Manufacture of Biochemicals (Lectures 37-40)

1 What are the basic differences between upstream and downstream processing?

Answer: The fermentation process is divided into two stages- upstream and downstream. The upstream process deals with the following-

- Inoculum preparation which involves screening and selection of production strain, genetic modification if required and preparation of pure culture at lab scale
- Media development and optimisation of growth parameters at lab scale
- Scale up of entire process i.e both inoculum and media preparation
- Inoculation

The downstream processing deals with the following:

- Post harvest product recovery- clarification, concentration, purification, polishing and formulation till packaging of the desired product.

2. How to assure that during manufacture of any targeted product no contamination has taken place when the process is a pure culture one.

Answer: Contamination free manufacture of targeted protein can be assured by the following:

- Maintenance of sterile condition- steam sterilization of the bioreactor, transfer lines and the filters, sterilization of media, use of sterile air, steam, WFI etc
- Proper designing of the rooms where the process of production, purification is taking place according to the rules of US FDA, WHO or any other international organization- with desired air flow rate, ventilation, particle count, pressure, humidity, temperature etc.
- Following proper SOPs (Standard operating Protocol) for both production and purification like-checking the filter integrity after each filtration process, use of WFI (Water for injection) which has very low amount of endotoxins etc.
- During fermentation proper maintenance of pH, temperature, DO etc is important along with foam control; regular sampling is important to monitor the growth and production and also to check for contamination by plating, microscopic examination etc.
- Regular sampling of WFI, air etc. and check for contamination like performing LAL(Limulus Amebocyte Lysate) test to estimate the amount of endotoxin in WFI(should be less than 0.25EU/ml)
- Maintenance of inoculum in pure form
- Performance of quality control tests on product
- Setting up quality assurance parameters
- Finally validation of the entire process is very important.

3. What are the essential steps for any biological product formation using plant cell as the cultivated cell line.

Answer: The essential steps for any biological product formation using plant cells as the cultivated cell line are as follows:

- Isolation and identification of protein of interest
- Characterization of protein of interest
- Identification of gene coding the protein of interest
- Construction of cDNA of the desired gene
- Construction of plasmid having the desired traits(reporter genes, enhancers, promoter etc)
- Ligation of gene of interest and the plasmid DNA forming a chimera or recombinant DNA
- Selection of host plant and the cells
- Culturing of cells and optimising culture parameters
- Development and maintenance of pure and contamination free cell culture
- Transfer of the chimera into host cells using suitable transfer technique
- Selection of stably transformed cells and their maintenance
- Scale up, if required and further culturing for production of desired protein
- Finally harvesting and recovery of product

4. What are the precaution one should take while handling plant cell and animal cell?

Answer: Different precautions to be taken while handling animal cells are:

- Maintenance of sterile condition- sterilizing the media properly using filters since steam sterilization can not be done in this case, use of pure and sterile air, WFI etc. Use of biosafety cabinets for culturing, transferring and other cell handling related purposes.
- Use of permanent cell lines- cell should be free of any infection, supplier should be certified one and check for contamination before starting the culture
- Use of serum obtained from countries where viral diseases associated with cows(like BSE) have not been reported or less reported, it should be obtained from certified supplier and regular check for contamination is necessary
- Maintenance and monitoring of all instruments, culture wares, critical apparatus and its proper documentation
- Proper training should be given to the people handling the cell cultures
- Animal cells are shear sensitive so vigorous aeration and agitation should be avoided. Use of low speed magnetic stirrer, microsparger is recommended and surface air is to be supplied for proper aeration
- Handling of two culture together should be avoided to prevent cross contamination
- Fast growing and slow growing cells should never be handled at the same time
- Regular monitoring of the culture is necessary along with proper documentation – number of passage, culture cell type, date etc.
- During recovery ensuring viral clearance is necessary
- Maintenance of humidity, temperature, air pressure inside the room according to the FDA norms is important.

Different precautions to be taken while handling plant cells are:

- Maintenance of sterile condition- media, glassware sterilization, use of filtered air and culture to be done under aseptic condition (LAFU).
- Selection of explant is very important depending on the requirement – mostly meristatic cells are taken for cell culture like apical buds, root tip etc. If the selection is not proper it may increase the risk of contamination and sometimes proper cell growth may not occur.
- Surface sterilization of explant is important as on the surface microbes are present. This can be done by using detergents, alcohol and mercury chloride. Some cases rinsing with a fungicide is also suggested.
- The composition of media should be optimised- proper composition of hormones and N sources according to the requirement.
- Use of antibiotics and fungicides at the correct dose is essential to prevent contamination during the culturing process.
- Maintenance of physical factors like pH, temperature, humidity, lighting for survival and proliferation of cells is required.
- The cells should be subculture at the right time at regular intervals to maintain the cell culture.
- Expertise in handling the culture is essential, thus proper training becomes indispensable requirement.

5. How microbial product formation is different from plan and animal cell?

Answer: In case of microbes, the difference includes both advantages and disadvantages.

- Since the biology of microbes is well understood, it is easy to grow them and also carry out genetic modification compared to plants and animal cells.
- Compared to animal cell the risk of contamination is low.
- It is less expensive and less labour intensive.
- Production is found to be higher in microbes
- In case of protein product the folding may not be proper- lack of disulphide bonds is seen when compared to animal and plant cells.
- Post translational modifications like glycosylation doesn't take place in microbes. This leads to changes in the protein of interest and may lead to immune respone in human.
- The protein product is often expressed in inclusion bodies which, makes the purification process very difficult. In case of animal cells the product is generally secreted out of the cell.
- High level of endotoxins also makes purification a difficult process and may lead to immune response in human.

Manufacture of Biochemicals (contd.)

1. Classify fermentation process

Answer: Depending on the mode of fermentation:

- **Batch fermentation** – A tank of fermenter is filled with the prepared media (60 to 70% of tank volume) .The temperature and pH for microbial fermentation is properly adjusted, and is controlled through out the fermentation process. The media is steam sterilized in a pure culture process. The inoculum of a pure culture is added to the fermenter and fermentation is carried out till harvesting.
- **Fed batch fermentation**- A tank of fermenter is filled with the prepared media till the minimum volume (40% of the tank).The temperature and pH for microbial fermentation is properly adjusted and controlled though out the process and occasionally nutritive supplements are added to the prepared media till the maximum volume is reached (80%). Then harvesting is done at the end of fermentation period.
- **Continuous fermentation**- In continuous fermentation, the substrate is added to the fermenter continuously at a fixed rate. This maintains the organisms in the logarithmic growth phase. The fermentation products are taken out continuously. The design and arrangements for continuous fermentation are some what complex.

Depending on oxygen demand-

- **Anaerobic Fermentation** - In anaerobic fermentation, a provision for aeration is usually not needed. But in some cases, aeration may be needed initially for

inoculum build up. The air present in the headspace of the fermentor should be replaced by CO_2, H_2, N_2 or a suitable mixture of these; this is particularly important for obligate anaerobes like Clostridium. In most cases, a mixing device is also unnecessary; while in some cases initial mixing of the inoculum is necessary. Once the fermentation begins, the gas produced in the process generates sufficient mixing. Recovery of products from anaerobic fermenters does not necessarily require anaerobic conditions.

- **Aerobic Fermentation** - The main feature of aerobic fermentation is the provision for adequate aeration; in some cases, the amount of air needed per hour is about 60-times the medium volume. Therefore, bioreactors used for aerobic fermentation have a provision for adequate supply of sterile air, which is generally sparged into the medium.
  In addition, these fermenters may have a mechanism for stirring and mixing of the medium and cells. Aerobic fermenters may be either of the following:

  (i) stirred tank type in which mechanical motor driven stirrers are provided or (ii) of air lift type in which no mechanical stirrers are used and the agitation is achieved by the air bubbles generated by the air supply.

  Generally, these bioreactors are of closed or batch type, but continuous flow reactors are also used such reactors provide a continuous source of cells and are also suitable for product generation when the product is released into the medium.

Depending on the physical state of media -

- **Submerged Culture Method** - In this process, the organism is grown in a liquid medium which is vigorously aerated and agitated in large tanks called fermenters. The foermenter could be either an open tank or a closed tank and may be a batch type or a continuous type and are generally made of non-corrosive type of metal or glass lined or of wood.
- **Solid State Fermentation** - In such fermentations, microbial growth and product formation occur at the surface of solid substrates. Examples of such fermentations are mushroom cultivation, mold ripened cheeses, starter cultures, etc. According to the physical state, solid state fermentations are divided into two groups:

  (i) low moisture solids fermented without or with occasional/continuous agitation, and

  (ii) suspended solids fermented in packed columns, through which liquid is circulated. The fungi used for solid state fermentations are usually obligate aerobes.

Solid state fermentations on large scale use stationary or rotary trays. Temperature and humidity controlled air is circulated through the stacked solids. Less frequently, rotary drum type fermenters have been used.

2. How solid state fermentation is different from submerged fermentation?

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3. What are the basic strategies of down stream processing?

Answer: The basic strategy for downstream processing is as follows:

- Separation of insolubles (cells, debris, precipitates etc)
- Extraction (removal of molecules that are dissimilar to product , removal of water etc)
- Purification (removal of molecules similar to product)

- Polishing (packing in a form easy to transport, handle and give stability to product)

4. What are the different techniques one can adopt for separation of insoluble after fermentation?

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

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

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

- **Flocculation and Floatation**. Flocculation, i.e., sticking together of cells, can be induced by inorganic salts, mineral hydrocolloids are organic polyelectrolytes. Since sedimentation rate of a particle increases with size, flocculated cells can be recovered by centrifugation.

  In cases, where flocculation is not effective, very fine gas bubbles can be created by sparging. The gas bubbles adsorb to and surround the cells, raising them to the surface of medium in form of foam (floatation); long chain fatty

acids or amines promote stable foam formation.

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

5. What is the importance of extraction?

Answer: Downstream processing is an integral part of any product development, and the final cost of the product depends largely on the cost incurred during purification techniques. The product cost increases and recovery decreases with increase in the number of steps of DSP. Thus, reduction in number of steps and increasing the efficiency of recovery becomes the main priority for any pharmaceutical company.

The process of extraction is important as:

- it substantially reduces the number of initial downstream steps and clarification, concentration, and partial purification can be integrated in one unit
- it is a fast, cost-effective, ecofriendly simple separation technique
- scale-up processes based on extraction systems are simple and a continuous steady state is possible
- an ideal technology, specially for proteins, since it is less time consuming and has the potential to give high yield and high purity, involving low investment, less energy, and lower labour costs.

Strategies for Biomolecules Separation (contd.)

1. Do you think that the separation of antibiotic and secondary metabolite of V*inta rosea* will have the similar type of down stream processing stragies. Explain.

Answer: Antibiotic are class of antimicrobial chemicals that are produced by micro-organism mainly the saprophytic molds and the bacteria of soil, while secondary metabolites are the organic compounds produced by the plant system, they can be alkaloids, terpenoids, phenols, glycosides etc. Plant cell is different from microbial cell in there structural organization, and even the site of accumulation of compounds are different as antibiotic are produced extracelluar while secondary metabolites are localized intracellularly. Downstream processing steps for antibiotics will be different from those of the plant metabolites.

Fermented broth
↓
Centrifugation
↓
Filtration
↓
Precipitation by solvent extraction
↓
Chromatography separation
↓
Crystallization

Vinca Leaves
↓
Drying and grinding
↓
Solvent Extraction
↓
Chromatographic separation

2. How to separate aloin from Aloe Vera?

Answer: Aloin is the main anthraquinone in aloe leaf, which occurs naturally as a mixture of two diastereoisomers, aloin A and aloin B, and currently served as one of the important control constituents in most of the commercial aloe products. High speed countercurrent chromatography (HSCCC) has been employed for the preparative separation of individual isomers combined with preseparation on silica gel chromatography.

3. How will you purify VitC from Amla fruit?

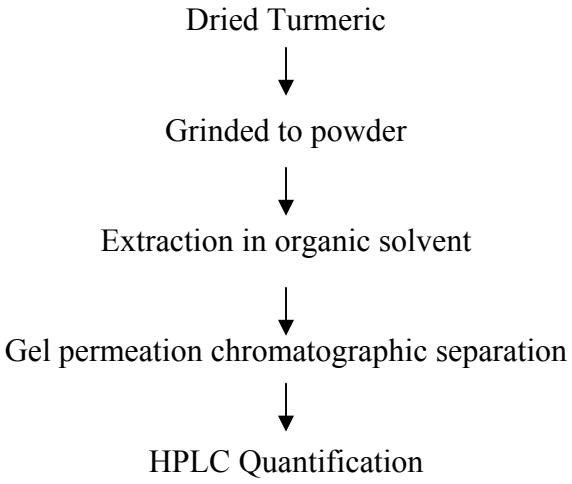
Answer: Pertraction (permeation through liquid membranes) is one of the new techniques can be applied for separation and advanced purification of VitC from amla fruit. Pertraction involves the extraction of organic compounds (volatile and non-volatile) from liquids (incl. water) with the aid of membranes. The used membranes have no selectivity. A hydrophobic micro-filtration membrane helps to realise a high contact area between the organic extraction product and the to-be-purified liquid. Further, it also prevents the two phases from mixing. This means the liquid and extraction product do not need to be separated, which saves time and money. The membrane also enables the flows of both phases to be regulated flexibly and independently, whereby it is easy to optimise the process. It then becomes possible to also bring small quantities of extractant in contact with large quantities of to-be-treated liquids. This keeps installations compact.

4. How will you separate plant growth hormone from any animal cell line?

Answer: Hydrophobic interaction chromatography is used to separate proteins with difference in hydrophobicity. This technique is ideally used as intermediate step in a purification process or to capture plant growth hormone from any animal cell line.

5. How to extract the anti carcinogenous compound from turmeric?

Answer: Turmeric (*Curcuma longa L.*) is a coloring agent, and it has been found to be a rich source of phenolic compounds, namely, curcuminoids. Curcumin, the principal natural yellow pigment used in the prevention of cancer and in the treatment of infection with human immunodeficiency virus.



Strategies for Biomolecules Separation

1. How to separate the biological macromolecules based on the biological properties?

Answer: Biological macromolecules bear properties which play a significant role in developing any purification strategies. Before starting it is required to have the knowledge about the nature of molecule regarding structure or functionality which will guide the system to maintain the favorable environment. Initial fractionation, concentration, and purification by chromatographic techniques in all these stages stability of molecule is of prime importance. The exact purification requirement during separation will depend upon the properties of the molecules. To overcome the loss of product and activity of macromolecules control of pH, time and temperature is also essential which affects the biological properties. The key requirements for separation of biological macromolecules are:

(a) Release of macromolecule from source material in suitable solvent

(b) Removal of solids and water to concentrate the molecule

(c) Removal of contaminants to achieve desired purity

(d) Stabilization of the target molecule

2. Differentiate between ion exchange and gel filtration chromatography?

Answer: Ion exchange chromatography works on the principle of separation of molecule on the basis of charge or the ionic interaction while in gel filtration chromatography,

separate the targeted molecule on molecular size basis, the solid phase matrix have a defined porosity due to which large molecule are unable to enter the pores and eluted out while smaller ones are retained.

3. Do you think that HIC is an affinity chromatography? How and why?

Answer: Hydrophobic Interaction Chromatography is a separation technique that uses the properties of hydrophobicity to separate proteins from one another. HIC can be an affinity chromatography. Since the molecular mechanism of HIC relies on unique structural features, it serves as an orthogonal method to ion exchange, gel filtration and affinity chromatography. HIC is widely used in protein purification in the research laboratory as a complement to other techniques that separate according to other parameters such as charge (ion exchange chromatography), size (gel filtration) or biospecific recognition (affinity chromatography).

4. What is ligand?

Answer: A ligand is a substance that is able to bind to and form a complex with a biomolecules.They can be a small molecules such as substrates for enzymes or larger molecules such as peptide hormones. For affinity chromatography the ligand selection on the basis of specificity, affinity, reversibility and stability is the critical factor to be considered.

5. Discuss briefly on mono specific ligand, group specific ligand and spacer arm?

Answer: Monospecfic ligands are those which bind to only one biomolecules. An example can be a monoclonal antibody.

Group specific ligands can bind to different molecules which have the binding moieties. Lectin is an example of group specific ligand which can bind to any protein which has carbohydrate moieties.

Spacer arm: A spacer molecule is employed to maintain the distance of ligand from the matrix backbone to avoid the hindrance between the ligand support (matrix) and the analyte. This accessory molecule is attached, when the low molecular weight ligand are used for separating respective analyte.