ISOLATION AND PURIFICATION OF ENZYMES

Enzymes are part of every living system. They are found in various sources like- plants, animals and microorganisms. Having known their importance and significance, procedures for isolation and purification of enzymes. Works established the need for isolation of enzymes.

In general isolation and purification of enzymes is required for the following reasons:

1 It is required for the study on various aspects of that enzyme.

2. It is required for identification of substrates, coenzyme or cofactors

3. It is required for the researchers to develop diagnostic components

4. It is required for therapeutic purposes

5. It is required to come up with industrial applications of enzymes and exploit their use in various areas

6. It is required to study application in leaching of ores.

7. Pure enzymes are also used in food processing and forensic science

Topics covered under enzyme isolation and purification are as follows:

1. Identification of sources of enzyme

2. General handling procedures for the source and crude extracts.

3. Methods of isolation and purification

4. Development of procedures for both qualitative and quantitative estimations

5. Stabilization and crystallization of enzymes

6. checking criteria for purity

7. methods of preservation of isolated enzymes

1. Identification of sources of enzymes:

i)Before going for isolation of enzymes one should have an idea of the richest source for the enzymes, their localisation-whether it is intracellular or extracellular, substrate specificity and an established essay procedure.

selection of the richest source is one important criteria for the isolation of enzymes.

Some of the sources and the enzyme that could be isolated include- salivary amylase- salivary juice is a source, beta amylases - sweet potato, Alpha amylase - germinating cereals, for lipases - germinating oil seeds or groundnut seeds, for proteases - germinating pulses etc.

Since few decades, microorganisms have also been used as sources for enzymes. They have to be grown using a production medium that should be inexpensive and the purification procedures must be economical.

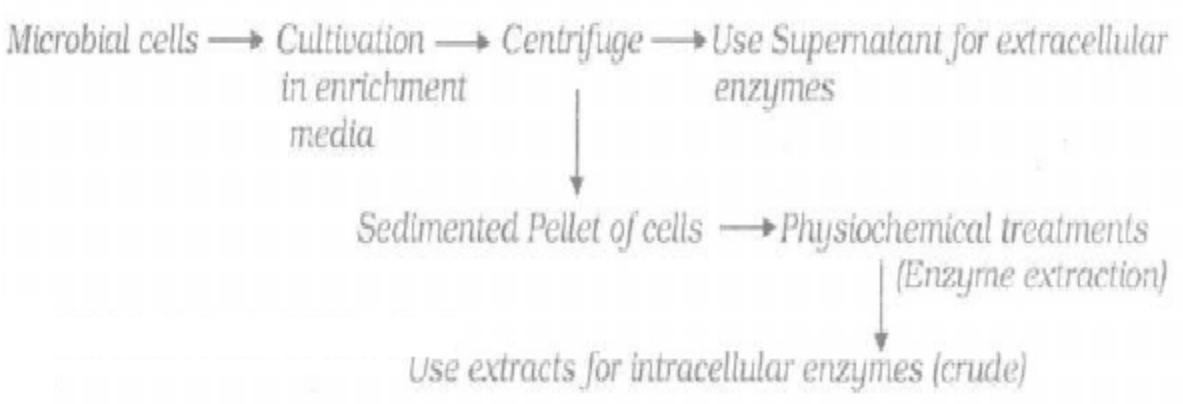
Some example include- Amylase from Bacillus subtilis, glucose oxidase from Aspergillus niger, lipase from rhizopus, cytochrome oxidase from Trichoderma sps, Glucose isomerase from Bacillus, inverter from saccharomyces etc

The other criteria to be considered include:

ii) Availability of sources: One can use plant materials that are readily available or cultivate microorganisms as sources for enzyme.
iii). The enzyme should be readily available. Else transportation charges if added, can lead to issues of delay and add to expenses
iv) The raw material chosen should be easy to store and handle.
v) The raw material should have longer shelf life and should not get perished easily.
vi) The enzyme of interest must be in enough amounts in the selected source
vii) During the processing of raw material the byproducts produced should not be toxic .

2. General handling procedures: The procedures vary based upon the source selected.
In general plant tissues are used for isolation of enzymes. Whole plant, leaves, roots or bark can be used. Protocol include the following steps
Harvestine plant tissue ---> Drying-----> Powder ----> Use
Or
Macerate with some suitable solvent ----> filter or centrifuge ---> Use

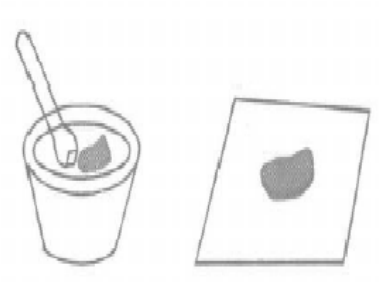
Microbial cell: When these are used, the cells are culture in liquid medium or on solid medium, harvested by certification and enzyme is isolated. Extracellular enzymes are found in the supernatant, and if it is an intracellular enzyme, the microbial pellet is taken and subjected to enzyme extraction procedures.



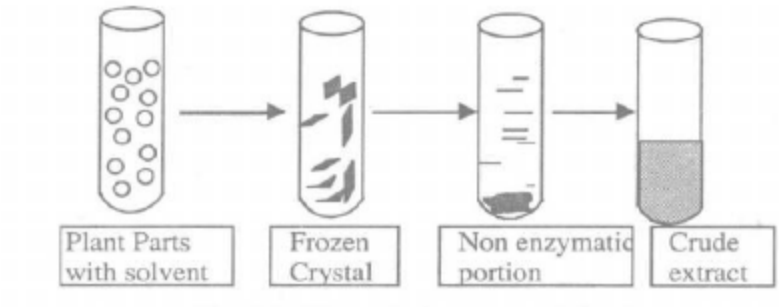
Animal tissues: These are used to isolate intracellular enzymes. The tissue is isolated and kept in cold or in deep freezer until further use. The material is homogenized and subjected to centrifugation. This generates crude extract that has enzymes. At times the cells are subjected to differential centrifugation to isolate enzymes from organelles.

3. Methods of isolation: There are many methods of isolation and some of them are discussed below:

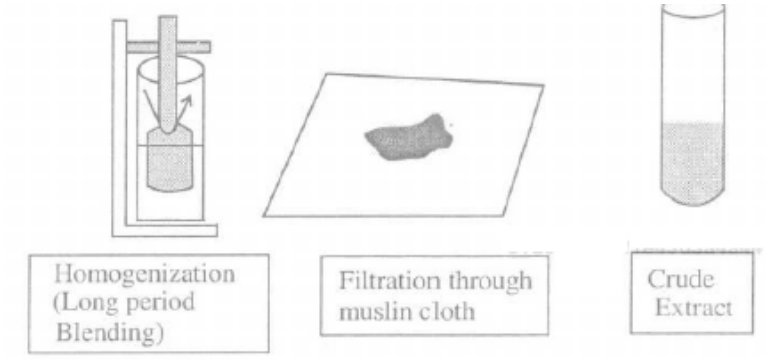
a) Grinding with abrasive: The plant tissues or the microbial cells are subjected to grinding with abrasive material such as Alumina and Silica. This can be done using simple mortar and pestle system or are in mixers and grinders. Abrasives disrupt the cell wall and release enzymes.



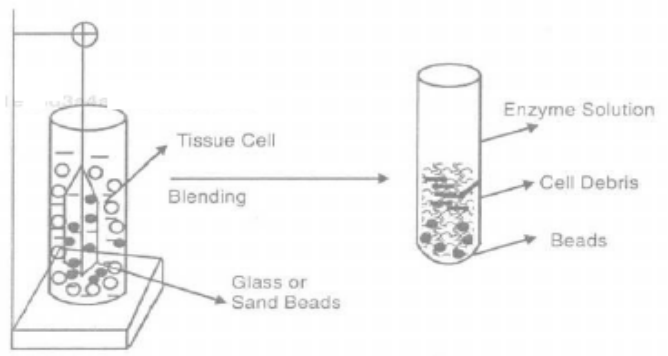
b) Alternate freezing and thawing: In this procedure the material is frozen that results in freezing of water present within the tissue. As tissue is thawed, the water undergoes anomalous expansion which leads to strain on the cell membrane and this intern disrupts the cells.



c) Blending: The tissue is subjected to blending, which leads to generation of mechanical forces upon the tissue. As it rubs against the wall of the blender, it disrupts leading to generation of crude enzyme.

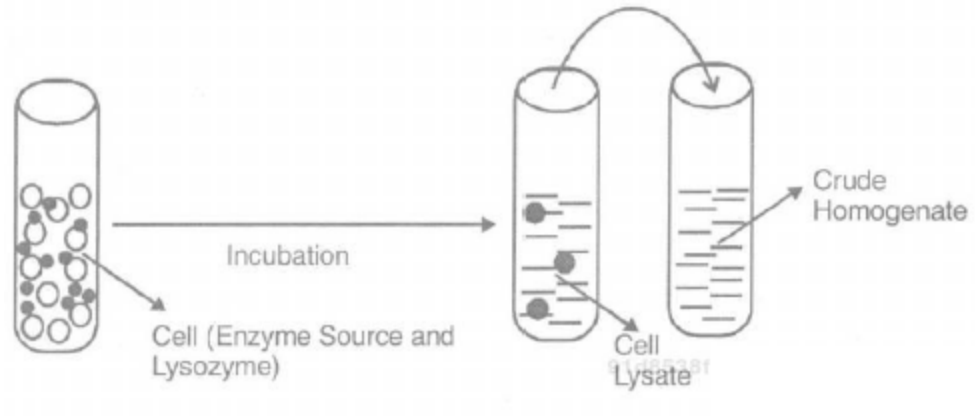


d) Addition of glass beads: During blending, acid washed glass beads ot acid washed sand is used to increase mechanical shear stress and make the process of isolation more effective.

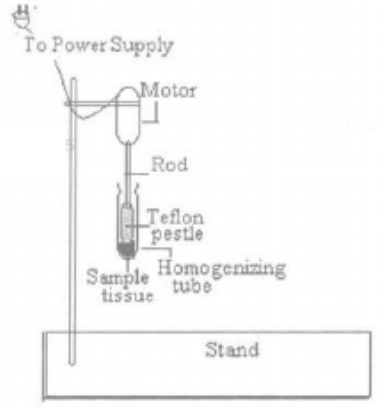


e) Use of hydrolytic enzymes: The tissue is subjected to hydrolytic enzymes like lysozyme proteases and lipases. These act upon the cell membrane and disrupt it leading to release of cell sap which consists of enzyme.

Enzymes at times are immobilized and the cells are passed through the column.This procedure saves investment on enzymes as they can be reused.



f) Homogenization using a high speed blender: This uses a glass tube and a Teflon pestle with a high speed motor. Used to treat animal tissues, they are subjected to a speed of 600 RPM for about 5 to 10 minutes using appropriate quantities of isotonic solution. The homogenate obtained is the source for enzymes.

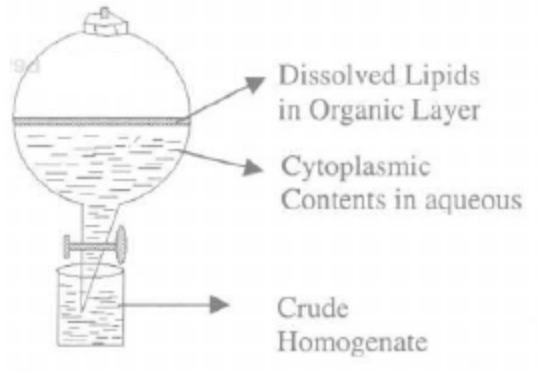


g) Treatment with hypotonic solution: Cell suspension is taken in hypotonic solution which leads to absorption of water. This creates turgor pressure on the cell membrane leading to its destruction. Sucrose solution or KCl solutions are used in this procedure .

h) Ultrasonic vibrations: The cells are subjected to ultrasonic vibrations that can disrupt the cell membrane and lead to release of the cell contents.

i) Alteration of pH of the solution: Changing the pH can cause denaturation of membrane proteins, which leads to formation of pores that leds to
Leakage of cell sap.

h) Usage of organic solvents: This is rarely used. Some solvents like chloroform and either are employed which dissolve cell membrane, leading to the formation of pores and cytoplasm oozes out as crude extract.



3. Methods of purification: Several strategiesStrategies are available for the purification of enzymes.

Property	Method	Scale
Size or mass	centrifugation	large or small
	gel filtration	generally small
	dialysis or ultrafiltration	generally small
Charge	Ion exchange chromatography	large or small
	electroforesis	generally small
	isoelectric focusing	generally small
Solubility	Change in pH	Generally large

	Change in ionic strength	large or small
	decrease in dielectric constant	generally large
specific binding sites	affinity chromatography	Generally small
	affinity elution	large or small

Methods depending on size are mass

Centrifugation: Enzymes, which are proteins of large molecular weight can be sedimented using high speed centrifuges. centrifugal force of over 300000g are obtained using high speed centrifuge. Initially cell debris is removed at low RPM followed by high speeds.

Density gradient or Zonal centrifugation can also be used.

Gel filtration or gel permeation technique: In this technique molecules are sieved through the gel. Large molecules come out of the column through the void spaces whli small molecules come out late. To separate proteins using this technique one has to know approximate molecular weight. The eluent from the column is collected at intermittent intervals and is analysed for the presence of enzymes.

Dialysis: This method is used to separate protein from small molecules; the procedure uses a semipermeable membrane which is filled with the sample and closed at both the ends like a sack. This sack is placed in a hypotonic solution and is allowed to stand until equilibrium is attained. The outside solution is changed repeatedly until then salt is diluted. This procedure is adopted to remove salt after salting out of proteins.

Methods based on charge:

Ion exchange chromatography: method employees Ion exchange resin. both cationic and anionic resins are available. The columns are packed with appropriate resins and the cell extract is added to the column. The protein exchange with counter ions and are eluted from the column by changing pH or by varying ionic strength.

In order to use this technique one has to know properties of protein on the enzyme to be isolated.

Electrophoresis: This technique is employed when small quantities of enzyme are to be isolated. zonal electrophoresis is used to check the presence of enzymes and characterize it.

Isoelectric focusing: this technique is used to find out the pI value of a protein or to separate the proteins based upon their pI value.

Isolation of enzymes based on solubility: Protein existence soluble form in the cytoplasm of cells. During isolation the pH of the solution and the ionic strength of a buffer helps to retain

them in their native form. During isolation change in pH can lead to precipitation of proteins. The precipitate is obtained as the source of enzyme .
Addition of a higher amount of salts can lead to precipitation of protein. This procedure is called salting out of proteins. Ammonium Sulphate is used which removes the water of hydration and precipitate protein.

Methods based on specific binding:
affinity chromatography: This is the best method for purification of enzymes. enzymes exhibit affinity towards their substrates and inhibitors. this specific binding is the principle used in affinity chromatography. Inert substrate is bound with a ligand to which the enzyme has affinity. Crude extract is added to the column and the column is left but sometime so that the enzyme binds to the legend. Elution of the bound enzyme is done by adding another strong ligand, by varying pH or by changing ionic strength of relation buffer.
This is a one step procedure for purification of enzymes.

4. Development of qualitative and quantitative estimation procedures: methods of estimation are the methods of assaying enzymes are to be developed before the isolation of enzymes.
It includes both qualitative and quantitative estimation. For example If amylase is being isolated, the qualitative test would be check starch hydrolysis adding gram Iodine. A qualitative test would be to check the amount of glucose or maltose released using the DNS method.

5. Stabilization and crystallization of enzymes: Isolated enzymes can easily loose their activity and get denatured. In order to prevent this, preservation of the proteins and stabilizing conditions like pH, ionic strength is done by using a suitable buffer.
Crystallization of enzymes is done by lyophilization which increases the shelf life of enzymes.

6.Checking the purity of enzymes: Purity of enzymes is determined by electrophoretic methods and also sedimentation methods. Electrophoresis of the purified enzyme should result in a single band. During sedimentation method using density gradient centrifugation a single band is seen which indicates the purity of the sample.

7. Methods of preservation of isolated enzymes: the purified sample generally stored as small aliquots. Cryopreservation is preferred to store the enzymes.