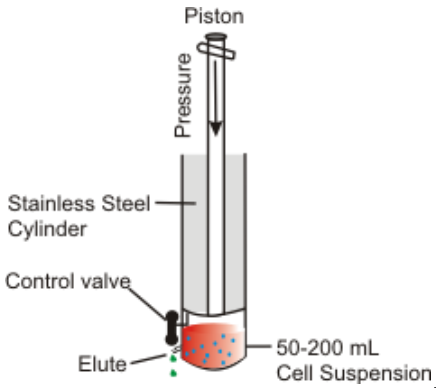

UNIT 2

EXTRACTION OF PROTEINS

Structure

2.1 Introduction

Expected learning outcomes

2.2 Cell disruption methods

2.3 Non-Mechanical Methods

Chemical Method

Treating with Detergent

Enzymatic Treatment

Osmotic shock

2.4 Mechanical Methods

Solid Shear methods

Bead Mill Method

French press

Liquid shear methods

Homogenisation

Ultra sonication

Freeze thaw

Potter-Elvehjem homogenizer with Teflon Pestle (PTFE)

2.5 Centrifugation

2.6 Summary

2.7 Terminal Questions

2.8 Answers

2.9 Further Readings

2.1 INTRODUCTION

In the previous unit we have discussed about the structure, function and biological importance of proteins as hormones, antibodies and other diverse important physiological functions. To use these proteins as therapeutic agents as well as to study and analyse the nature of these proteins we need to extract proteins from their subcellular locations. These subcellular locations could be biological membranes vital subcellular organelles like mitochondria, golgi complex and endoplasmic reticulum etc. Moreover life exists in different forms like eukaryotic and prokaryotic cells. Irrespective of eukaryotes and prokaryotes these cells are made up of different types of protecting layers like membranes or cell walls to maintain their structural integrity.

In order to extract the proteins, present inside these cells we need to treat cells with some chemical agents or enzymes or apply certain external forces that induces changes later breaks the entire cell membrane or create minute pores on the cell wall so that the cytosol present inside the cell will come out along with the proteins and other bio molecules.

Proteins can be either or intracellular and can be extracted from different locations like membrane, cytoplasmic and nuclear. By performing cell disruption or lysis we are going to extract and retrieve the desired biological product from a cell. These extraction methods are very much helpful in the recovery of natural as well as recombinant biological products (synthetic) like proteins enzymes and hormones.

To begin this unit, we will be discussing with solubilization of proteins followed by some of the simple non-mechanical and mechanical methods that are widely used for preparing crude extracts of cells. You will also learn about routine methods like homogenization and grinding that are used to prepare cell extracts. At the end of the unit, we will be discussing about Ultra sonication, French press and Centrifugation to further extract proteins from cells and their subcellular locations.

Expected Learning Outcomes

After studying this unit, you should be able to:

- describe the purpose of protein extraction;
- enlist non-mechanical and mechanical methods of extraction;
- explain the principle behind each extraction method; and
- distinguish between extraction methods with respect to their applications.

2.2 CELL DISRUPTION METHODS

Cell disruption methods can be broadly divided into two types one is non-mechanical and the other one is mechanical disruption (Fig. 2.1). Let us begin the discussion of non-mechanical method for cell disruption. Learners are advised to go through the video link provided for additional information https://www.youtube.com/watch?v=lKOtqLfv0kA .

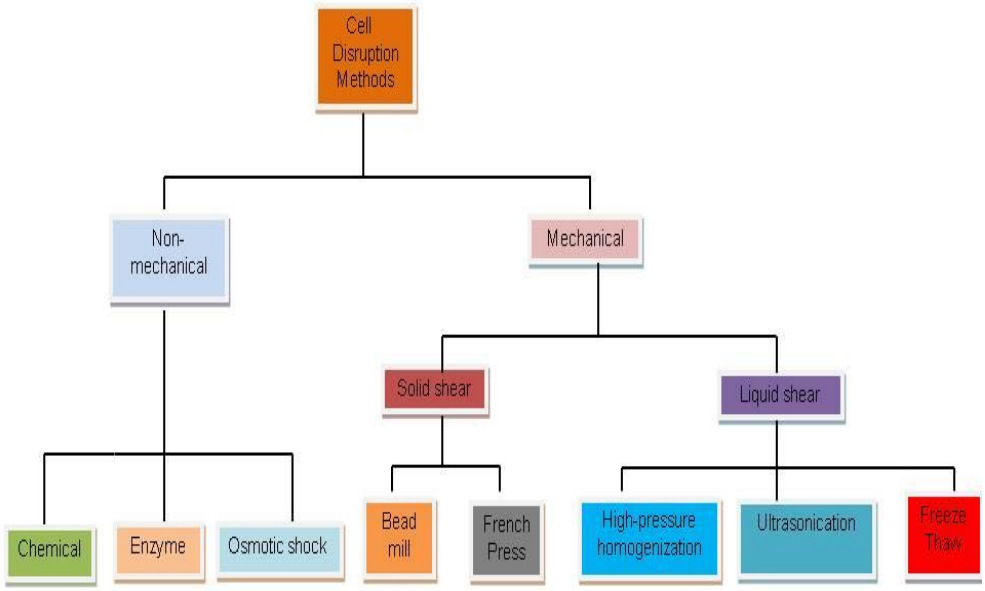

Fig. 2.1: Schematic representation of various methods used for the preparation of cell extracts.

2.3 NON-MECHANICAL METHODS

The first type of ***non-mechanical*** cell disruption method includes treatment with acid or alkali or with organic solvent, hence known as chemical method.

2.3.1 Chemical Method

Under this method there are three subtypes like using i. Acid/alkali/ detergent, ii. enzymatic and iii. osmotic shock.

Treating with Alkali: In this cells or tissues are suspended in alkaline solution with a pH range between10- 12. This alkaline solution digests the cell membrane in few minutes and allows the flow of cytosol to come out of the cell.

In some other conditions, organic solvents like alcohols, dimethyl sulfoxide, methyl ethyl ketone or toluene could be added. These solvents also act upon the external cell membrane and dissolve the membrane allowing the cytosol and other cellular ingredients to come out of the cell (Fig. 2.2).

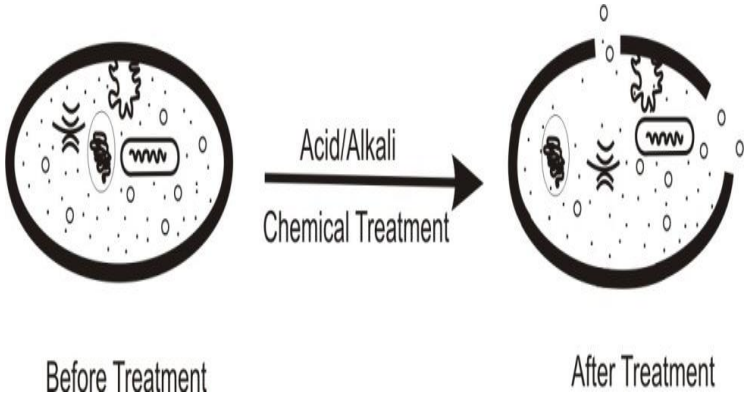

Fig. 2.2: Cell disruption method using chemical treatment.

2.3.2 Treating with Detergent

The other way of disrupting cell membrane is by adding detergents. Detergents are a class of molecules whose unique properties enable manipulation (disruption or formation) of hydrophobic–hydrophilic interactions among molecules in biological samples. In biological research, detergents are used to lyse cells (release soluble proteins), solubilize membrane proteins and lipids, control protein crystallization, prevent nonspecific binding in affinity purification and immunoassay procedures, and are used as additives in electrophoresis. **Triton x 100**, **sodium dodecyl sulfate (SDS)**, and ethyl dimethyl ammonium bromide are the commonly used detergents to achieve cell disruption by detergents. Triton x 100 is a non-ionic detergent that can solubilize membrane proteins. Whereas sodium dodecyl sulfate being an anionic detergent, denatures proteins by disrupting the non-covalent protein bonds making the proteins lose their native conformation and shape. Hence this detergent is used in electrophoresis to perform SDS page electrophoresis to disrupt the protein-protein interactions and separate protein into individual subunits. Ethyl dimethyl ammonium bromide, a cationic detergent act upon the cell membrane especially on lipopolysaccharides and phospholipids and creates minute pores which further lead to the development of differences in the osmotic pressure and allows the cell to disrupt (Fig. 2.3).

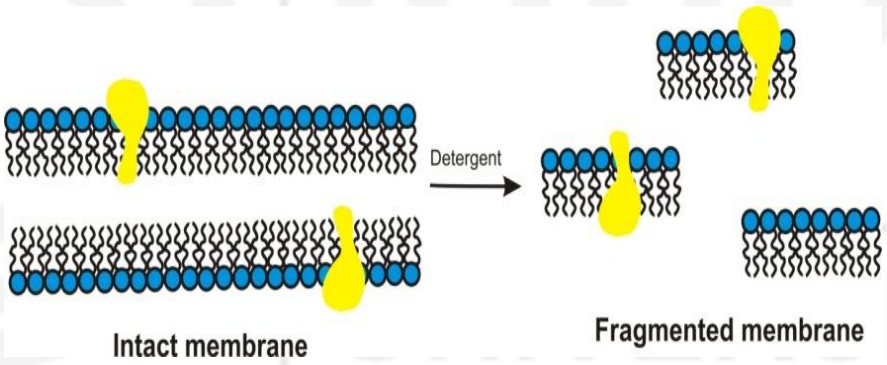

Fig. 2.3: Disintegration of bio membrane after treatment with detergent.

2.3.3 Enzymatic Treatment

The second category of non-mechanical cell disruption is an enzymatic method. Let us explore the details of this method by taking some commonly used enzymes.

Cellulases popularly known to digest the cellulose present in the plant cell wall. By exploiting this property of the enzyme one can apply this enzyme on the surface of plant cell and incubate for sometime (Fig. 2.4). After desired incubation time one can observe the partially digested cell wall. This allows the disintegration of cell wall and releases internal subcellular organelles and other biomolecules including proteins to come out of the plant cell.

Enzymes like pectinases, xylanases and chitinases are the enzymes that are also used to disrupt cell wall of Yeast and Fungi cells. Lysozyme is another

best example to digest the cell wall of gram positive bacteria. Lysozyme hydrolyses β-1,4-glycosidic bonds in the bacterial cell wall. Similarly, zymolase enzyme is used for the degradation of Yeast cell wall.

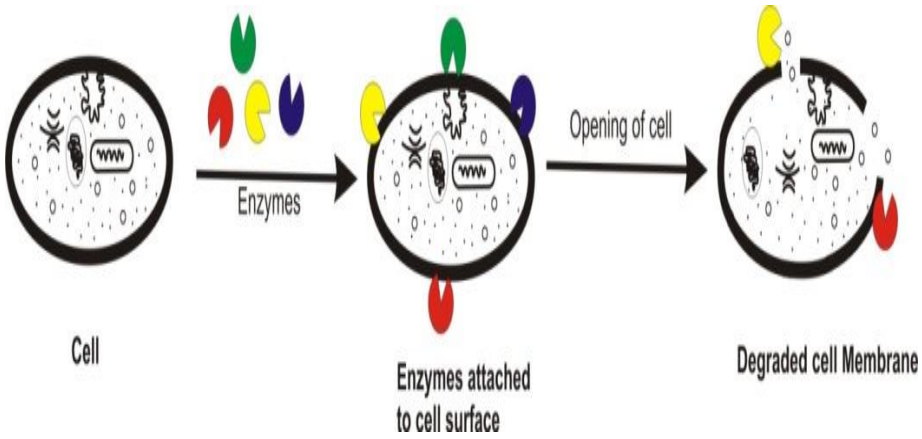

Fig. 2.4: Effect of enzyme on biological membrane of a cell.

However, chemical digestion has certain limitations, like cost of downstream processing (recovery of biological product) towards neutralizing the effect of enzymes or chemicals after performing the chemical digestion is an unwanted expenditure. In addition to this being enzymes biocatalysts they may act on other products or molecules of the cell which in turn may break those cells and molecules leading to the decrease in the overall product quantity and which will in turn affect the quantity of the final desired product.

2.3.4 Osmotic Shock

This is third method of non-mechanical cell disruption. This method is based on the principle of 'osmosis' (is the diffusion of water across a semi-permeable membrane. Where, water flows down a concentration gradient and toward an area that has a higher solute concentration) to disrupt the cell (Fig. 2.5). For example, if a **cell is placed in a hypertonic solution**, water will leave the **cell**, and the **cell** will shrink (**exo-osmosis**). In an isotonic environment, the relative concentrations of solute and water are equal on both sides of the membrane. There is no net water movement, so there is no change in the size/shape of the **cell**. When **kept in hypotonic solution**, the **cell** will swell and become turgid/swollen. Because the water potential outside the **cell** will be lesser than that of inside the **cell**. So, water will flow from out of the **cell** to inside the **cell (endo-osmosis,** Fig. 2.5**).**

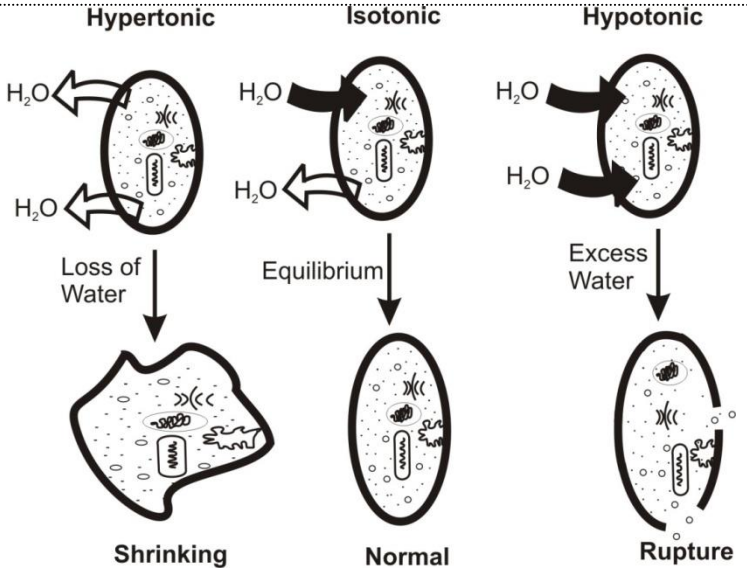

Fig. 2.5: Cells exposed to different types of salt solutions (osmotic shock).

SAQ 1 Do as directed:

1. Enlist the detergents that are commonly used for cell lysis.
2. Lysozyme hydrolyses _____bond of bacterial cell wall.
3. What will happen to a cell when it is shifted from isotonic solution to hypotonic solution?
4. _____enzymes are used to digest the cell wall of Yeast cells.

2.4 MECHANICAL METHODS

Mechanical methods of extraction can be broadly divided into two type of ***solid shear*** and ***liquid shear***. In both these methods external stress and movement is created by using solid particles or liquid layers.

In both the categories of cell disruption methods cells are subjected to high pressures that are created by the external forces like ultrasound and physical forces like rapid agitation with beads.

2.4.1 Solid Shear Methods

Let us start the discussion with the first type of mechanical method for cell disruption i.e., solid shear beginning with bead mill method.

2.4.1.1 Bead Mill Method

The instrument includes small rotating cylinder which is partially loaded with small beads, generally these beads are made up of zirconia (made of zirconium a hard & durable material). When we rotate the cylinder in a clock wise direction as shown in the figure (Fig. 2.6) the cylinder will transfer the kinetic energy into the beads present in it as mechanical energy. This mechanical energy creates a shear force and generates collisions among the beads and as well as the cells present inside the rolling cylinder. According to the needs of the disruption one can opt for larger are smaller beads.

However, the optimum loading capacity of this bead mill is around 80 to 85%. One of the advantages of bead mill method is that it can be used for high disruption efficiency, regulated external temperature control and high biomass loading capacity.

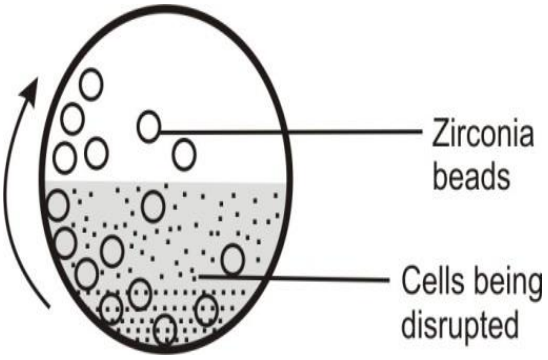

Fig.2.6: Schematic presentation of Bead mill.

Mortar and pestle is a commonly used grinding device in our kitchen to grind small quantities of spices. This is also used in laboratories for preparing tissue homogenate by adding some suitable buffer under controlled temperature.

2.4.1.2 French press

This is second method of solid shear extraction. Pressure generated by the piston is the force working behind this disruption method. Sudden drop in the pressure or fluctuations in the pressure levels inside the steel cylinder will lead to exploding of cells. In this method a pressure up to 1500 bar can be applied. Figure 4.7 shows the structure of cylinder along with piston being used in this method. At the bottom of the container there is a space to load the cell suspension. Pressure is applied through the piston and a sudden release of pressure induces some stress on cells enabling them to explode. The cell extract will elute through the outlet tube (see the Fig. 2.7). This method is widely used in in dairy industry for preparing homogenous mixtures.

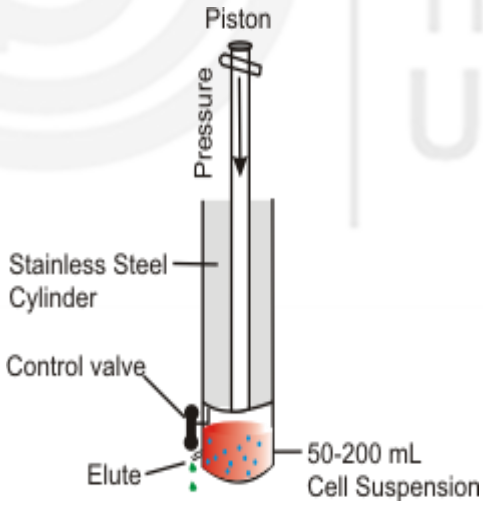

Fig. 2.7: French press.

2.4.2 LIQUID SHEAR METHODS

So far we have discussed about sold shear methods now, we will explore about liquid shear methods that are commonly employed in research work as well as in industry. An external liquid force or stress is imposed on the cells to

induce the lysis of cell walls or membranes. Let us first start the discussion of homogenization through high pressure.

2.4.2.1 Homogenisation

The literal meaning of “homogenous” means “uniform or similar in nature” i.e., by employing this method we are going to make uniform mixture of cells or tissue. This can be compared with grinding mill, where the grains are crushed to obtain soft uniform flour.

This is one of the most widely used mechanical cell disruption method in industry. Popular for the isolation of recombinant proteins produced inside bacteria or yeast cells. Pharmaceutical industries use this method in downstream processing for product recovery. Look at the figure 4.8 where target cells are passed through a narrow tunnel which is having a wide opening on both sides and a narrow passage. An external pressure around 1200 PSI is applied that will create a set of shear force while cells passing through these narrow tunnel. Once cells reach the other end of the tunnel cells will start expanding and this phenomenon called cavitation (formation of a cavity inside the cell) this further leads to cell disruption. You can observe the small particles of cell extract around 1-4 µm in size coming out of the tunnel as shown in the (Fig. 2.8)

The advantage of this method is its speed and ease of doing without any interruption. However this method is having some disadvantages with regard to isolation of heat labile products/molecules like enzymes.

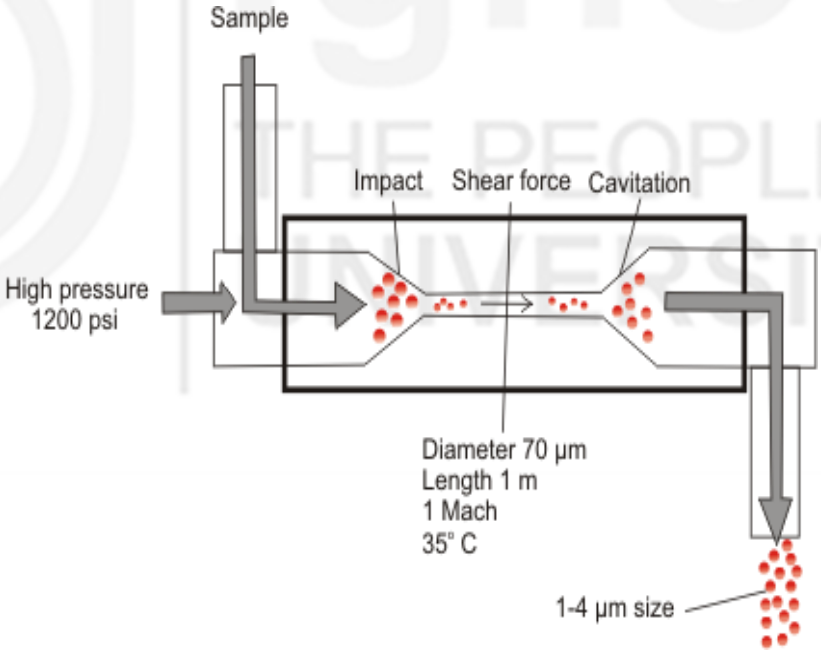

Fig. 2.8: Flow of cells inside a high pressure homogenizer.

2.4.2.2 Ultra Sonication

In the above section we have studied about a method that is based on the pressure generated by an external pump. In this section we will be studying about Sonication that is dependent on the pressure waves that are generated by ultrasound (sonic waves of 25 kHz). This pressure wave further creates

impact of cavitation. Oscillating cavitations collapse after some time generating through implosions. These implosions will cause a localised shock wave and rise in temperature (Fig. 2.9). Sonication is used for small volume samples of around 1 mL to 2 L.

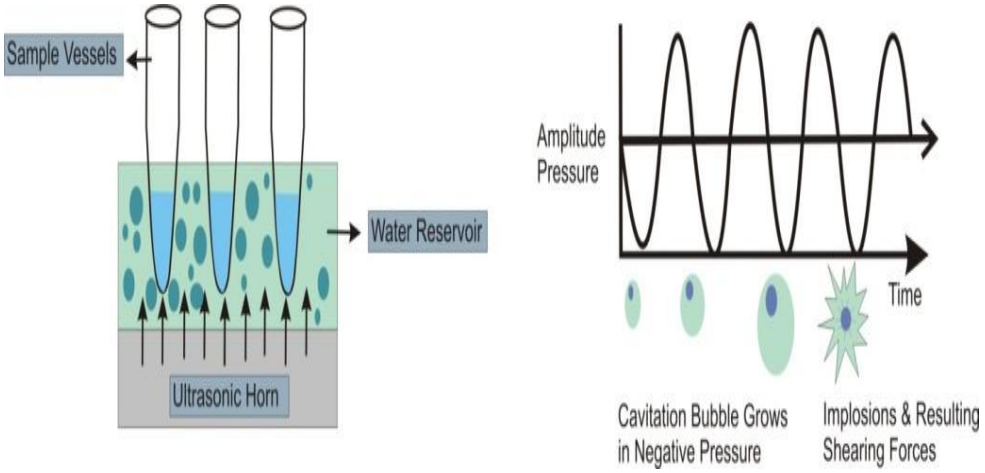

Fig. 2.9: Impact of ultrasonic waves through cavitation bubble.

Downstream processing is the process of harvesting biological products like proteins, enzymes, hormones, pharmaceutical products etc., from the natural sources/producers like plant/animal/microbial cells and sometimes recombinant cells.

Look at (Fig. 2.10) where you observe a control unit connected with a blender like structure which is known as sonicator. This sonicator amplifies and transmits the vibrations to the solution. Here it is important to remember that we need to maintain cold conditions while performing sonication to avoid the loss of activity protein of due to the heat produced. Sonication instrument can be compared with a blender used in kitchen to crush the soft fruit and prepare fruit juice or extract. However, blender operates through fine blades and sonication is based on the ultrasonic waves. But broadly the purpose seems to be similar, however blender is used to cut and collect the extract from a fruit whereas sonication device is used to lyse the cells and collect the cell extract.

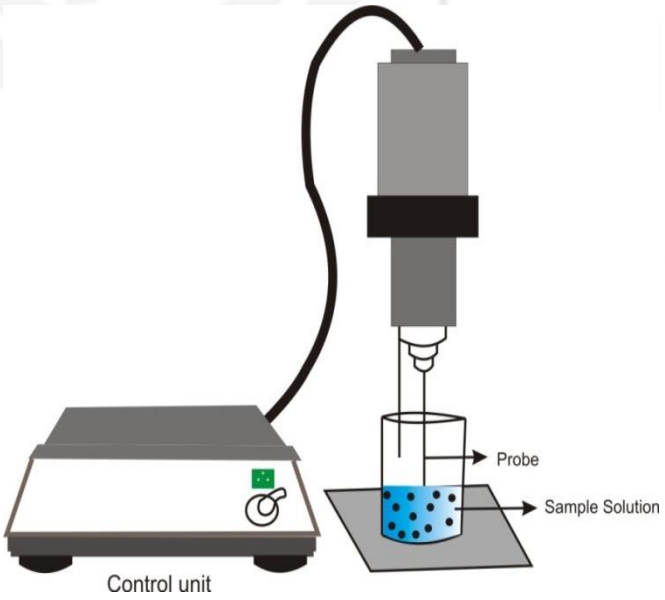

Fig. 2.10: Sonicator.

Sonication is comparatively having more advantages over other cell disruption methods like low operating cost and less denaturation of the cellar extract as

we can perform this method at low temperatures. Apart from this, the cost of post extraction or recovery (Downstream processing) are comparatively low when compared to other methods i.e, there is no need to remove any unwanted chemicals or beads that are used to disrupt the cell. However, continuous sonication may produce excess heat and generate certain free radicals which may damage the biomolecules present inside the extract hence it is advised to perform sonication in spells or under optimised conditions.

2.4.2.3 Freeze thaw

This method can be considered as temperature induced lysis, as a sudden change in the temperature causes cell disruption. In brief bacterial and or mammalian cells are submerged in a suitable cell storage solution/buffer and placed in dry ice or in ethanol or placed in deep freezers with extreme low temperatures using specially designed vials (small containers/ eppendrof). To perform freeze thawing these vials are taken out and then gently thawed (defrost) at room temperature. By performing this there will be raise in temperature and induce disruption of the cells by inducing sudden change in temperature. However, continuous thawing is not advised as this will raise the temperature and denature heat sensitive molecules like proteins, enzymes and nucleic acids. Hence to achieve an effective isolation this procedure needs to be repeated in multiple cycles consisting of freezing followed by thawing" (Fig. 2.11). In this process cell undergoes swelling and ultimately breaks and releases internal subcellular components along with the cytosol. This method is quite time consuming as it needs to be performed in multiple cycles for efficient cell lysis. Frequently, used while performing molecular biology research work to isolate the recombinant proteins from cloned and cultured cells.

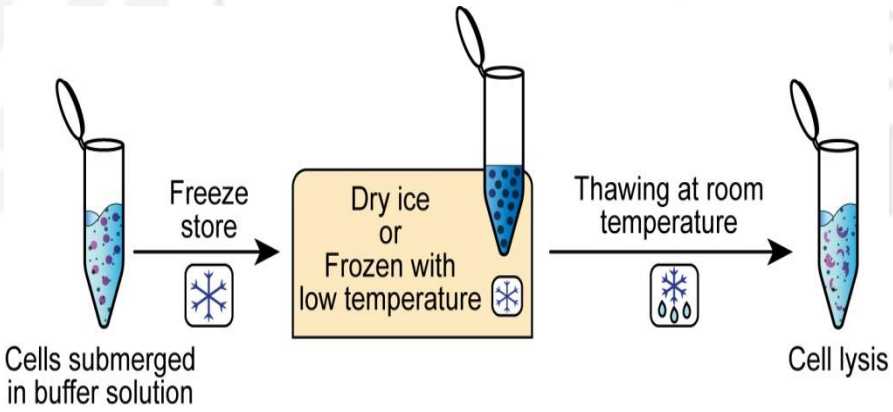

Fig. 2.11: Steps involved in Freeze thaw procedure (need to be modified).

2.4.2.4 Potter-Elvehjem homogenizer with Teflon Pestle (PTFE)

Potter-Elvehjem homogenizers can be used for both grinding tissues and shearing cells. Teflon (PTFE} pestle is effectively used as a shearing homogenizer (Fig. 2.12). For shearing, the pestle can be connected to a variable speed lab motor. For sample processing, soft tissue is placed in the sample and the pestle is rotated at 600-750 rpm. The tube is repeatedly pressed up on the pestle where shearing forces disrupt the sample. Potter-Elvehjem homogenizer is relatively inexpensive. They are easy to use and clean, and samples can be kept cold on ice during processing. The

homogenizer is effective for disrupting soft tissues and animal cells for the generation of subcellular components.

Fig. 2.12: Homogenizer with Teflon Pestle.

2.5 CENTRIFUGATION

Till now you have studied about Non-mechanical and Mechanical methods for disrupting cells and prepare cell extract. Now in this section we will be studying about “centrifugation technique”, which is widely explored for separation and isolation of cells and subcellular organelles. Especially to separate the solid particles present in a liquid suspension.

However, you have studied about this technique in unit-2 of cell biology course BBCCT-103.In this section we will be recollecting and discussing in brief about the principle and applications of this technique. Centrifuge is the instrument used in this technique and as mentioned above it is used to separate and purify the mixtures of biological samples. A table top model of centrifuge is shown at (Fig. 2.13). You might have observed using such centrifugation instrument in a diagnostic lab where the technician will be separating the serum from blood. Before moving further learners are advised to watch the video available at the following YouTube link
https://www.youtube.com/watch?v=He5YgfRNZAk

Fig. 2.13: Centrifuge (source Wikimedia commons).

In the year 1920 a scientist named as Svedberg, developed this technique. **Relative centrifugal field (g) abbreviated** as RCF is the key force working behind this technique. Revolutions per minute (rpm) is the unit used to measure RCF. Different types of centrifuges like preparative, analytical and ultracentrifuge are available for performing distinct functions like separation of cells from tissues, separation of subcellular organelles and isolation of biomolecules. Both the analytical and ultracentrifuge have the provision to operate at low temperatures to avoid loss of activity of proteins due to the heat produced by high speed rotations.

To convert rpm to RCF, the following formula is used:

$RCF = (rpm)^2 \times 1.118 \times 10^5 \times r.$

RCF is dependent on the speed of rotations in rpm and distance of particles from the centre of the rotation.

The centrifugation is based on the size, shape, and density of the particles, viscosity of the medium, and the speed of rotation. The principle is that the denser particles are forced to the bottom and the lighter particles stay at the top when spun rapidly. In centrifugation technique a cell extract prepared by either mechanical or non- mechanical methods will be taken into a centrifuge tube (Fig. 2.14 A). This centrifuge tube is placed into a rotor which allows solution mixture to rotate at a high speed during this process the solid particles settles down at the bottom of the tube according to their densities (Fig. 2.14 B). In simple words "*the particle with high density settles first followed by the particle with medium density and then by the particle with low density on the top layer".*

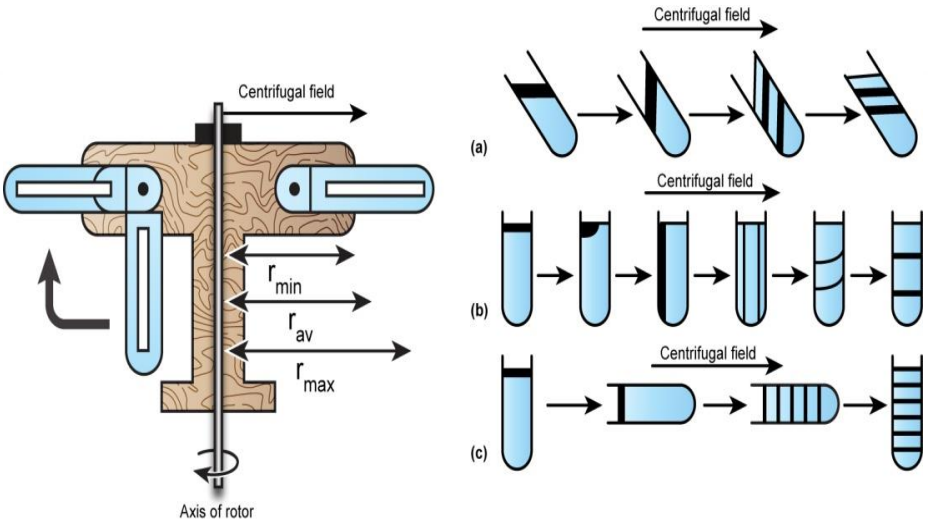

Fig. 2.14: A) Internal arrangement of Centrifuge B) Sedimentation of particles.

Centrifugation techniques have wide range of applications like, table top centrifuge is widely used in diagnostic laboratory to isolate blood cells and Yeast cells. Whereas high speed centrifuge is used to isolate subcellular organelles like mitochondria, golgi complex and other cell precipitates like antigen-antibody complexes (Fig. 2.15). Analytical centrifugation is having an additional provision to examine the changes in the molecular confirmations of proteins while they are being isolated.

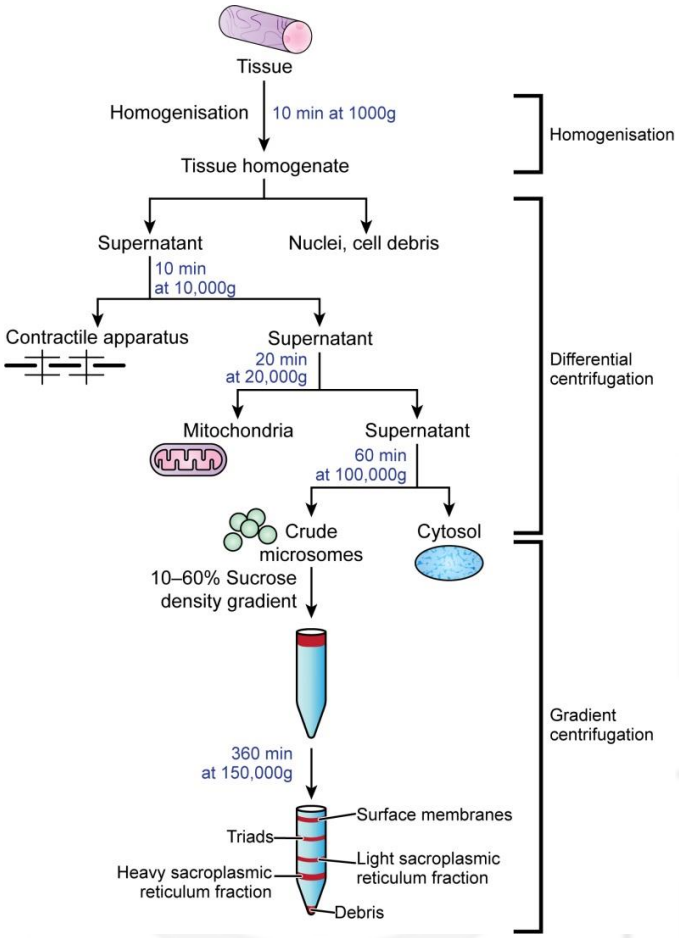

Fig. 2.15: Stages of subcellular fractionations using centrifugation

SAQ 2

Fill in the blanks

1. In bead mill method _____ force generated by cylinder is converted into _____force.
2. French press method come under _____ type shear technique.
3. What is the major advantage of sonication over other methods with regard to downstream processing?
4. _____ Organic solvent is used in freeze thaw method.
5. _____ is working force behind centrifugation.
6. Expand rpm.

2.6 SUMMARY

- In this unit you have studied about various methods that are used to prepare cell extracts. These methods include simple grinding to high end sonication.
- We have observed that there are different methods to isolate different types of cells according to the needs of the research to be conducted. To lyse the red blood cells simple osmotic shock method is sufficient. Whereas to break the bacterial and plant cell walls one can apply specific enzymes.
- Similarly, for industrial applications we have high pressure homogenisation and bead mill method. Where as to perform cell disruption at the level of a research laboratory both freeze thaw and ultra sonication are very much useful.
- At the end of the unit we have discussed about centrifugation technique which is popularly used to separate the cells, organelles and purify molecules based on their densities.
- It is important to remember that all these techniques are useful to prepare the initial cell extracts and to some extent the isolation of the subcellular organelles and bio molecules.
- The techniques that are going to be discussed in the next two units will be fulfilling the needs of isolation and purification of the proteins and other essential biomolecules.

2.7 TERMINAL QUESTIONS

1. Distinguish between non-mechanical and mechanical methods of cell disruption.
2. Describe the liquid shear methods with a special emphasis to their advantages.
3. Give a note on a non-mechanical method that is used for the lysis of β-1,4- glycosidic linkages of a bacterial cell wall.
4. What will happen when we subject a protein mixture containing 500 kDa, 350 kDa, 280 kDa and 200 kDa for centrifugation?

2.8 ANSWERS

SAQ 1

1. Triton X-100, SDS and Ethyl trimethyl ammonium bromide are commonly used for cell lysis.
2. β-1,4-glycosidic bond
3. Uptake water and ions from the surrounding solution and swells finally bursts.

4. Zymolase.

SAQ 2

1. Kinetic force to Mechanical
2. Solid shear
3. Ethanol
4. Relative centrifugal force
5. Revolutions per minute
6. User friendly and cost effective

Terminal Questions

1. Non-mechanical methods mainly depend on chemicals/enzymes/detergents, whereas mechanical methods are depended on solid and liquid shear forces (refer fig 2.1 and section 2.2).
2. Both homogenization and sonication comes under liquid shear methods of cell disruption. Swiftness and cost effectiveness are the major advantages of both Homogenisation and sonication. (Refer section 2.4.2)
3. Lysozyme enzyme (enzymatic method, refer section 2.3.3) is used to cleave the β-1,4- glycosidic linkages of a bacterial cell wall.
4. Centrifugation is a separation technique, hence when a protein mixture containing 500 kDa, 350 kDa, 280 kDa and 200 kDa issubjected to centrifugation proteins will settle down or sediment according to their densities i.e., 500 kDa being high density protein will first reach the bottom of the tube followed by 350, 280 and 200 kDa on the subsequent upper layers. (Refer section 2.5)

2.9 FURTHER READINGS

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