INTRODUCTION TO FERMENTATION PROCESS

The range of fermentation processes - Strain, culture collection management, Inoculum preparation, Scale up of the inoculum - Sterilization, Batch and Continuous sterilization of medium, Aseptic operation.

Unit – I - The Ranges of Fermentation Processes

1. Processes that produce microbial enzymes
2. Processes that produce microbial metabolites
3. Processes that produce microbial cells (biomass) as the products
4. Processes that produce recombinant products
5. Processes that modify substrates (transformation process)

1. Processes that produce microbial enzymes

- Microbes, plants and animal are the major source of enzymes
- Commercial production of many enzymes exploiting these sources have been achived
- As being produced in large quantities by the fermentation processess, microbial enzymes have the enormous economic potential
- Microbes are more prone to change in its genetics to enhance its productivity compared to plant or animal system
- It is possible to produce enzymes of eukaryotes into the prokaryote systems with the help of recombunant DNA technology
- It is possible to control and improve microbial enzyme production by introducing inducers and activators in the production medium
- It is also possible to increase the copy number of gene coding for the a specific enzyme using principles of recombinant DNA technology

2. Processes that produce microbial metabolites

The growth of a microbial culture can be divided into major four phases. These are.

1. Lag phase
2. Log phase
3. Stationary phase
4. Death phase

1. Lag phase

- Once the inoculation of the cells into fresh medium is done, the bacterial population remains temporarily unchanged
- There is no cell division during this phase
- The cells grow in volume and mass by synthesizing the population remains temporarily unchanged etc.
- Metabolic activity is at high rate
- This period is known as the period of adaptation
- **T**here are various factors that affects the this phase are size of inoculum, time required to recover shock in the transfer, time required for synthesizing essential coenzymes and other factors
- Time required for synthesis of necessary new enzymes to metabolize the substrates present in the medium

2. Phase of exponential growth

- This period is also known as the phase of exponential growth
- During this period, the growth rate of the cells gradually increases
- The cells grow at a constant, maximum rate
- Cells are growing in geometric progression dividing by binary fission
- The incubation conditions and composition of the growth medium control the rate of cell division

3. Stationary phase

- During this phase growth cease
- In a batch culture (in test tube or EM flask) , exponential growth cannot be continued forever
- Various factors like exhaustion of available nutrients, accumulation of inhibitory, metabolites or end products and lack of biological space limit the growth during this phase
- During this phase the number of dividing cells equals the number of dyeing cells
- This is not a quiescence period like lag

4. Death phase

- This phase is the reverse of the log phase
- The viable cell population declines exponentially during this phase

Base on the various products produced, the phases of bacterial growth can be categorized into two phases. These are

(i) The Trophophase

(ii) The Idiophase

1. Tropho phase

- Metabolites which are essential to the growth of the cells like amino acids, nucleotides, proteins, nucleic acids, lipids, carbohydrates are produced during the log phase of the growth
- The products (metabolites) produced during this phase (log phase) are known as **primary metabolites** and the phase in which they are produced(equivalent to the log, or exponential phase) is referred to as the ***trophophase*** (Bu'Lock *et al.,* 1965)
- The primary metabolites are also known as central metabolites
- Several primary metabolites are of economic importance and can be produced in large quantity by fermentation process
- The synthesis of primary metabolites by wild-type micro-organisms aims to meet the requirements of the organism
- The industrial production these metabolites can be achieve by providing appropriate cultural conditions to the wild-type organism to increase and improve the productivity of these compounds
- Productivity can also be improve by modifying interested genes by the help of recombinant DNA technology
- Following are few economically important primary metabolites which can be produced at large scale

Idiophase

- During the stationary phases several microbial cultures produce certain compounds (these compunds are not produced during the "***trophophase"*** and which do not appear to have any obvious function in cell metabolism). These compounds are called the secondary compounds of metabolism. The phase during which these compounds are produced (equivalent to the stationary phase) as the "***idiophase***" (Bu'Lock et al., 1965)
- The secondary metabolism is also known as "**special metabolism**"
- The products of secondary metabolism are not absolutely required for the survival of the organisms
- All microorganisms do not undergo secondary metabolism. It is common amongst the filamentous bacteria and fungi and the spore forming bacteria
- The taxonomic distribution of secondary metabolism is different from that of primary metabolism
- The physiological role of secondary metabolism and hence secondary metabolites in the producer cells has been the subject of considerable debate
- The large scale production of secondary metabolites focus on the importance of these metabolites on organisms other than those that produce them
- Secondary metabolites play an important physiological role several ways. Many secondary metabolites possess antimicrobial activity, some acts as specific enzyme inhibitors and growth promoters and many have pharmacological properties
- Thus, due to a huge economic potential, the industrial production of these metabolites have formed the basis of a number of fermentation processes
- As the wild-type microorganisms produce very low concentrations of secondary metabolites, the large scale synthesis can be controlled by induction, catabolite repression and feed-back systems
- Following is the outline of inter-relationships between primary and secondary metabolism and their respective products

3. Processes that produce microbial cells (or biomass) as the product

The commercial microbial biomass production can be divided into two major processes:

1. The production of yeast to be used in the baking industry and
2. The production of microbial cells which can be used as human and/or animal food (single-cell protein)

- Bakers' yeast has been produced on a large scale since the early 1900s and yeast was produced as human food in Germany during the First World War
- However, it was not until the 1960s that the production of microbial biomass as a source of food protein was explored to any great depth
- A few large-scale continuous processes for animal feed production were established in the 1970s. These processes were based on hydrocarbon feedstocks which could not compete against other high protein animal feeds, resulting in their closure in the late 1980s

4. Recombinant products

- Recombinant DNA molecules are also known as chimeric DNA, as they consist genes (DNA) of two different species
- The nucleotide sequences used in the construction of recombinant DNA (rDNA) molecules can be from any species. For instance, plant or human DNA may be combined with bacterial DNA, or human DNA may be joined with fungal DNA
- Genes from higher organisms can be inserted into microbial cells in such a way that the recipients are capable of synthesizing ***'foreign'*** proteins
- The advancement in the application of rDNA technology has made possible to produce a range of recombinant products by the fermentation process
- A wide range of microbial cells have been used as hosts for such systems including Escherichia coli, Saccharomyces cerevisiae and filamentous fungi
- Recombinant DNA is widely used in research, agriculture, medicine and biotechnology
- Several products that result from the use of rDNA technology are found in almost every pharmacy, medical testing laboratory, doctor's as well as and veterinarian's office, and biological research laboratory

- Following are the recombinant products that produced by genetically engineered organisms

Human Growth Hormone (rHGH)
Biosynthetic Himan Insulin (BHI)
Envelope protein of the Hepatitis B virus
Follicle Stimulating Hirmeone (FSH)
Blood clotting Factor III
Erythropoietin (EPO)
Granulocyte Colony-Stimulating Factor(G-CSF)
Alpha-galactosidae
Alpha-L-iduronidase
N-acetylgalactosamine-4-sulfatase
Dornasealfa
Tissue Plasminogen Activator(TPA)
Glucocerebrosidase
Interferon (IF)
Insulin-like growth factor 1 (IGF-1)
Bovine somatotropin (bST)
Porcine somatotropin
Bovine chymosin

5. Processes modifying substrates (Transformation Process)

- Many microbial cells may be exploited to convert a compound into a structurally related, financially more valuable compounds
- As microbes can behave as catalysts with high positional specificity and stereospecificity, microbial processes are more specific than purely chemical ones
- These microbial processes enable the removal, addition and/or modification of various functional groups at predefiend specific sites on a complex molecule without the use of chemical protection
- The reactions which may be catalyzed include *Dehydrogenation*, *Oxidation, Hydroxylation, Dehydration and Condensation*, *Decarboxylation*, *Amination*, *Deamination and Isomerization*

- As microbial processes can be operated at a relatively low temperatures and pressures have the additional advantage over chemical processes which require high temperatures, more pressures and presence of heavy-metal catalysts-a potential environmental pollutant
- Production of vinegar is the most well-established microbial transformation process (conversion of ethanol to acetic acid)
- Many transformation processes have been rationalized by immobilizing either the whole cells, or the isolated enzymes on an inert support which catalyze the reactions
- The immobilized cells or enzymes may be reused many times

Table 4.4: Examples of International Depository Authorities for the deposition of cultures for patent purposes under the Budapest Treaty

American Type Culture Collection (ATCC) Rockville, Maryland, USA	Algae, pathogenic and nonpathogenic bacteria, protozoa, fungi, phages, plasmids, cell lines, hybridomas, animal and plant viruses
Northern Regional Research Lab. NRRL) Peoria, Illinois,USA	Fungi and bacteria that can be freeze-dried (no human or plant pathogens)
Centraalbureau voor Schimmelcultures (CBS) Baarn, Netherlands	Fungi and actinomycetes
Deutsche Sammlung von Mikroorganismen (DSM) Braunschweig, Federal Republic of Germany	Bacteria, fungi, phages (no human pathogens)
Fermentation Research Institute Ibaragi, Japan	Fungi, bacteria (no human pathogens)
National Collection of Yeast Cultures (NCYC) Norwich, England, UK	Yeasts that can be freeze-dried (no pathogens)
National Collections of Industrial and Marine Bacteria (NCIMB) Aberdeen, Scotland, UK	Bacteria and phages (no pathogens)
National Collection of Type Cultures (NCTC) London, England, UK	Bacteria pathogenic to man or animals
The Culture Centre of Algae and Protozoa (CCAP) Cambridge, England, UK	Algae (but not large seaweeds), free living and parasitic protozoa (no pathogens)
Culture Collection of Commonwealth Mycological Institute (CMI), Kew, England, UK	Fungi (no human pathogens)

The Preservation of Industrially Important Microbes

Microbes are required for the production of fermentation products. They are very valuable for specific product. One product produced efficiently by specific microbe will not be given by all the microbes.

The isolation of a desired organism for a fermentation process may be a time consuming and very expensive procedure and it is therefore essential that it retains the desirable characteristics that led to its selection. Also, the culture used for the fermentation process should remain viable and free from contamination. Thus, industrial cultures must be preserved and maintained in such way as to eliminate genetic change, protect against contamination and retain viability.

Different techniques are used for maintenance and preservation of different organisms based on their properties. Selected method should also conserve the properties of the organisms.

Techniques for the Preservation of microbes are broadly divided into two

1. **Methods where organisms are in Continuous metabolic active state**
2. **Methods where organisms are in Suspended metabolic state**

Continuous metabolic active state preservation technique

In this technique organisms are preserved on nutrient medium by repeated sub-culturing. In this technique any organisms are stored by using general nutrient medium. Here repeated sub-culturing is required due to depletion or drying of nutrient medium. This technique includes preservation by following methods.

- **Periodic transfer to fresh media**

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

- **Overlaying culture with mineral oil**

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

- **Storage in sterile soil**

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

- **Saline suspension**

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

Methods where organisms are in Suspended metabolic state

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

- **Drying in vacuum**

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

- **Lyophilization**

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

- **Use of Liquid nitrogen**

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

- **Storage in silica gel**

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

Note:

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