure .stirred tank bioreactor

2- Solid-State Fermentation

Solid-state fermentation (SSF) is used for the **production of bioproducts from microorganisms under conditions of low moisture content for growth**. The main **difference between submerged and solid-state fermentations is the amount of free liquid in the substrate** .The **medium used for SSF is usually a solid substrate (e.g., rice bran, wheat bran, or grain),** which **requires no processing**. In order to

optimize water activity requirements, which are of major importance for growth, it is necessary to take into account the water sorption properties of the solid substrate during the fermentation . **In view of the low water content, fewer problems due to contamination are observed**. The power requirements are lower **than submerged fermentation. Inadequate mixing, limitations of nutrient diffusion, metabolic heat accumulation, and ineffective process control renders SSF generally applicable for low value products with less monitoring and control.**

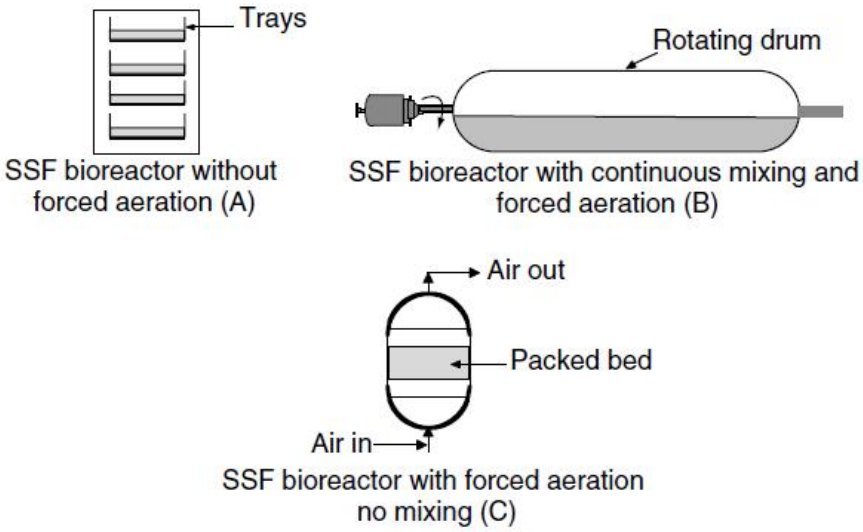


Figure 3.2 Solid state bioreactor systems

STAGES IN A FERMENTATION PROCESS

A- Upstream Processing

The upstream processing in a fermentation process includes preparation of the **fermentation medium, sterilization of air and fermentation medium and inoculation of the fermentor**.

1-Fermentation Medium

Metabolic activity can only be maintained if the necessary nutrients are available to the cell. **A medium is defined as the substance surrounding the cells**, which enables the microorganisms to grow and form products. While the single components of a medium are defined as substrates, generally, **only the carbon source is called the substrate. Formulation** of proper medium is an essential requirement for the success of a fermentation process. Medium used for industrial fermentations should have the following criteria : Relatively expensive substrates, such as yeast extract, brain heart infusion, peptone, casamio acids, and tryptone are often used for complex medium. In order to maximize the yield and selectivity of serine alkaline protease production by recombinant *Bacillus subtilis*, based on the amino acid compositions of the enzyme, the fermentation medium was designed with complex nutrients to supply the most needed amino acids to increase the yield ., for the production of pectinase by solid-state fermentation using *Aspergillus niger* .

2- Optimization of Fermentation Medium

Optimization of fermentation medium is done by using an experimental design considering the variables, mostly medium ingredients, that may have a significant influence on the

3-Components of Industrial Fermentation Medium

In industrial processes, the cheapest substrates are used to keep the production costs low. Some of the cheap substrates available include barley, barley malt, blood meal, corn meal, cotton seed meal, rice bran, rice flour, soybean meal, etc .The composition of the medium has to reflect the demands necessary for the growth and synthesis of products other than cells.

Generally, media consist of an aqueous solution containing dissolved nutrient salts and a gaseous component (O2). With the economics of fermentation playing an important role in a successful fermentation .Metabolic engineering of *S.cerevisiae* for efficient xylose fermentation to ethanol has been reported

4- Sterilization

Sterilization is a process by which microorganisms are either killed or removed from the material or equipment. Sterilization consist of :

A- **Medium**(fermentation media) **Sterilization Techniques ;**

1- by high temperature achieved by direct or indirect steam or electric heating .

2. by Sterilization of the medium for animal cell culture by membrane filtration with absolute rating of 0.1–0.04 μm.

3-Microwave irradiation, which is used commonly in food industries and has been reported to cause cell death .

4 Newer techniques, like photosemiconductor powders which involve the rupture of the cell membrane by increasing the transmembrane electric field strength beyond a certain threshold.

B -Sterilization by Heat

When a medium containing microorganisms is heated above a certain temperature limit, the microorganisms are unable to survive. However, because endospores have higher heat tolerance, inactivation of spores is a good indication of the sterilization process efficiency. In the testing of a heat sterilization process, *Bacillus stearothermophilus* spores, which are the most temperature resistant, are widely used as test organisms . Batch sterilization: Sterilization of medium in the fermentor can be carried out in batch mode by passing steam through the available heat transfer area or through direct steam or with electrical heaters. The highest temperature to be operated for batch sterilization of medium is 121°C.

C- Sterilization of Air

Aerobic fermentation processes require significant quantities of sterile air. The sterilization of air in the strict **bacteriological sense means the complete elimination of all viable microorganisms.** The removal of bacteria by means of depth filters consisting of granular carbon or fibrous media has been almost universally adopted. In most fermentation systems, a prefilter usually made of cellulose, or glass wool is installed prior to the absolute filter to remove dust, oil droplets, and moisture from the process air. After air filter sterilization, the filters are held under positive pressure until the end of fermentation. Pharmaceutical grade cartridges are processed with deionized, pyrogen free water and evaluated by bacterial challenge in accordance with

international ecommendations using standard test organisms (e.g., *Pseudomonas diminuta, Acholeplasma laidlawii*).

5- Inoculation

The process of inoculation is the transfer of seed material or inoculum into the fermentor. Inoculation of a laboratory fermentor is generally done using presterilized tubing and a peristaltic pump. Heat susceptible substances such as amino acids and **some vitamins must be dissolved in small volumes of water, sterilized by filtration and added** separately to the final medium aseptically. It is necessary that a standard is set for cultural conditions needed for development of the inoculum. **growing biomass determined by parameters such as turbidity, dry weight, wet weight, or morphological characteristics** . The inoculum age at three time intervals, **namely early log, log, and declining phases of growth determined online by the carbon dioxide production** rates were carried out to determine **the best inoculum for fermentation. the log phase inoculum gave a significan**tly higher product concentration compared to the other two in the fermentor.

2- Fermentation Process

The fermentation process involves actual growth of the microorganism and formation of the product under agitation and aeration, to provide uniform environment and adequate oxygen to the cell for growth, survival, and product formation.

2.1 Modes of Operation

A fermentation system is usually operated in one of the following modes**: batch, fed batch, or continuous fermentation. The choice of the fermentation mode is** dependent **on the relation of consumption of substrate to biomass and products** .for continuous fermentation are **1-Chemostats 2- auxostats**.

1-Chemostats:- The commonly used **auxostats include turbidostats the pHauxostat and the nutristat**.

Chemostat: Presently, the chemostat is the most widely used apparatus for **studying microorganisms under constant environmental conditions**. It is a **continuous fermentation process performed in a maintaining a growth rate through c**ontinuously feeding **a growth limiting nutrient and withdrawing part of medium at the same rate, thereby** achieving steady state growth. The growth limiting nutrient may be **carbon, nitrogen, phosphorus, or any other essential nutrient,** which influences the specific growth rate. A schematic of chemostat (single stage) cultivation with and without cell recycle is shown in Figure 3.5 and Figure 3.6 respectively.

2- Auxostat : An auxostat is a continuous culture technique wherein the dilution rate is regulated based on an indication of the metabolic activity of the culture.

A chemostat is essentially used **for operation at moderate to low dilution rates**, but an auxostat is used **at high dilution rates**. Population **selection pressures in an auxostat lead to cultures that grow rapidly**. Practical applications include high rate propagation, destruction . In a **A** -pHauxostat**, the feed rate is regulated by measurement and control of the pH of the fermentation medium**. This can be applied only if there is a change in pH consequent to the growth of microorganism. The PHauxostat has been used for continuous mass cultivation of bacteria for isolation of intracellular products

B-Turbidostat: The turbidostat controls the feed rate **depending on the optical density (turbidity) of the fermentation broth, which is proportional to the biomass concentration .** In this mode of operation, the culture cannot washout as in a chemostat. This mode of operation is ideal only when operated near maximum growth The isolation of acid tolerant baker's yeast variants was developed in a turbidostat.

C- Nutristat: The **nutristat involves regulation of the feed rate to maintain the residual substrate concentration during fermentation**. The use of specific sensors for monitoring the residual substrate level during fermentation is employed. Ion selective electrodes (NH4 have been used for control in the nutristat. However, the lack of reliable and accurate sensors for common substrates is a bottleneck to nutristat operation baker's yeast, vinegar, gluconic acid, acetone, butanol, and ethanol **.**

2-2Agitation

In stirred tank bioreactors, mixing and dispersion of air in the fermentation broth is achieved by mechanical agitation.

2-3 Aeration

The cheapest mode of supplying oxygen to the fermentation media is air. The aeration system essentially comprises an air compressor, an air filter (prefilter and sterile filter) and a sparger.

2-4 Process Monitoring and Control

The complexity of metabolic biosynthesis involved in fermentation necessitates the use of process control parameters for monitoring and control to ensure optimum process performance.

B-Downstream Processing

The products of fermentation are usually found in complex mixtures of dilute solutions and must be concentrated and purified. The separation of the product of interest from the fermentation broth depends on the accumulation of the product, which may be intracellular or extracellular. The typical downstream operations and the unit operations involved in the processing of fermentation broth are:

1. Cell separation (settling, centrifugation, dead end filtration, and cross flow filtration)
2. Cell disruption (high pressure homogenization, wet milling, and lysis)
3. Clarification of extract (centrifugation, extraction, dead end filtration, and cross flow filtration)
4. Enrichment (precipitation, batch adsorption, ultrafiltration, and partition)
5. High resolution techniques (ion exchange, affinity, hydrophobic, gelfiltration, adsorption chromatography, and electrophoresis)
6. Concentration (sterile filtration, diafiltration, ultrafiltration, freeze drying, spray drying, and precipitation). While downstream processing is an important part of fermentation process development, a detailed discussion on the subject is outside the scope of the chapter and hence not dealt with.

Types of Microbial Culture

Microbial culture procedures can be carried out in the desired vessel in different ways. Sometimes these types of culture methods are suitable for the growth of a particular type of organism to produce the desired metabolites . Some Microbial culture procedures have been given in Table 1

	Batch Culture	**Fed-batch Culture**	**Continuous Culture**
Closed/ Open	Closed culture system	Open	Open
Nutrients	Limited amount of nutrients	Continuously and sequentially fed with fresh medium	One of the nutrients is in limited quantity. Just before the nutrient is fully exhausted, fresh medium containing the limited nutrient is added.
Removal of culture	**No** removal of growing culture	**No** removal of growing culture	cells and products are **removed at intervals**.
What is achieved	Only low cell densities can be achieved	High cell densities can be achieved in the same reactor.	Culture is constantly removed from the vessel and formation of new biomass by the culture is balanced by the loss of culture from the vessel.
Examples	Culturing microbes in an ordinary flask in the laboratory is a batch culture.	Fed-batch culture is preferred when high substrate concentration causes growth inhibition.	Continuous culture is most suitable for production of biomass or metabolites. Continuous culture is widely used for the treatment of liquid waste wherein waste organic materials are converted into microbial biomass.
Types	One Type	One Type	Two Types. **1-**In a chemostat, constant chemical environment is mained. **2-** In a turbidostat, constant cell concentration is mained.

Applications of Microbial Culture Technology

Microbial culture has immense potential for the production of very useful compounds. Once the microbial culture is established, depending on its metabolic activity it can be used for the production of numerous compounds. In general, microbial cultures can be exploited primarily in six different ways for the production of metabolites. They are listed below :-

1-Production of whole microbial cells :-

A- For the production of fermented food such as curd and cheese **.**

C- The whole bacteria are used as starter cultures.

D- Used for preparations such as bacterial vaccines, e.g. vaccine for typhoid and tuberculosis **.**

E- Single cell protein is used a source of protein.

2- Production of primary metabolites examples are **acids and** alcohol **.**

3- Production of secondary metabolites from different microorganisms **for produced antibiotics.**

4- Biotransformation reactions examples are **enzymatic and steroid.**

5- Exploitation of metabolism :

A-microbial leaching such as production of vitamin C.

B- waste treatment is means convert unsuitable substrates to useful products. Examples are Extraction of metals and treatment of liquid waste .

6-Recombinant proteins by using genetic engineering techniques .**:-**

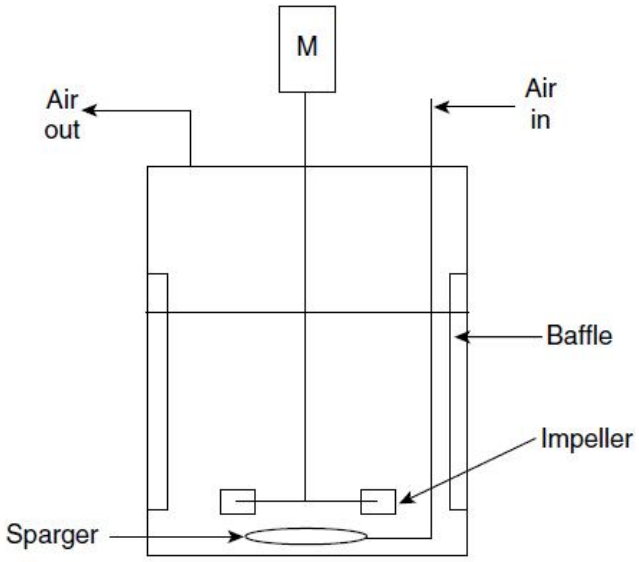
A-therapeutic proteins They uses of microorganisms as hosts for production of recombinant proteins. Expression of human insulin in ***coli*** and hepatitis B vaccine in **Yeast**

B- gene delivery vectors/DNA

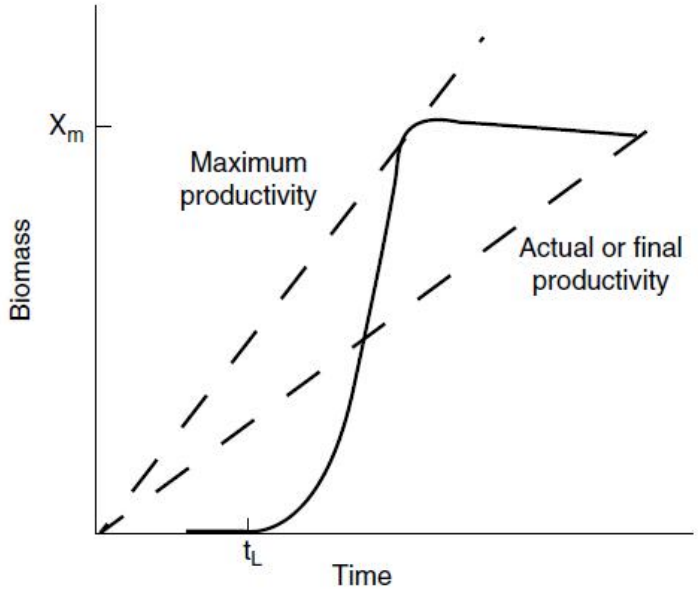
Some specific examples of products derived from microbial cultures have been given in Table 1.

Microorganisms	Products
Saccharomyces cerevisiae	Ethanol
Aspergillus niger	Citric Acid
Penicillium chrysogenum	Penicillin
Streptomyces griseus	Streptomycin
Corynebacterium glutamicum	L-Lysine
Propionibacterium shormanii	Vitamin B_{12}
Aspergillus oryzae	Amylases,
Leuconostoc mesenteroides	Dextran
Escherichia coli (via recombinant technology)	Insulin, growth hormones, interferons,
Saccharomyces cerevisiae (via recombinant technology)	Hepatitis B surface antigen
Alcaligenes eutrophus	Poly 3-hydroxybutyrate

Table 1 Some microbial species used for producing commercial products



ıre 3.3 Batch fermentation system



ıre 3.4 Productivity in batch fermentation

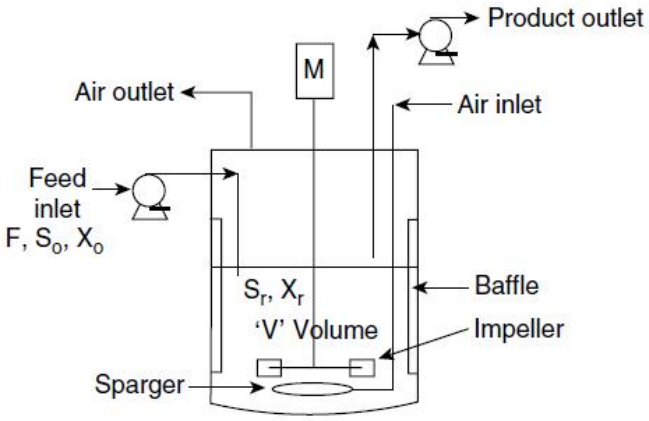


Figure 3.5 Continuous fermentation system

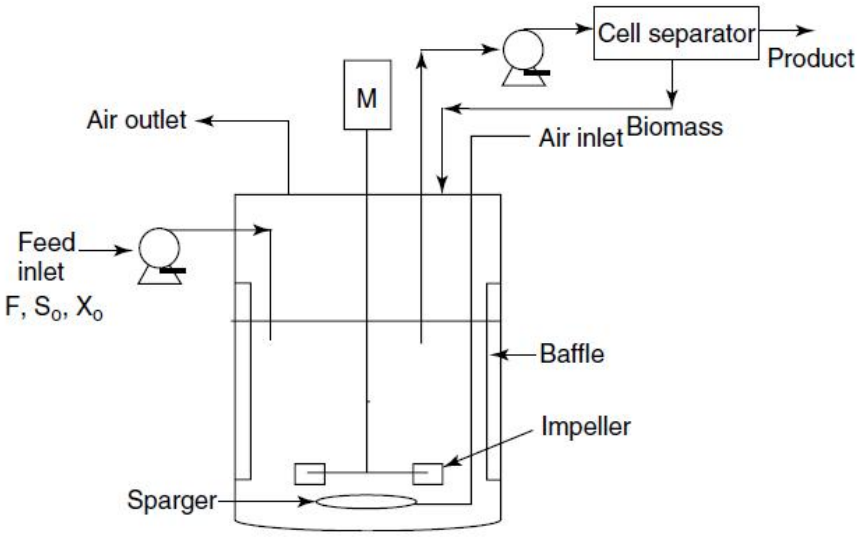


Figure 3.6 Continuous fermentation system with cell recycle