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Analytical Characterization of Biopharmaceuticals During Upstream and Downstream Processes: A Review

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ABSTRACT

Biopharmaceutical drug products have transformed the treatment of various categories of diseases, since last three decades. These industries are widely growing and developing with all branches of medicines. Biopharmaceutical drug products will play a vital role for the development and enhance the safety and efficacy by the altered Active Biopharmaceutical ingredients. These products are widely used for the treatment of various types of diseases such as cancers metabolic and hormonal disorders, etc. During the drug product development and manufacturing of biopharmaceutical products various analytical tests are performed for the determination, identification and consistency for the test results as per established parameters which are developed by the research and development methods. For the new drug application, clinical and non-clinical studies are backbone for safety and efficacy of product. Analytical characterization of various techniques based on properties of the that product or reference product. Critical quality attributes are defined constructed on the risk assessment data based on its impact on Pharmacokinetics and Pharmacodynamics therapeutic activity, safety and immunogenicity. These studies are performed for the determination or to identify the structural and functional elements of the formulation. As per the regulatory and territory norms, these products vary in there the strength of the products. For the same the analytical tests will be performed accordingly.

Keywords: Upstream processes, Downstream processes, Chromatographic Techniques, Purification of proteins

INTRODUCTION:

In recent years, biopharmaceutical market has developed much faster than the market for all drugs and believed to have the great potential for further dynamic growth because for the tremendous demand for the biopharmaceutical drugs in all over the world in upcoming few years. These products play for the vital role for its site specific target actions with less side effects. For public health purposes, it is essential that the standard of evidence supporting the decision to license a biosimilar is sufficiently high to ensure that the product meets acceptable levels of quality, safety and efficacy. [1]

As per the various regulatory authorities, these are anticipated to deliver globally tolerable standards for the licensing of biopharmaceutical products that are claimed to be similar to biological products of assured quality, safety and efficacy.

Biopharmaceuticals are extracted from living cells, whereas synthetic drugs are the products of chemical processes. The expression system of biopharmaceuticals is different batches of same product if the same conditions are applied for manufacturing. [2][3]

Definitions:

Biosimilar: A biological product that is revealed to be extremely comparable in relations of its quality, safety and efficacy to an already accredited reference formulation.

Drug product: A finished dosage form of pharmaceutical or biopharmaceutical product that typically consists of a drug substance formulated with excipients which are approved for commercialization.

Drug substance: the active pharmaceutical ingredient which is anticipated to deliver the physiological activity and bonded with additional excipients or molecules that are characteristically expressed just before produce the drug product.

Efficacy study: a clinical trial to compare the efficacy of the biosimilar to the reference product.

Excipient: an essential component of a medicine other than the drug substance which does not have any therapeutic action. Some additives can have a well-known physiological actions or effect in certain conditions. The excipients may differ for a biosimilar and its reference product and need to be declared in the labelling and package leaflet of the medicine to ensure its safe use.

Upstream Process: Fermentation process incorporating dispensing, media preparation and cell culture.

Downstream Process:

Equivalent: equal or highly similar in the parameter of interest. Equivalent quality, safety and efficacy of two medicinal products denotes that they can be expected to have similar (no better and no worse) quality, safety and efficacy, and that any observed differences are of no clinical relevance.

Characterization and evaluation of the biopharmaceutical is the first step for the development. It includes the various analytical methods which are very sensitive and sophisticated and assays to demonstrate the structural, functional and clinical identification.

These characterization methods and evaluation of biopharmaceuticals are proposed for use in the calibration of bioassays and are available for a wide range of substances including hormones and modified/long-acting proteins and monoclonal antibodies (mAbs). It may contain the other excipients which provides the stability for the desired lifespan of the biopharmaceutical product but which may also interfere with its physicochemical stability. [4]

Upstream processing of biopharmaceutical manufacturing processes is based on microscopic and living cells. By targeting the specific protein by providing the necessary environment to grow the cells. By this method various products such as antibiotics, therapeutic proteins enzymes, antigen or antibodies, hormones, amino acids, blood constituents, etc. are altered active pharmaceutical ingredients are produced for desired targeted action. The manufacturing of such products by intracellular or extracellular cells extraction. Cloning of cell lines with anticipated properties used for the bulk manufacturing in biopharmaceuticals. For the appropriate growth and further processing of the cells culture or bacterial based protein products was used. For the microbial fermentation process mammalian cell culture was used. The medium which are used for the produce the cells provides all the nutrition to cells within a short time span of required conditions.

The specific cell lines are used for the production of the specific target protein or microbial antigen antibodies or hormones. These cell lines are stored in frozen state in specific solution (e.g. Liquid Nitrogen). The most biopharmaceuticals organizations they have their own host cells or cell lines which are required for the manufacturing of specific products. [5][6]

Methodology:

This process includes the set of operations which recover the bioproduct (i.e., target proteins, specific antigen or antibodies, hormones and blood components, etc.). The separation of unwanted materials by various analytical methods, disintegration and separation of solids, separation recovery of soluble contents. For the recovery of large amount of bioproduct various crystalizing agents and buffer are added. These processes progressively increase the purity of the target proteins. Downstream processes involve the harvesting and clarification steps to remove the contaminations such as low molecular weight and high molecular weight viruses, particulates, substitutes, oils, salts and carbohydrates from the entire bulk. Also involves the various polishing steps which refines the target product for its purity, safety and efficacy.

Downstream processes operations are having several steps for the manufacturing of desired biopharmaceutical product such as cell disruption, purification and polishing

1. Removal of insoluble
2. Product Isolation
3. Product Purification
4. Product Polishing

Separation of biomass:

The bulk is prepared by fermentation process. Fermentation is a chemical process in which the chemical changes in metabolic pathways or processes of biological substances. In this process, the energy is generation takes place with the help of carbohydrate and other cell components. [7] By this method production of large scale processes of microbial fermentation of chemicals, biofuels, enzymes and proteins are carried out which are used as the active pharmaceutical ingredients in the various types of pharmaceutical and biopharmaceutical products. These processes are carried out by aerobic and anaerobic methods. [8]

The cells are obtained from the yeast which are adaptable to any environment for supporting the microbes or cells or their growth. The cells collected from the Yeast contains the high amount of carbohydrates with fatty chains, it breaks down the carbohydrate to form the energy and produces the ethanol and carbon dioxide (CO^2) as by products.

Similarly, the cells collected from various sources such as mammalian, plants, fungi, bacterial, insects, transgenic animals, etc. The mammalian cells such as rodents cell lines such as CHO (Chinese Hamster Ovaries), V79 (Male Variant of CHO), CHL (Female Variant of CHO), etc. The specific cells lines have its own chemical compositions of proteins and enzymes in its basic structure. By performing the various chemical and analytical characterization processes, the specific target protein from the cells are extracted. [9]

Separation of such whole cells from the culture medium or broth which contains the soluble and insoluble ingredients after the separation of desired product. The separation techniques are as below:

1. Centrifugation
2. Release of intracellular products
3. Solid-liquid separation
4. Concentration
5. Purification by chromatography

1. Centrifugation:

Centrifugation is a mechanical process which involves the use of the centrifugal force to detached elements from a solution according to their size, profile, density, standard viscosity and rotor agitations. The denser mechanisms of the blend transfer away from the axis of the centrifuge, while the less impenetrable components of the mixture transfer in the direction of the axis. Technician or operator may growth the operative gravitational influence of the vessels so that the blend (pellet) will portable rapidly and entirely to the bottommost of the vessel. The remaining liquid that lies above the precipitate is called a supernatant or supernatant. [10]

It is widely applicable in pharmaceutical and biopharmaceutical industries but not only is this method used to distinct miscible ingredients, but also to characterise the solid-liquid miscible properties of very small molecules.

Various centrifuges are used based on the sample sizes:

1.1 Microcentrifuges:

These centrifuges are widely used in laboratory scales. The microcentrifuges having the high speed up to 15000 rpm. These devices are very low weight and having maximum acceleration, used for volume of 0.2ml – 2.0ml samples for centrifugation.

1.2 Low Speed Centrifuges:

These are used for storage of various type of cells organelles for longer duration of time. The speed of these centrifuges ranges around 10000 rpm.

1.3 High Speed Centrifuges:

These centrifuges are having highest speed around 30000 rpm. These centrifuges widely used for the separation of large volume cell organelles. The roters of these centrifuges are having adaptability to hold the various sizes of bottles, bottles or flasks.

1.4 Ultracentrifuges:

These centrifuges are having high centrifugal forces for the separation of biological and suspended insoluble materials from the solution or harvest. These type of centrifuges having the speed more than 100000 rpm. These are widely used for nucleus contents such as DNA, RNA, plasmid, blood components, etc. For the studies of density gradients purification, ligand binding separation studies and various analytical separation and purification techniques. [11]

2. Release of Intracellular Products:

Cells are having its own structure and composition, which contains various intracellular organelles such as nucleus, mitochondria, golgi apparatus, Lysosomes, vesicles, Endoplasmic reticulum and plasma membrane, etc. These components are having its specific chemical structure and it stores the essential components which provide the adequate growth to the cell. Organelles contains the enzymes or vitamins which are located intracellularly. The cells and cell organelles are disrupted or ruptured by various mechanical or chemical methods. The selection of processes depends on the type of cells to be separated or purified.

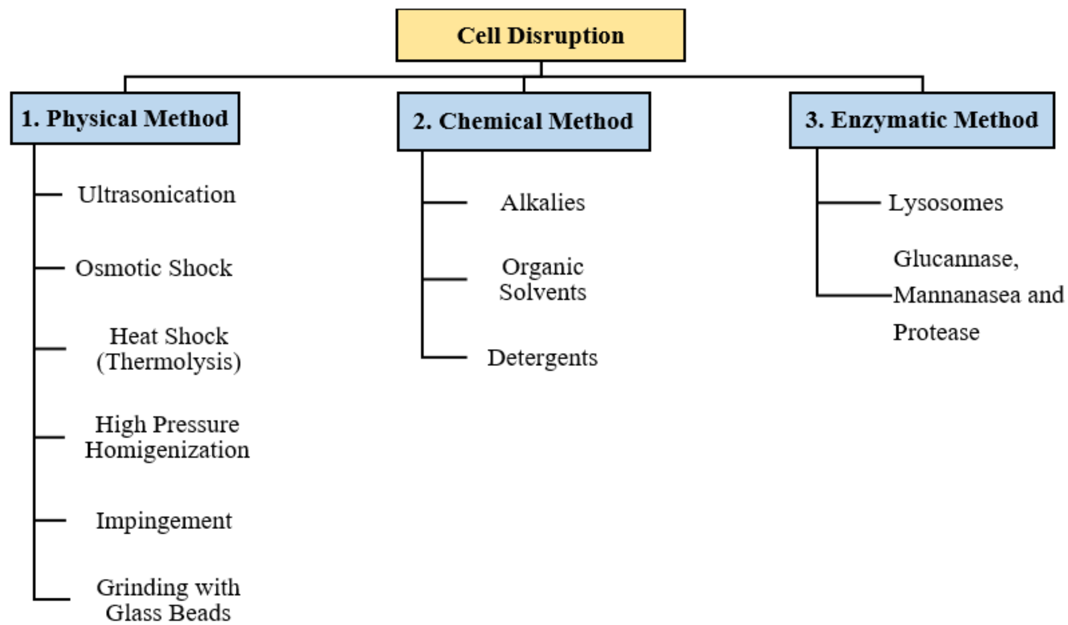


Figure: Methods of Cell Disruption

A. Physical Methods of cell disruption:

a. Ultrasonication:

It is the process of breakdown of the cells by applying the sound energy by producing the air bubbles, these air bubble cavities are then bombarded on cell membrane and the energy released from the air cavities will raptures the cell membrane.

b. Osmotic Shock:

Osmotic shock is the method, by a sudden change in the concentration on the solute around the cell. Due to this change rapid movement of solvent happens across the cell membrane.

c. Heat Shock (Thermolysis):

In this method the heat is applied for the rupturing the cells. By increasing the stress by introduction of the higher temperature, oxidative stress and heavy metals. This method only implies for the heat stable intracellular cell organelles.

d. High Pressure Homogenization:

In this method, suspension like oil/fluids which are relocated from the very miniature orifice with higher pressure and about 1nm particles are produced.

e. Impingements:

By this method the cells are breakdown or lysis by applying extreme stress or pressure. The pressure applied by using beads or ultrasound waves. [12]

f. Grinding with Glass Beads:

Bead technique have been used for the cell disruption for small and large scale production. The principle of grinding of cells with grinding clamber with rotating shaft which is rotating at its central axis. The agitators present inside the chamber provides the kinetic energy to beads will colloid with one another. Due to this collusion the energy formed by these cells will efficient of the cell disruption. [13]

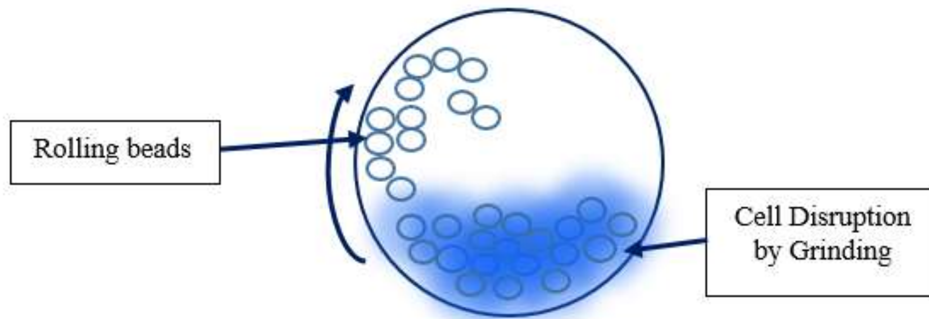


Figure 1: Cell Disruption by grinding

B. Chemical Methods of cell disruption:

Below enlisted methods are used for the separation or extraction of specific targets protein according to its affinity to binding with the chemicals which are added further it will get lysed/ breakdown and get desired product is very critical.

i. Alkalies:

Examples: Sodium Hydroxide,

ii. Organic Solvents:

Examples: methanol, ethanol, isopropanol and Chloroform

iii. Detergents:

Examples: Sodium Dodecyl Sulfate (SDS), Triton, Tween and Sodium Lauryl Sulphate (SLS)

C. Enzymatic Methods of cell disruption:

Enzymatic cell disruption method is the method used for the lysis of specific cell organelles. Selection of enzyme depends on the type of cells (Gram – positive cells or Gram – Negative cells) to be lysed to provide the extraction of target protein. By this method the breakdown mucopeptide bond of cell wall is susceptible by enzymatic method. [14]

a. Lysosomes

b. Glucannase, Mannanasea and Protease

3. Solid-liquid separation:

It is the initial step of separation of recovery of cells from the other insoluble ingredients. It is the part of quantitative analysis key method for the following treatment of cell biomass and wastewater mixture, to remove solids from bulk slurry and to make both solid and liquid phases more appropriately for further formulation processes. [15]

It includes the several methods are as below:

A. Flotation:

In this method the dissolved air or gas is bubbled out from the liquid and it forms the layer of foam. By this method the oil or solids are separated. By applying the pressure, the bulk solution, these bubbled foam layer get collected and removed. The foam might be including the long fatty chain acids and amine derivatives, etc. [16]

Examples: Long fatty chain acids and amines.

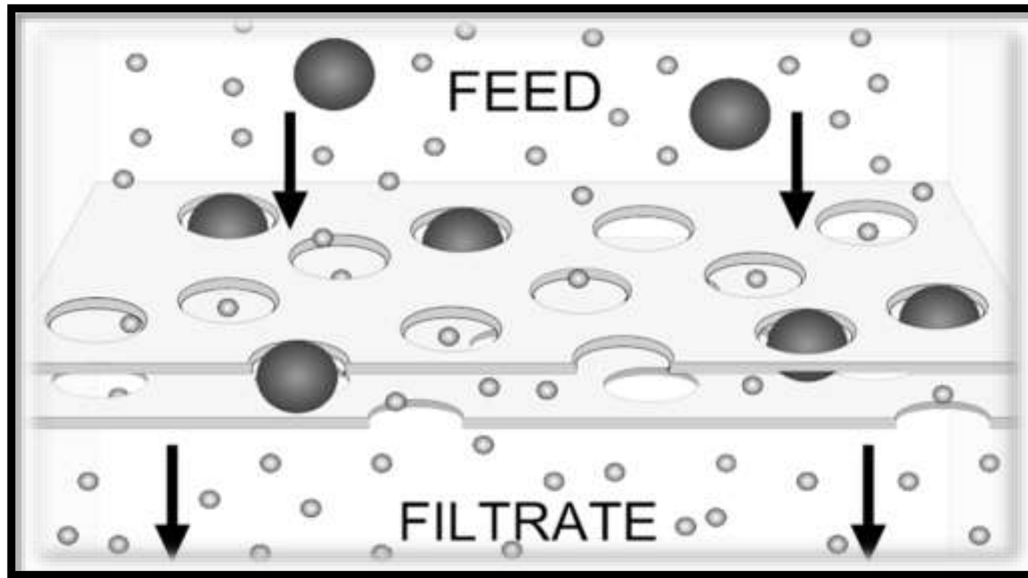
B. Flocculation:

Flocculation is the process by which the separation colloidal particles and to form the flocs, this process is happened due to addition of any chemicals or clarifying agents or buffers. These suspended particles are get settled according to time and particle size. By adding the coagulating agents such as Potassium Bromide, Calcium Chloride and Magnesium Bromide.

C. Filtration:

Filtration is the process by which the separation of solid substances and liquids from the mixture by using the filter sheaths. The filter sheaths or membranes are having the specific pore sizes for the separation of particles. The mixture solutions are passes through this membrane, the larger particles

than the filter membrane are get entrapped or blocked between the pores and liquid is passed from the small pores. Oversize particles are form the filter cake over the filter membrane. (Refer Below Picture)



It is the most common method used and most widely used technique for the separation of cell biomass. The efficiency of filtration depends on size of organelles, other parts of cells, viscosity of medium and temperature.

For the separation various type of filters or depth filters, rotary drum filters, vacuum filters and membrane filters.

D. Centrifugation:

Centrifugation is based on the principle of differences between the density of the solute and solvent or the particles to be separated from the medium. These method is widely used in laboratory scale. The centrifugal force is applied for the separation of particles according to speed which facilitates the sedimentation. The relationship between the particle size and density of particles associated in the mixture by the force applied that of gravity. [17]

4. Concentration:

The filtrate is free from the suspended particles such as cell, cell debris and other components. The desired content should be water soluble usually contains 80 to 98%. Desired constituents are available in very minute quantity. To get the desires content, the removal of water or any other solvent has to be removed from it. For the concentration of content following methods are used:

a. Evaporation:

Evaporation is the simple process of vaporization that occurs liquid surface changes into the gas phase. The cell components are dissolved and suspended in the broth culture and water. The content which are to be removed by simple evaporation process. Evaporation is most widely used technique for the various pharmaceutical and biopharmaceutical processes of purification. The heat is supplied to the solvents by applying the steam by uniform manner it will remove the water from the solvent by condensing vapour.

For these processes various types of evaporators are used:

i. Plate Evaporator

ii. Falling Film evaporator

iii. Forced Film evaporator

iv. Centrifugal forced film evaporator

b. Liquid – Liquid Extraction:

Liquid – Liquid Extraction is based on the principle of partitioning of the solubilities of the two different immiscible liquids are going to separate. The one liquid phase to another phase (solvents) which contains the amount of dissolved biological product (solute). These phenomenon is referred as Liquid – Liquid Extraction. It is the widely used technique for the purification. The extraction of the various immiscible liquids broadly categorized by various techniques which are performed on the high molecular weight and lower molecular weight products. [18]

The various processes are as follow:

i. Physical extraction – For non-ionic compounds

ii. Dissociation extraction – For non-ionic compounds

iii. Supercritical Fluid Extraction (SCF): For Gases and Liquids

c. Precipitation:

It is the most common methods used for the concentration of the molecules, proteins, enzymes and polysaccharides. Most of the types proteins get precipitated by adding the various salts into the cell mass or culture broth. As the salting concentration increases, the stability of the proteins get changes by formation of salts. During this chemical reaction, the hydrophobic reagents are get reacted with other hydrophobic group of protein composition and by formation of such cluster of hydrophobic components. It is the very less expensive method which are performed on small scale (in laboratories) as well as on large scale (in industries). [19]

Examples: Sodium Dodecyl Sulfate (SDS), Ammonium Sulphate, Triton etc.

Precipitation is used for the removal of unwanted byproducts such as pigments and nucleic acids. Due to precipitation, there is formation of additional non-protein specific components happened this are separated by affinity precipitation or ligands precipitation.

- Neutral Salts: By addition of Ammonium Sulphate
- Organic Solvents: By addition of Ethanol, Acetone and Chloroform, etc.
- Non-ionic polymers: By addition of Polyethylene glycol (PEG) used for nonionic polymeric compounds
- Ionic Polymers: By addition of Poly-acrylic acid and Poly-ethyleneimine
- Change in pH: For Protein precipitation
- Affinity Precipitation: For Antigen and Antibodies
- Precipitation by ligands:

d. Adsorption:

Adsorption is the concentration of an ingredient at the superficial interface of compressed and liquid or gaseous level remaining the operation of superficial atoms. After the fermentation of biological products, the product is further proceeded for the concentration. The product is concentrated by the addition of solid adsorbents. Cellulose based adsorbents are used for the protein concentration. Low molecular weight and high molecular compounds such as proteins, antibiotics and peptide chains matrices based on polystyrene, methacrylate and acrylate and the bed of adsorbent column is prepared. Activated charcoal is also used as the adsorbent material. [20]

5. Extraction of components by using various Chromatographic techniques:

Chromatography is the technique of separation for the mixture of compounds. Mixture of various compounds have different affinity as per its chemical constituents. The mixture of components is dissolved in solvent (gas or liquid) known as mobile phase, which carries through the stationary phase by capillary action known as stationary phase. Based on the constituents of mixture have the different affinities to the stationary phases. Due to different affinities the lengths of timing depends on the interactions between its stationary surface. The portioning between the mobile phase is based on the differential partitioning. [21]

By chromatography, various products such as proteins, biopharmaceutical and pharmaceutical products, diagnostic compounds, proteins, enzymes, etc. were identically successfully purified by various chromatographic separation techniques. It is the analytical technique by which the separation of closely associated compounds. The stationary phase is porous solid texture packed in column, by loading the mobile phase into the column. Various types of chromatography methods are used for the partitioning for different types of principles. [22]

Refer the below mentioned table for reference:

Technique	**Stationary phase**	**Mobile phase**	**Basis of separation**
Size exclusion chromatography or Gel	solid (microporous beads of silica)	liquid	size of molecules
Ion-exchange	solid (cationic or anionic resin)	liquid	ionic charge of the molecules
Affinity chromatography	solid (agarose or porous glass beads on to which are immobilized molecules like enzymes and	liquid	binding affinity of the analyte molecule to the molecule immobilized on the stationary phase
Gas chromatography	liquid or solid support	gas (inert gas like argon or helium)	boiling point of the molecules

A. Size exclusion chromatography or Gel Permeation Chromatography:

Size extrusion chromatography is the polishing step chromatography of bulk formulation. This method of chromatography useful for the determination of tertiary or quaternary structure of purified proteins. Molecules are separated based on their size, shape and molecular weight (macromolecules and micro-molecules). The bed of porous membrane having sponge / gel like structure which function as a sieve. The mixture of molecules is passed through gel beads bed, then the large molecules present inside the mixture are not get passed through the pores and small molecules are entrapped into the pores and clear solvent is eluted out from the pores.

B. Ion Exchange chromatography:

Ion-exchange chromatography based on the charge presents on the respective analytes. This chromatographic method used for the separation of anions, cations, amino acids, peptides proteins and amines, etc. Based on the charge, there are two types of chromatographic techniques: Cation Exchange Chromatography and Anion Exchange Chromatography. In cation exchange chromatography, the stationary phase having the negative charge and exchangeable ion is cation (Examples: Carboxymethyl and sulphones). However, in the Anion exchange chromatography, the stationary phase contains the Positive charge and exchangeable ion is anion (Example: Diethylaminoethyl). It is widely used technique for purification of various pharmaceuticals as well as biopharmaceutical ingredients. In this method the pH is very critical, due to the anions and cations are present in the mixture. This method in widely used various industries for antibiotics and protein based formulation. [23]

C. Affinity chromatography

This method is widely used for the purification of high molecular weight protein, antigen-antibodies and amino acids. Columns are effectively prepared for this chromatographic technique. By reacting with the immobilized ligands the specific proteins and amino acids are separated. Various ligands such as specific antibody, antigen, substrate, or inhibitors. During this type of chromatographic processes, the various buffers are used to achieve the pH of the solution. The buffers having ionic components in its basic chemical composition due to this the pH range of the buffers are changing. For altering such issues of ionic strength the ligands are used.

Conclusion:

Analytical Characterization of the Biopharmaceutical Products is performed at various manufacturing processes such as Upstream and Downstream Processes. Various types of products are prepared by using these methods of characterization and purification of targeted protein, hormones, enzymes, etc. These methods are widely used for the detection, identification and removal of the toxic components from the prepared formulation which is having potential impact on the human health. This review gives the overview of the purification, separation and characterization of biopharmaceutical formulation.

There are lots of methods are available to determine and characterization of the biopharmaceutical components in various types of samples (plant, animal, Bacteria, fungi, enzymes and viruses, etc.) including chromatographic methods (HPLC), Spectroscopic methods (UV and IR), Immunoassays, Centrifugation, Filtration, etc. But, even though of their higher analytical characteristics such as high sensitivity, selectivity, accuracy and ability to analyze a wide range of sample conditions, these methods have some disadvantages incudes high expendable of organic solvents, complicated processes of purification and separation of target molecules, time taking sample preparation and expensive instrumentations.

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