Molecular Biology
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Module - 01
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Lecture-05 Cellular Fractionation (Part 2)

Hello everyone, this is Dr. Vishal Trivedi from Department of Biosciences and Bioengineering IIT, Guwahati. And what we were discussing, we were discussing about the cellular structures of the prokaryotic cell and the eukaryotic cell. And in the previous two, three lectures, we have focused more on to the structure and as well as function of the different parts, what is present in these type of cells. So when we were discussing about the prokaryotic cell, we have discussed about the plasma membrane, we have discussed about the cell membrane, we have discussed about the plasmids and we have also discussed how you can be able to isolate the plasmids and how the plasmids can actually be able to exist in three different forms. And subsequent to that, we have also discussed about the eukaryotic cell.

When we were talking about the eukaryotic cell, we have discussed about the plant cell and as well as the animal cell, we have discussed about the differences between the plant cell and the animal cell. And we have also discussed about the different types of membrane-bound organelles what are present in the eukaryotic cell. So we discuss about the nucleus, we discuss about the mitochondria, chloroplasts, glycosomes, Golgi bodies, endoplasmic reticulums. And at the end, we have also discussed about the plasma membranes.

And while we were discussing about these cell organelles, we were also discussing about their structures, functions, and what is their contribution in regulating the different events within the cell. So in today's lecture, we are going to discuss about how you can be able to study these individual organelles. So although the membrane-bound organelles are not present within the hyperkaryotic system, we can still be able to isolate the different fractions from the prokaryotic cell and we can be able to study those fractions. Now the first question comes how we are actually going to do the fractionation of the prokaryotic cell. So within the prokaryotic cell, what are different zone where the protein is present, right? So if you see a prokaryotic cell, what you have is a cell membrane, right? You have a capsule, you have a cell membrane, then you have a cytosol and within the cytosol, you have the chromosome and other kinds of things, right? And if I show you the subcellular localizations, so you can actually have the five different fractions.

You can have the periplasmic fractions, you can have the protein which are present in the outer membrane of the cell wall, then you can have the extracellular media, you can actually have the protein which are present in the inner membrane and then you also have the protein which is present in the cytoplasm, right? This means the proteins which are present in the outer membrane, right? Protein which are present in the outer membrane, the protein which are present in the inner membrane, the protein which are present between the outer and the inner membrane, which means the periplasmic space and the protein which are present in the outer media or the protein which is present within the cytoplasm. Now, if you see the relative abundance of the proteins, now total number of protein what is present in the periplasmic fraction is 129, whereas the protein, the total number of protein which is present in the cytoplasm is 183, right? So, this means the major localization of the different proteins is either in the periplasmic space or into the cytoplasm. So, first we understand that how you can be able to get these fractions and if I want to get these fractions, what I supposed to do, right? So, this is a bacteria, right? I have to actually devise a mechanism so that I should be able to break this bacteria so that all these content should be released into the environment, right? Or into the my tube, right? And then only I can actually be able to separate them and I can actually be able to collect them separately. So, to breaking these is required are different types of disruption methods, right? So, you can actually have the different types of disruption methods to break the cells, right? So, what are different types of disruptions method and the disruption methods are specific to the prokaryotic cell or specific to the eukaryotic cell. So, the cell disruption method.

So, cell disruption method is actually works on the basic principle of modulating the physical parameters, chemical parameters or the biological parameters. Now, if you see what we have is we have the different types of cells. We have the prokaryotic cell, eukaryotic cell, we have the plant cells, right? So, they all are very different. They all are very different in terms of the structures like for example, the bacterial cell they are very small, they are having the cell wall. So, they are actually very resistant for breakage, right? Compared to that the animal cells they do not have cell wall and their size is very large.

So, you can actually be able to use them and you can actually be able to use a delicate matter. And for the animal cell they are big, for the plant cell they are big, but they also have the cell wall. So, you can actually be able to play the three different parameters. You can actually be able to play with the physical strength such as the cell wall, right? Or you can actually be able to work with the structural organizations such as whether the plasma membrane is present or not, whether the cell wall is present or not, whether the how thick the cell wall in that or not. And then you can also use the chemical compositions.

So, you can actually be able to have the composition of the plasma membrane, you can have the composition of the cell wall and that could be different and all these can be exploited in the different types of cell disruption methods. So, considering these parameters such as physical strength, chemical composition and the structural organizations, you can also have the different types of cell disruption methods. So, what are the different cell depression methods? You can have the physical method. So, you can where you are actually going to vary the physical parameters. You can also have the chemical or the enzymatic method where you are going to use the different types of chemicals or you can also have the mechanical method which are actually going to use the different types of machines.

Now, let us first talk about the physical methods. So, in a physical method, these methods play with the physical parameter to damage the cell to release the product. So, number one is the thermolysis. This method is easy, economical and require no additional specialized equipment. It can be used only if the your protein of interest which you are interested is thermostable.

This method gives a heat shock to kill the organism and as a result, it disturb the cellular integrity without affecting the product. The effect of heat shock depends on to the ionic strength, presence of chelating agents such as DDTA and the presence of other mutilating enzymes. Then we also have the other kinds of physical methods such as osmotic shock. So, most of the mammalian cells have the plasma membrane with an active transporter to maintain the osmotic balance. Maintaining an osmotic balance is an active process with the expenditure of energy.

Prolonged exposure to the cell with a hypotonic liquid such as water causes the osmotic imbalance and ultimately causes the lysis of the cell. In this process due to the inflow of water, the cell swell and ultimately burst to release the product. According to the Hoffman's equation, osmotic pressure is directly proportional to the concentration of the solute and the temperature. So, each mammalian cell has a different susceptibility towards the osmotic shock. For example, I have given you an example of red blood cells.

So, red blood cells will life with the addition of a tiny drop of water, right. Plant or bacterial cells are more registered towards the osmotic lyses due to a thick cell wall, right. And so, what will happen is that if you have a cell, right and if you keep them under the hypotonic condition, so, for example, if you take the RBC and put it into the water, what will happen is the water molecule will start entering, right, start entering into the cell because you know that as per the principle of osmosis, the solute is actually going to enter from the lower concentration to higher concentrations. So, this is actually

going to enter and as a result, it is actually going to swell. So, it is actually going to be increase in size.

So, it will increase in size to a certain capacity. After that, what will happen is that it is actually going to break, right and when it is going to break, it is actually going to release its content and that is what exactly is happening here, right. So, osmotic shock is good for the mammalian cells, but it is not good for the plant or the cell. Then we also have another physical method which is called as sonications. So, a sonicator generates the ultraviolet waves of frequency more than the 20 kilohertz to cause the cell disruption by the cavitation.

The interaction of the ultrasound with the liquid causes the compression and decompression very rapidly. The bubbles are formed in the liquid compresses several thousand atmosphere and gives the shock wave to the cell wall or the plasma membrane to cause the cell lysis. Generation of ultrasonic waves in liquid causes a rapid change in temperature and may cause the thermal denaturation. Hence, ultrasonication media need to be cooled and very long duration should be avoided. So, this is the sonication, right and we have prepared a very small demo like where the students might have shown where students are actually going to show you how you are actually going to perform the sonication as well and what are the different types of precaution you should take.

Because as I said, you know sonication means where you are actually going to you know send the sonic waves and when the sonic waves will enter, it is actually going to cause the compression and decompression very rapidly and as a result, it is actually going to create the cavitation bubbles and these cavitation bubbles when they were actually going to interact with the cells, they are actually going to cause the lysis of the cell. But it will not going to happen only with the sample, it is also going to happen with your body as well and that is why you are actually going to keep this sonic probes within a cabinet and on the other hand, you are also going to take the lot of precautions so that it should not affect your sensory organs such as ears and the eyes. Now we will talk about the chemical and the enzymatic methods. So chemical enzymatic method where you are going to use the alkali treatment. So this is a harsh but effective chemical treatment to lyse the cells.

Alkali treatment cause the liquid saponification which disturb the lipid packing and affect the cell's integrity. Then you can also use the detergents and detergents are actually going to dissolve the lipid membranes and as a result, it is actually going to cause the lysis of the cell. So when you add the lipid molecules, if it is a low concentration, it is actually going to cause the pores into the cell and that process is called as the permealizations. But if you are going to add the high quantity of the lipids,

then it is actually going to cause the complete removal of the plasma membrane. As a result, it is going to cause the solubilizations.

And then we have the enzymatic digestion. So enzymatic methods are specific, gentle and most effective but costly. Lysozyme is commercially available to treat the bacteria to release the intracellular product. In the lysosine, there are three other types of bacteriolytic enzymes, glycosidases, acetyl muralimyl-L-enulin neuradase and endopeptidase. Propriotases are also fine to be the bacteriolytic.

Each cell requires, each cell lysis require a mixture of different enzymes such as prokinesis, proteases, menaces or chitinases. Plant cell can be liked by the cellulose and the prokinesis. In most of the enzyme mediated cell lysis method, the rupture of the cell wall depends on to the osmotic pressure of the external media. In few cases, the enzymatic digestion is performed to remove the external cell wall and then in the second step, protoplasts is disrupted by the gentle solutions. So in some cases, for example, in the case of plant, you can actually be able to treat it with the enzyme.

And as a result, the plant is actually going to be without cell wall. Once the plant is without cell wall, then you can just put it into the water and it is actually going to be experienced osmotic shock and as a result, it is actually going to release the content. Then we have the mechanical methods. So we can use the different types of mechanical method. Many of these mechanical methods are very oftenly being used in our household.

For example, you are actually going to use the mixer grinder, right. The mixer grinder is nothing but the warring blender, right. So you can actually be able to use the mixer grinder, right. So what mixer grinder is doing, it actually has a blade, right. You have seen right, they have actually blade right and these blades are running at a very high speed and as a result, what will happen is the cell is passing through these blades and when they are cells are passing through these blades, they are getting being cut, right and once they are going to be cut, they are actually going to release the content.

Then we also have the pestle mortars. Both are effective towards animal and plant tissue as well as the filamentous organisms. So you might have seen your mother or your housemaid that they are actually using the mixer grinder and as well as petal mortar for making different types of chutneys, right. That chutney is nothing but the cell extract, right. In industrial scale, cell disruption is carried out by the two different types of mechanical method, one is called bead mill and the other is called as the high pressure homogenizers.

So bead mill is bead mill disruption. The bead mill consists of a grinding cylinder with a central shaft fitted with a number of impellers which can be moved in clockwise or anticlockwise direction with the help of a motor. The grinding cylinder is filled with the beads made up of the glass, aluminia, titanium, carbide or zinconium oxide or the zinconium silicate. There is an inlet to supply the cell suspension and at the outlet to collect the sample after the process and the bead mill runs, cell experiences the shear forces between produced between the moving beads and the cells. So this is what the bead mill disruptor where you have a jacket, right and in this you are actually going to have the impellers and the impellers actually can run in clockwise direction or the anticlock direction and it is actually going to have the beads.

These beads are made up of the glass or silica or titanium or the kinds of metals and when it moves these beads are also hitting the cells, right. So it is actually hitting the cells. So when the cells are getting crushed between these two beads, it is actually going to get broken down and as a result it is actually going to release the content and that release content can be collected from here, right after a certain time period. Then we have the high pressure homogenizers. So in a high pressure homogenizers, you are actually going to have the moving plates, you are actually going to have the impact rings and all that and you are actually going to feed this high pressure homogenizers with it from the feeders and when the cell is passing through these narrow gaps, because this is actually going to go up and down and as a result what will happen is that the cell will actually going to pass through with this particular narrow gap for multiple times, it is actually going to get broken down into the smaller pieces and you are actually going to change the pressure for 200 to 1000 osmosis pressure and that change in pressure is very high and that actually causes the destruction of the cells.

So this is all about the cell disruption. So once you are actually going to select the proper cell disruption method, for example, for a prokaryotic cell, you can actually be able to use the sonications, you can actually be able to use the detergents, you can actually be able to use the enzymatic method and a combination of these depending upon the product what you are trying to isolate and depending upon the stability of that particular product and so on. Now once you have done the cell disruption, you are actually going to, for example, if you are actually going to disrupt the bacterium, bacterial cell, right, you are actually going to have all the 5 fractions which we have just discussed, right. You can actually have the outer membrane, you can have the inner membrane, you can have the periplasmic fractions, you can have cytosol and you can also have the external media, right. All these 5 fractions are available and now you are actually going to devise a mechanism if you want the protein from here, whether you want the protein from here, here, here and here.

So this is what we are going to discuss. So first is how you are actually going to do the isolation of the periplasmic fractions. So what you do is you harvest the bacterial cell by centrifugation at 3000 G for 20 minutes at 4 degree Celsius, right. Remember that all these procedures, majority of the molecular biology procedures are always being performed at 4 degree Celsius except that in some specific cases where you are actually going to be asked to do it at room temperature. Then you discard the supernatant and carefully remove the last few drops of liquid with a pipette because that is actually going to contain the media. Then you gently resuspend the palette in 1 ml of the TSE buffers using a wire loops.

Then you do the 30 minute incubation on ice. So when you do the 30 minute incubation on ice, you are actually going to swell the bacterial cells. Then you transfer the cells in a micro centrifuge tube and centrifuge at 16,000 G for 30 minutes at 4 degree Celsius. Then transfer the supernatant to the new centrifuge tube and this supernatant which you are actually going to get is actually going to be the periplasmic fraction. And the periplasmic fraction you can actually be able to use.

Now the second question is how you are actually going to do the isolation of the protein from the cytosolic fraction. So what you are going to do is you are going to take the bacterial cell. What you can do is you resuspend them, resuspend in resuspension buffer. So you can actually be able to suspend them in for example phosphate buffer saline. Now you do a solication because we are not interested in getting the protein from the periplasmic fractions or other places.

So you can actually be able to, we can very easily be able to do the solication. Once you do the solications after that you are going to do a centrifugation at for example at low speed first right to 3000 G for 5 to 10 minutes at 4 degree Celsius that is actually going to give you a pellet and going to give you the supernatant. That supernatant again you are going to spin at 16000 G okay for 25 minutes at 4 degree Celsius. And again you are going to get the pellet and you are actually going to get the supernatant. The supernatant is actually the protein which are present in the cytosolic fractions.

Remember that in this pellet you are actually going to have the cell wall which means outer and inner cell wall. And whereas in this pellet is also a cell wall components right because you know that the bacteria does not contain any kind of membrane bond organelles. So basically you can actually be able to get this. From this cytosolic fractions you can be able to isolate most of the proteins. Now let us talk about the fractionation of the eukaryotic cell and remember that the fractionation of the eukaryotic cell is more complicated than the prokaryotic cell because it has a very different types of the membrane bond organelles.

So here we are discussing about the fractionation of the animal cell and as well as the plant cell. So fractionation for most of the organelles are going to be the same except that the some of the fraction some of the exclusive organelles are only present in the plant cell. So for them we are going to discuss in detail in differently. So for example purpose I have taken the example of how you can be able to isolate or fractionate the plasma membrane mitochondria cortisol and as well as the chloroplast from a eukaryotic cell. And if you want to do the fractionation of a eukaryotic cell you should also be able to or you should be very familiar with the different types of centrifuges which you are going to use for fractionation.

So we have the different types of centrifuge. You can have the microfuge or I will say low speed centrifuge. So it actually is going to be used for very low speed centrifugation and this is not having a low temperature and this is the high speed centrifugation. This is also a high speed centrifugation and this is a ultra centrifuge. So ultra centrifuge actually can go more than 1 lakh g RPM. So it actually can go and this is actually the ultra centrifuge rotor and this rotor is made up of a metal which is called as titanium and this is very sturdy.

And this is the same high speed centrifuge but it is actually going to be a low temperature high centrifuge tube. So these are the different types of centrifuges what we actually require if you want to do the cell fractionations. Now as far as the technique is concerned the centrifugation can be done in two different modes. It can actually be able to do a differential centrifugations or it actually can be done in a density gradient centrifugations. Differential centrifugation means that you are actually going to run the sample at a different speed and you are actually going to do the fractionation.

Similarly in a density gradient centrifugation you are actually going to change the density of the media and as a result it is actually going to separate. So we will discuss both of these aspects or different mode of the. So let us first start about the differential centrifugation. So differential centrifugation is based on the differences in the sedimentation rate of the biological molecules because of their different size, shape and density. For example you have different types of biomolecules which are actually different in terms of size, shape and density.

So what will happen is that their centrifugal power is actually going to be different and it is actually going to be affected by the solvent. It is actually going to affected by the different types of particle size and it is actually going to be different by the other kinds of things. So what will happen is that when you do the centrifugation at a particular wave layer, particular speed that speed may not be able to provide the different enough force

to settle down the small size particle or medium size particle. So as a result the large size particle are actually going to be get pelleted down first. Medium size particle are actually going to be pelleted down second and the small size particles are actually going to be pelleted down at later stage because the small size because they are small size they require more force to get sediment.

Now let us understand this by example. For example you have the different types of molecules right. You have a iron block which is 100 kg, you require have a stone which is 30 kg, you have also an iron block which is 10 kg, you have another stone which is 10 kg, then you also have a cotton which is 8 kg and then you also have iron which is 1 kg. So what will happen is that when you do the centrifugation or when you are going to do the differential centrifugations irrespective of the weight right, irrespective of the weight the iron is actually going to get settled down first right. And then you are going to have the stone which is actually of the different density, this is actually of the highest density, this is the medium density and this is the lighter density right. So because the cotton is the lightest density it is actually going to be pelleted down at a very very high speed compared to the iron or the stone.

Same is true for the different types of biomolecule also. For example if you see the density versus sedimentation coefficient what you will going to see is that the sedimentation of the different types of organisms. For example the nucleus, mitochondria, microsomes, virus, soluble proteins all they are actually going to have the different sedimentation coefficient and as a result they are actually going to be get pelleted down at a different time points. For example the DNA is if you see the sedimentation right this is actually going to be different, RNA is going to be different, DNA is going to be different and soluble protein are actually going to be different. And this is can be exploited in a differential centrifugations to isolate in the different types of cell organelles and as well as the different types of fractions. Let's take an example of how you can be able to do the fractionation of the different organelles from organ.

So we have taken an example of the liver. So liver you know that the liver is made up of the cell which is called as hepatocytes. Apart from that it also contains the comfort cells and other kinds of blood cells but we assume that it is actually a pure hepatocyte. So the first thing what you are going to do is you are going to do the homogenization which means you are actually going to break the tissue so that it is actually going to form the single cell suspension in the first stage and then that single cells are also going to be broken down by the homogenizers. And as a result it is actually going to have the mixture of the whole cell and as well as the mixture of the different types of organelles. So imagine that these are the nucleus, endoplasmic reticulum, mitochondria and all that.

And all these organelles are different in terms of their sedimentation coefficients, in terms of their density, in terms of their size. So that actually is going to be affect their sedimentations. So what will happen is that you are going to have a mixture of organelles in the beginning of the start. Now what you are going to do is first you are going to do a very low speed homogenized sedimentation. So what you are going to do is you are going to run a centrifuge at 6,600 G for 10 minutes and because this is very, very low speed you will not be able to pallet down the other material but you will be able to pallet down the nucleus, right.

Or you will be able to pallet down the unbroken cells because they are also going to be very heavy. So this is going to be a nucleus and as well as the unbroken cells. Now this portion you can actually be able to take out and put it into another tube and then you are actually going to run this at 15,000 G for 15 minutes. And when you do that it is actually going to pallet down the subsequent molecules. So subsequent to that it is actually going to pallet down the mitochondria, lysosomes and paraxosomes because they are actually going to be of heavy in nature in terms of density and in terms of size also.

But it is actually going to release the endoplasmic reticulum, it is going to leave the plasma membrane and it is also going to leave the ribosomes and other kinds of small vesicles. So this is actually going to be present here. Now if you take out these and put it into another tube and run it at a differential certification for 1 lakh G for 60 minutes then what will happen is that it is actually going to pallet down the plasma membrane, it is going to pallet down the ER, it is actually going to pallet down the small vesicles. Now still it is not, it has not pallet down the ribosomes, it has not pallet down the micro macromolecules like the ribosome machinery and RNA polymerase and those kind of multimeric proteins and it also has not pallet down the virus which is very very very small right. Now what you do is you take this supernatant into another tube and run it for 3 lakhs G for 2 hours right.

So when you do that it is actually going to pallet down the ribosomes, viruses and the large macromolecules and this is what it is actually going to be left and that is called as the cytosol okay. That cytosol is only containing the monomeric proteins right. So this is the power of the differential centrifugation through which you can be able to isolate the different organelles and you can be able to test them for their martensines and other kinds of things right. So you can actually be able to use the different types of analytical tools to characterize that you have isolated the nucleus or you have isolated the mitochondria or the phatopodia or the phytoplasm chloroplasts and all that.

I have taken another examples of cell fractionation. The scheme is almost the same that but the source is different. So here I have taken the muscle tissue. Again you are going

to do the homogenization that is actually going to give you the mixture of the cell lysate and also going to contain the unbroken cells when you do the you know the 1000g for 10 minutes it is going to remove the contacryl ductile then you are going to take supernatant if you run it at 20000g it is actually going to separate the mitochondria when you take the supernatant and run it for 1 lakh g it is actually going to produce the crude microsomes and cytosol is different and then if you run that the stigadient centrifugation you can be able to separate out the other organelles. So this is all about the differential centrifugations. Now because these biological molecules and as well as the organelles are different in terms of density you can also be able to use the density gradient centrifugation.

So what you see here is the densities of the biological molecules are different. You have the pocratic cells, you can have the mammalian cells, organelles, proteins, DNA, RNA all these are very very different right. And what happened is that they are actually going to be get sedimentated when the sedimentation force is higher than the sedimentation rate right. So what happened is that if you actually going to have the water as a buffer right if you take a tube right and fill it with the buffer it is actually going to have the same density right. So this is actually going to have the this layer also has the same density, this layer also has the same density, this layer also has the same density.

This means it will not going to create a barrier. But what will happen is that if I take a tube and if I put the solution of different densities. So what will happen is that if the molecule is having a sedimentation coefficient which is good enough to break this barrier because you will not be able to enter inside this then you are actually going to be focused here right. Similarly if you pass this layer but you will not be able to pass this layer then you will focus here. If you pass like that that is how it is actually going to have the differential centrifugation. So what you are going to do is you prepare a density gradient centrifugation which you are going to take the tube and you can actually have the increasing density gradient or you can actually have the decreasing density gradient.

And what will happen is that you will overlay the sample right on top. So all the three samples are ready right and when you spin them you are actually going to push them in the lower side. So when they push them they will actually going to use their density as a force right. So because of that they are actually going to break the layers which are being formed and as it will go further down the density will be keep increasing keep increasing right. So it will be of lower density on the top and will be on a higher density at the bottom. And as a result it is actually going to be localized at their respective density band which means this yellow one is of a very high density and this blue ones are of low density.

And as a result they will be getting localized in that particular density zone. And now what you are going to do is you are going to take out these densities by using the different types of techniques. So you can actually be able to you know do the fractionation you can actually be able to you know poke the tubes from the bottom and you can be able to collect the blue cyan and as well as the yellow bottles right. Apart from that you can also be able to use different types of methods. So there are different methods of collecting the fractions from the density gradient certifications.

One is manual collection with the help of the pipette. So what you see here this is the pipette and I am sure majority of you actually know how to run how to use the pipette. And if you are not aware of then you can actually be able to follow one of my MOOCs course which is on the experimental biotechnology where I have explained and as well as given you a demo about how you can be able to use the pipettes, how you can be able to measure the liquids properly and dispense the liquids. And this is the different types of pipettes what we have. Then you can also use the automatic fraction collector for unstable gradients.

So and you can also be able to use the freeze drying technique. In a freeze drying technique what you are going to do is you are going to take this tube where you have the different zones right and then you freeze them. And after the freezing you can actually be able to collect the slices just like as we cut the slices of ice cream brick right. If you have an ice cream brick and if you have your friends in your home what you do is you cut these brick into small pieces and then it is actually going to be taken up one slice to each friend. Same is true here. You can actually be able to make the freeze then you break and you cut the slices and then each slice you collect it into a separate tube and that you are actually going to test for the presence of the different types of organelles.

So with this I would like to conclude my lecture here. In our subsequent lecture we are going to discuss some more aspects related to the biological system. Thank you.