Genetic Engineering- Theory and Applications
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Module 1
Introduction and Basics of Biological System
Lecture 3
Cellular Structure (Part III)

Hello everybody, this is Dr. Vishal Trivedi of Department of Biosciences and Bioengineering, IIT Guwahati. And so far what we have discussed? We have discussed about the structure of prokaryotic cells as well as the structure of eukaryotic cells and also what we have discussed? We have discussed about the different organelles what are present in the eukaryotic cells and we have also discussed their functions in the cell. And today what we are going to discuss?

We are going to discuss about how you can isolate these different organelles so that you can use them for downstream applications. In case of prokaryotic cell as well as in eukaryotic cells, you have different regions which could be important for recovery of the product and in this context, let us discuss what are the regions are present in a prokaryotic cell for isolating the proteins.

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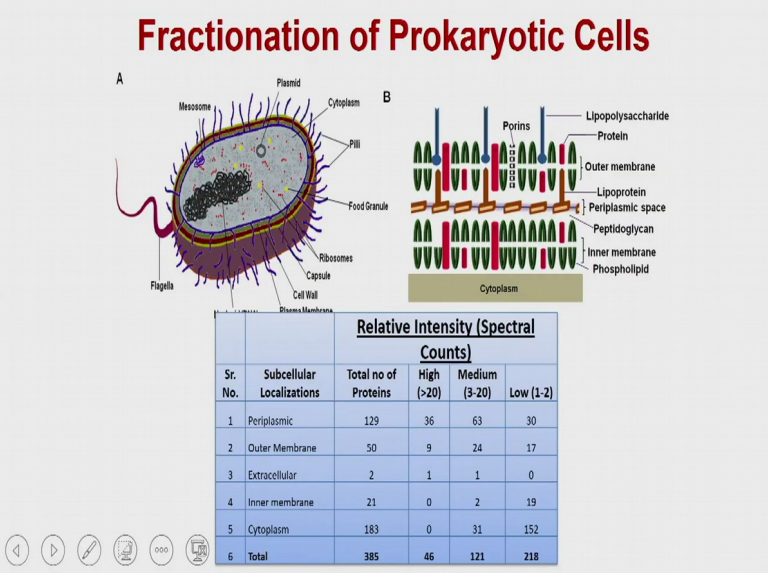

Sr. No.	Subcellular Localizations	Total no of Proteins	Relative Intensity (Spectral Counts) High (>20)	Medium (3-20)	Low (1-2)
1	Periplasmic	129	36	63	30
2	Outer Membrane	50	9	24	17
3	Extracellular	2	1	1	0
4	Inner membrane	21	0	2	19
5	Cytoplasm	183	0	31	152
6	Total	385	46	121	218

So as you can see, in a typical prokaryotic cell, what you have? You have the capsule and then below the capsule you have the cell wall and then below the cell wall, you have the plasma

membrane and inside, you have the cytosol or the cytoplasm. So in the case of prokaryotic cell, you have the 2 major places where you can actually use to extract the proteins. What are these places? You have the cytoplasm and the other place what you can use also is the periplasmic space. So what is the periplasmic space?

The periplasmic space is the space which is present in between, so between the cell wall, you have the outer membrane and then you have the inner membrane. So within the cytoplasm, you have in between there is a space and this space is called the periplasmic space. And this space can be used for storage of different types of proteins. Now you can see the relative distribution of the proteins in the different cellular structures. So what you have is in the periplasmic space, you have the 129 proteins and whereas the protein which are present in the outer membrane or the inner membrane, you have the 50 in the case of outer membrane and 7you have 21 in the case of the inner membrane.

And then the major fraction of the protein which is present in the cytoplasm that is the 183 different proteins. So this means you have the choice of overexpressing your protein or you have the choice to isolating the protein from the 2 regions, one is the cytoplasm, the other one is the periplasmic space.

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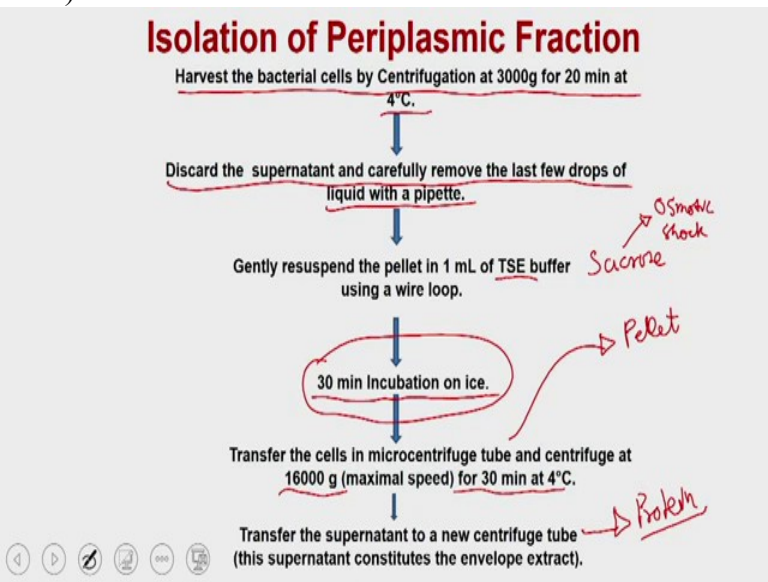

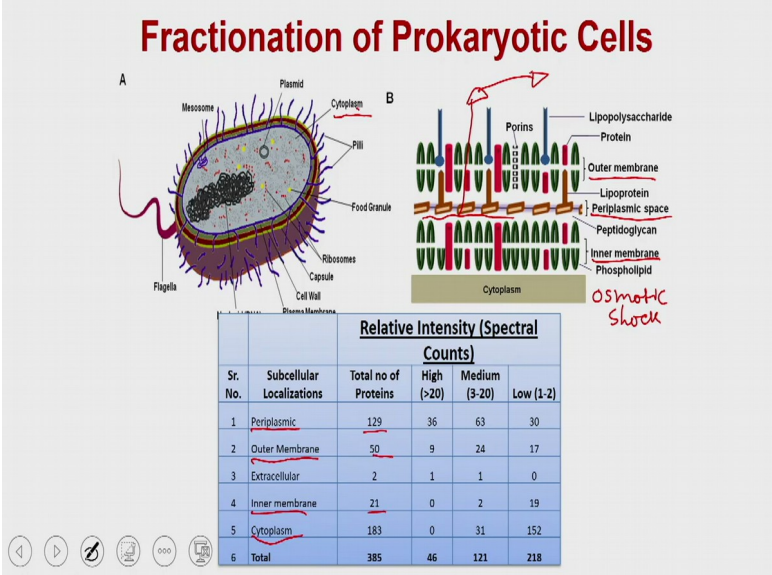

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So let us see what are steps required to isolate the periplasmic fractions. So as I said, the periplasmic fraction is present within the cell wall and in the cell wall you have the 2 layer. One is called outer membrane, the other one is called the inner membrane. So within this cell wall, there is a region which is called as the periplasmic space. So if you give some kind of osmotic shock, if you give the osmotic shock to the bacteria, what will happen is the protein which are present in this region will come off and can be used for the downstream applications.

So this can be done simply by following a similar protocol or simple protocol. In this protocol, what you have to do is, 1st you harvest the bacterial cell by centrifugation at 3000 g for 20 minutes at 4 degree. Then once you got the and then you carefully remove the media as well as the supernatant and with the help of the pipette and once you have removed the supernatant, you can gently re-suspend the pellet in 1 ml of tris cross EDTA buffer which is called as the TSE buffer using a loop or using a pipette.

And once the cells are re-suspended you incubate them for 30 minutes in ice. So this TSE buffer contains sucrose and the sucrose is going to and this also contains tris and EDTA. So the sucrose actually gives the osmotic shock to the cell and as a result what will happen is, the material which are present in the periplasmic fractions, comes out into the supernatant. Once they comes out into the supernatant in this 30 minutes, then what you do is, transfer the cell in a micro-centrifuge and then centrifuge at a very high speed, at 16,000 g.

Once you spin at 16,000 g, it will give you the supernatant as well as the pellet. So it will give to the pellet which is actually the bacterial pellet which contains the bacterial cell and the cytosol and other things. And then you are going to get the supernatant and this supernatant is going to contain the proteins which are present in the periplasmic fraction. Now for the isolation of the proteins from the cytosolic fractions, what you can do is you can take the cells and then you sonic it. And by doing the sonications, you could be able to achieve the breaking of the cells.

Once you break the cells, what will happen is the cytosolic content will become out into the supernatant and then what you do is, you do a centrifugation as it was done for the periplasmic fraction as well. So once you do the centrifugation at 16000 g for 30 minutes at 4 degree, that actually will give you a pellet as well as the supernatant just like it was being discussed for the periplasmic fractions and then the supernatant will contain the proteins from the cytosol and the pellet will contain the dead or the damaged bacterial cell which you can discard. So this is for the prokaryotic cells.

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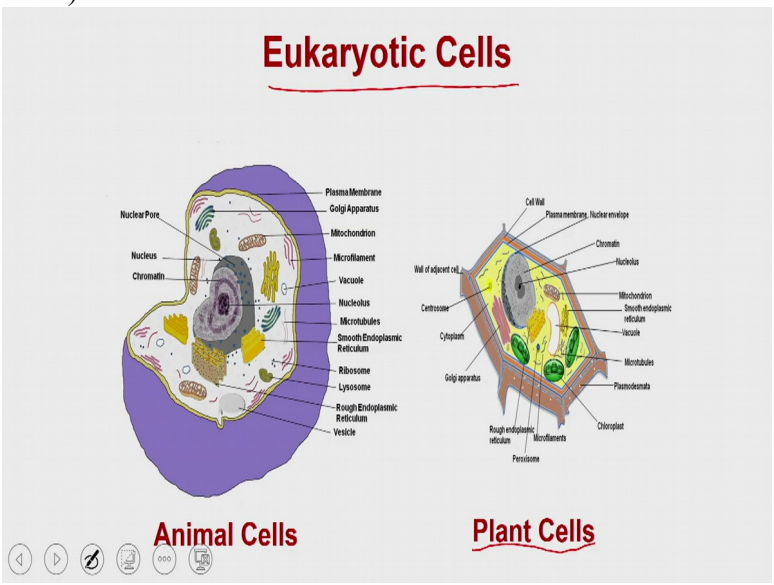

Now talking about the eukaryotic cell, as we discussed previously also, the eukaryotic cells are much more complicated compared to the eukaryotic cell and they contains well-defined membranous organelles and most of these organelles are useful for, both for the study in terms of if you want to study the function of a particular protein as well as some of the organelles are also being used for protein production. So whether it is an animal cell or a plant cell, in both the

cases, you can use some of the dedicated organelles for producing the proteins. Let us see what are these organelles.

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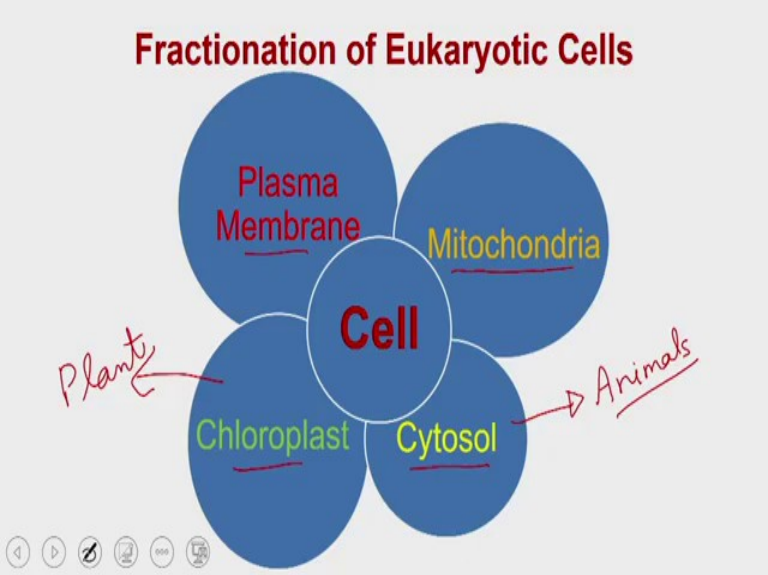

So in a typical eukaryotic cell, what you have is the cases where you can actually get the proteins in larger fractions or the organelles which are having the high importance in terms of the research as well as for product recovery, those are called as, so there are 4 or 5 place organelles which are having the very high importance as far as the eukaryotic cell is concerned. What are these organelles? One is called mitochondria, plasma membrane, chloroplast and cytosol.

So chloroplast is actually the protein production machinery as in the case of plant where as the indication of animal cell, the cytosol is the cyte for the protein production. But if you know the isolation of these organelles, you could be able to isolate either these organelles or you could be able to removes of these contaminating organelles from the cytosol and that actually will give you a very high purity in terms of extracting your specific proteins and that actually also helps in terms of the downstream processing and make the purification of these proteins simpler.

Before getting into the details of how to fraction the eukaryotic cells and how to use them, let us see what are the technical tools are available to you to achieve the organelle separation from the eukaryotic cells. So most of the fractionation of eukaryotic cell is done by the centrifugations or by separating these organelles based on the some of the basic physical properties and there are 2

physical properties which people are using. One is called density, the other one is called as the ability of these organelles or the molecular weight or the mass of these organelles.

This mass of the organelle is indirectly related to the ability of these organelles for their sedimentation in a given buffer or the given microenvironment.

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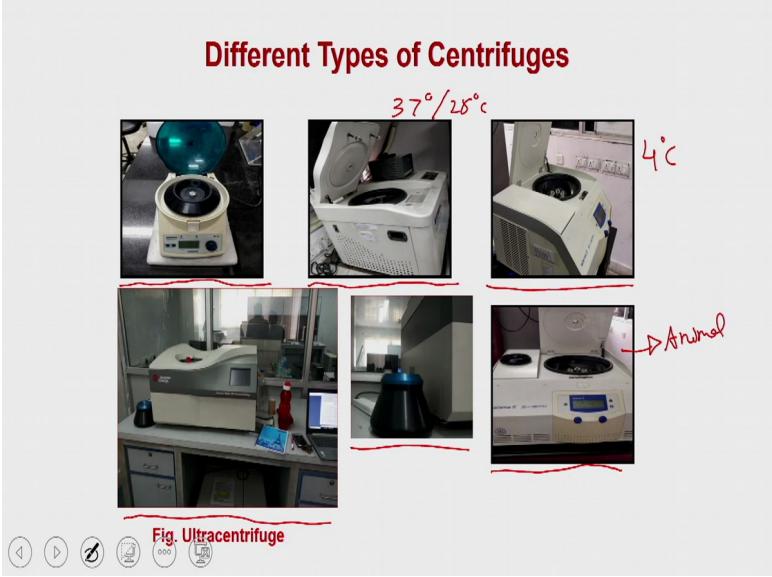

So the instrument what you can use for the extraction, for the isolation of these organelle is known as the centrifuge. So you have different varieties of centrifuges which are available for RND as well as for industrial purposes. You have the micro-centrifuge where you can use the smaller samples like the eppendorf, then you have the centrifuge with the larger capacities, the centrifuges can be used for 2 purposes. One, for pelleting down the bacteria which are being grown in the large cultures like 1 litre, 2 litres.

And then also these can be used for culturing, for removing the damaged cells from the cell lysate which remains these centrifuges can be used for clarification of your sample as well. These centrifuges are available which are either the temperature stable, means you can use them at 4 degree or you can use at room temperature, the 37 or the 25 degrees Celsius and apart from that, you also have the ultra-centrifuges. These ultra-centrifuges can be used to spin the sample at a very very high speed such as the 1 lakh g or more than 1 lakh g.

The advantage of these centrifuges are that this allow you to separate the organelles which are very very light or which are having the coefficient, this is the typical rotor what people are using for the ultracentrifuge and this is another centrifuge where you can spin the animal cells and this is been used in most of the cell culture applications where you pelletate the cells and you do the soft culturing of these cells using the these kind of cell culture, using these cell culture specific centrifuges. Apart from the centrifuge machines, you have the freedom of doing 2 different types of centrifugation.

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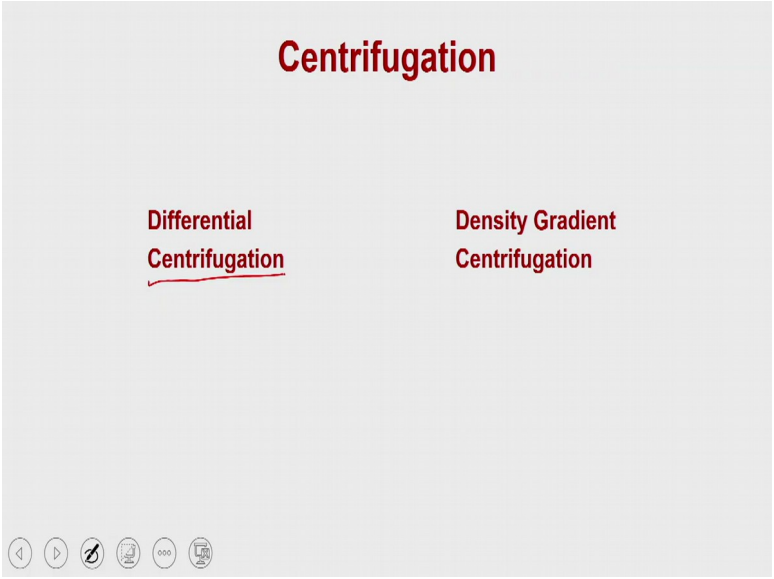

You have the differential centrifugations or the density gradient centrifugations. In the differential centrifugations what you do is, you run the sample at different speeds or different RCF values and because of that, when you spend down, the molecule which is corresponding to that RCF value get pelleted down and you will find that particular organelle or the substance in the pellet fraction whereas the supernatant will contain all other molecules.

Once you take the supernatant, you can spin again at the slightly more high-speed and that is how you can be able to separate the molecules based on their sedimentation coefficient or the sedimentation rates. Whereas in the case of density gradients centrifugations, what you do is to exploit the inability of or you exploit the density of a particular solvent system or the buffer actually.

So what to do is, you vary the density of the external buffer and because of that, what will happen is, the biomolecules are getting, biomolecules or the organelles are getting separated because they holds the varying density and whenever they matches to their corresponding density, they form a band or the zone and that is how you can have the, you can develop the different zones within the density gradient centrifugations and every zone you can actually isolate later on and can be used or can be represented by a particular organelle. So let us discuss these 2 techniques how these 2 techniques can be used to separate the different organelles.

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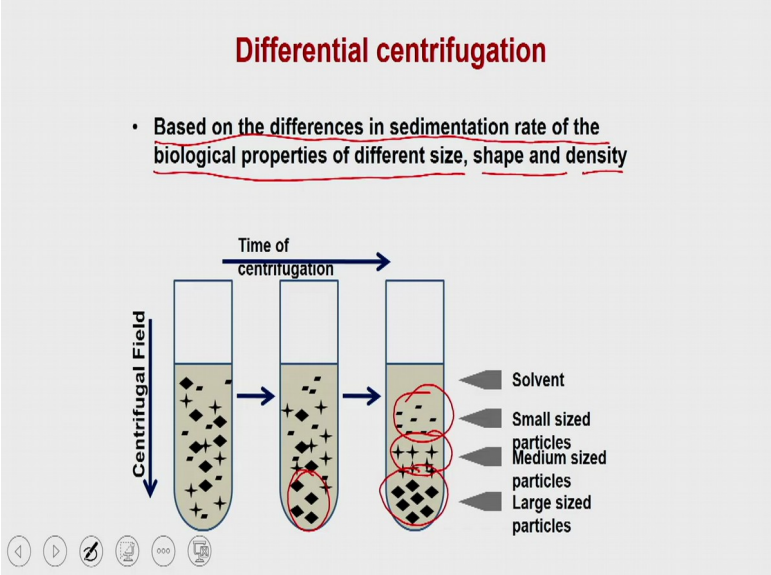

So as I said, in the differential centrifugation, differential centrifugation is based on the differences in the sedimentation rate of the biological properties of a different sizes, shape and the density. You can imagine a situation where you have the particles of different sizes, shape as well as the density and when you do the differential centrifugation, in one round of centrifugation, these particles are going to settle down in the pellet fraction. Whereas the all the remaining sample will be present in the supernatant.

And the subsequent fractions when you do this supernatant, you will get this fraction in the palette and the 3rd time, you will get this fraction in the palette and that is why it is called as the differential centrifugation because in one round of centrifugation, you have isolated these molecule, in the 2nd round, you have isolated these molecules and the 3rd round, you have

separated these 3 molecules and that is how you can actually separate if the samples are suspended in a particular solvent system, the small size, medium-sized and large size particles.

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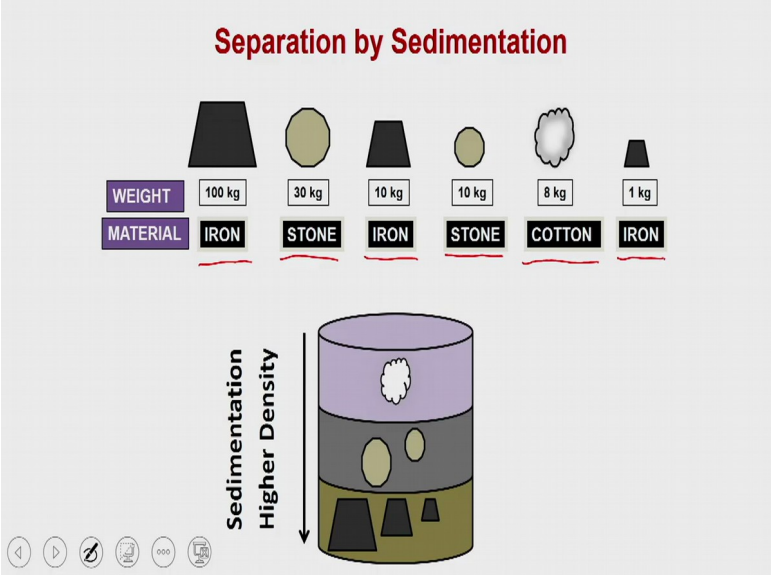

Let us take an example. Suppose you have the different substances and the different material of different sizes, different density. For example, you have a iron block of 100 kg, you have a stone of 30 kg, you have the another iron block of 10 kg, you have a stone of 10 kg, then you have cotton of 8 kg and you have another iron block which is of 1 kg. So you know that this cotton is going to have the lower density or it is going to be the lowest density compared to all other samples.

Next to that is the stone which is going to be slightly higher density to the cotton. Then you have the iron which is actually going to be the highest density. So if you do this differential centrifugation and what you will see is that the 100 kg block is going to be settled down 1st followed by the 10 kg iron block and the followed by the 1 kg block okay. Onto that, if you further doing that, then you will see is that 30 kg stone block will be pelleted, next to that you are going to have the pelleting of 10 KG.

At the end what you are going to see is that the 8 KG cotton palette is going to be settled down which means it actually not only depends on the molecular weight or the density, it depends on the both. It depends on the size, it depends on the density and it depends on the actual weight of that particular object. So you can actually exploit these properties.

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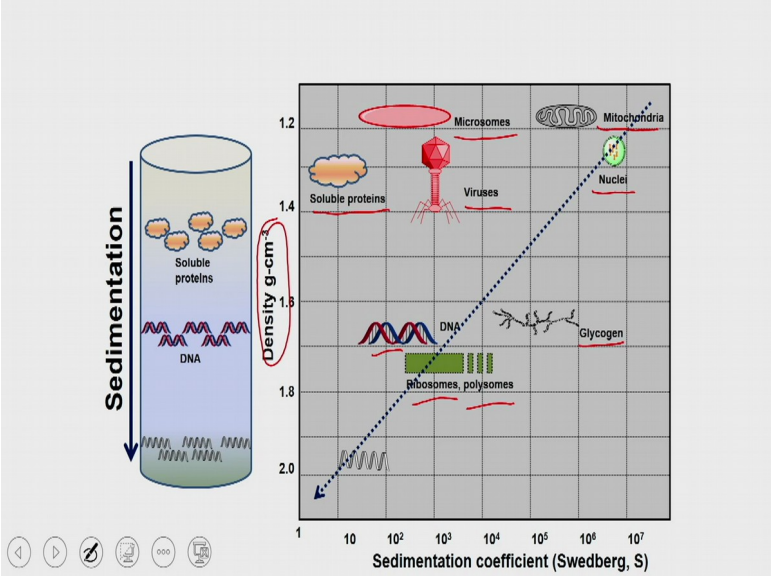

Now you can see another example where we have the different types of biological molecules. You have the microsomes, you have the mitochondria, you have nuclei, you have viruses, you have soluble proteins, you have DNA, you have ribosomes, you have polysomes, glycogen which is another biopolymer and what you can see is that all these molecules have the different sedimentation coefficient, different density.

So if you spin down them, what you will see is all these biomolecules will separate to be tethered because they will settle down at different steps. So 1st round, this molecule is going to be settled down. In the 2nd round, the DNA will be settled down and ultimately in the 3rd round, because the soluble protein is going to be settled down.

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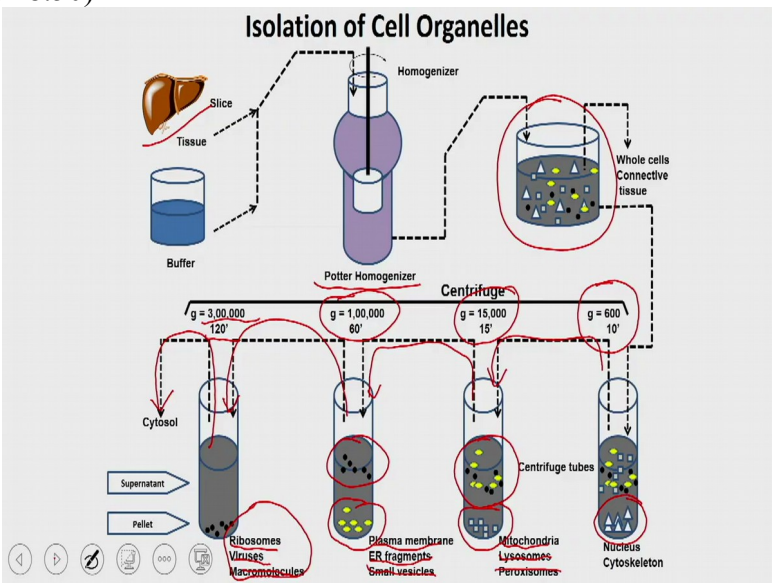

Let us take a real example. So when the real example, suppose we are isolating the different organelles from the liver and what you can see is, you take a liver from a animal. Let us 1st step, you do the homogenisation. That will give you the different cells and once you do the homogenisation, that will break down the organ and it will give you the individual cells.

If you do the further homogenisation, the individual cells will be broken down and then they will give you a combination of organelles which will be present in your vessel and these different organelles can be separated because what you do is 1st you will spend down at a very very low speed, that is the 600 g for 10 minutes and that actually is going to give you only for the nucleus or the heavy particles. So that will settle down the nucleus in the pellet fraction and all other molecules will be in the supernatant.

Once you take that supernatant and put it into another round of centrifugation and then you centrifuge at 15000 g for 15 minutes at 4 degree, then it will pellet down the another heavy particles. These are called mitochondria, lysosomes and peroxisomes, whereas the remaining organelles will be present in the supernatant.

Once you take these molecules which are present in the supernatant and do another round of centrifugation at 1 lakh g which you will do in ultracentrifuge for one hour, what you will see is now it has pelleted down the plasma membrane, ER fragments and the smaller vesicle such as the, which are called the light membranes and now if you take the supernatant and do another

round of centrifugation and now you do a centrifugation at 3 lakh g for 2 hours which also you were going to do in the ultracentrifuge, what you are going to get?

You are going to get the very very small particles like ribosomes, you are going to get the viruses and you are going to get the free proteins. And if you take this supernatant, you are going to get the cytosol. This cytosol will contains the proteins as well as the salt and all other, the liquid material which is present in this typical cell.

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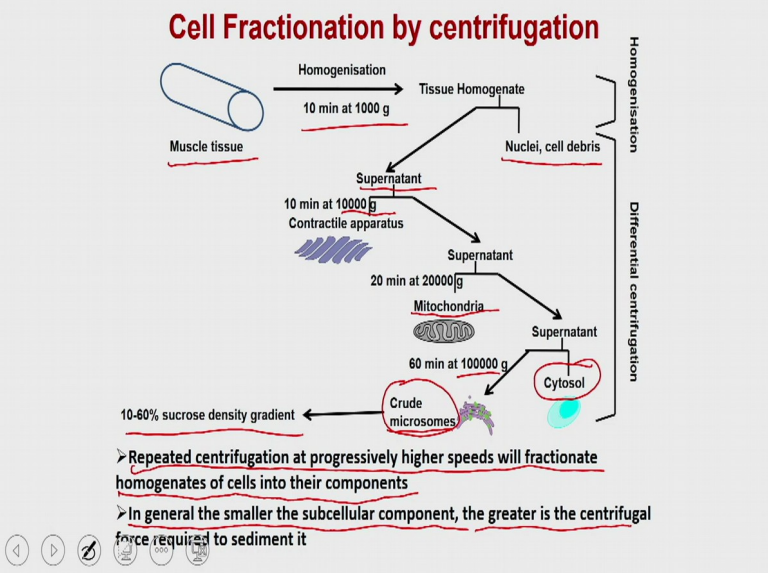

Now take another example. Suppose you started with the muscle tissue, exactly the same way, you are going to do homogenisation, then if you do a centrifugation for 1000 g for 10 minutes, in 1st lot you are going to get the nuclei as well as the cellular debris. Once you take the supernatant and do another round of centrifugation at 10,000 g for 10 minutes, that actually will give you the mitochondrial fraction and the supernatant if you spin down at 1 lakh g, that will give you the microsomes as well as the cytosol.

And if you take these crude microsomes and run it for the density gradient, you could be able to even isolate or further divide or purify many material from these microsomal fragments. So what is, what we are doing actually is that we are doing a repeated centrifugation at progressively high-speed, will fascinate a very very complex homogenates of cell into their individual components which means the individual organelles and in general the smaller the organelle, the greater is the centrifugation force is required to sediment it which means if you are working with

a lighter and lighter particles, just like as we have taken an example of the iron and stone and cotton, you might have seen that we have supposed, the cotton got pelleted at the end because cotton is having the very very low density compared to the stone as well as the iron.

So the light of the organelles, the larger will be your centrifugation speed. Because of this limitation, you can also exploit the densities of these particular organelles or different molecules which are present inside the cell.

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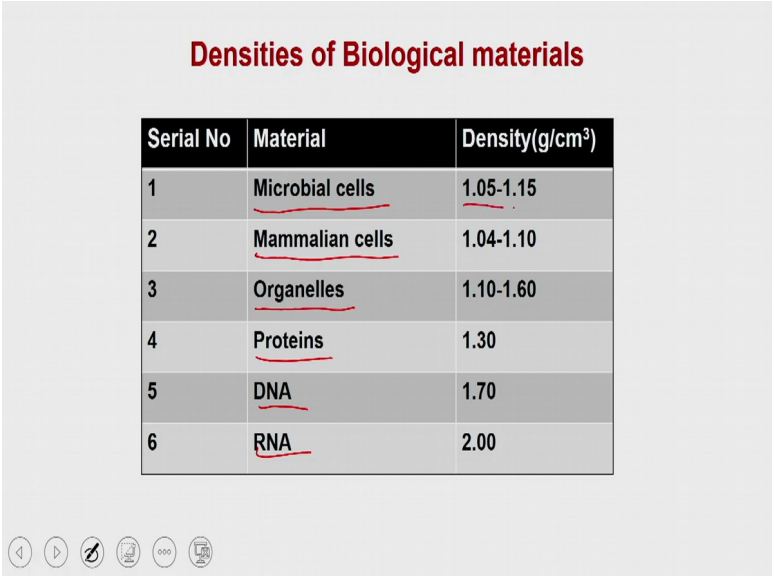

So in a typical different types of cell which are present in the biological system, you have the microbial cell or the prokaryotic cells, you have the mammalian cells, you have the organelles which are present in the mammalian cells, then you have the proteins, DNA and RNA. And as you can see, all these molecules have the very very different densities. For example in the prokaryotic it is a density between 1 to 1.15. Whereas the mammalian cells are slightly different in density from the prokaryotic cells.

Similarly, the organelles are very much different from the whole cell and they are in the range of 1.1 to 1.6. Whereas the proteins, DNA and RNA are also having the different density which means, since these biological samples are having the different densities, you can also use this particular property as well to exploit and to separate them in a density gradient centrifugation.

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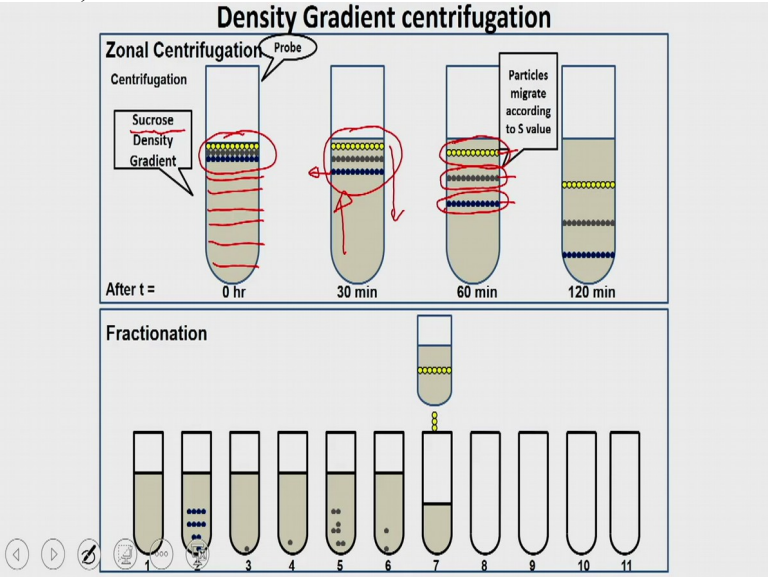

So in a density gradient centrifugation, what you are supposed to do is you make a density gradient media. What is meant by density gradient media? For example, in this case we have taken a sucrose as a material. So what you do is, you put the sucrose, different concentration of sucrose starting from 0 percent to 30 percent and as a result what will happen is that all this, this media is going to be separated in different zones of different densities according to the concentration of sucrose present in each layer and then what you do is, you overlay your complex solution which is going to be separated.

Now you can see that I have used the 3 different types of beads. One is blue, another one is green, another one is cyan and what these beads and then you do a centrifugation. Once you do the centrifugation, these beads will move towards the liquid and because this liquid is containing a media with the different densities, it will actually going to create a fiction or it will oppose the entry of these molecules and the place where these molecules will stop is the place where the opposing forces as well as the centrifugation forces are going to be equalised and that place is actually the place where the density of the media is going to match with your sample.

For example, in this case the blue got matched here, the green got matched here and the yellow got matched here. So it means, if you have the sample with the variable densities, you can be able to separate simply by centrifugation of these samples in the density gradient. And as you run it, you could be able to make the better separation between the molecules.

And once the separation is over, you could be able to isolate the individual components from this particular tube and you can take out the individual bands from these tubes and you can be able to purify these individual bands into the individual fractions and that actually individual fraction is going to give you the pure organelles or the pure material from the cell. How to collect this material?

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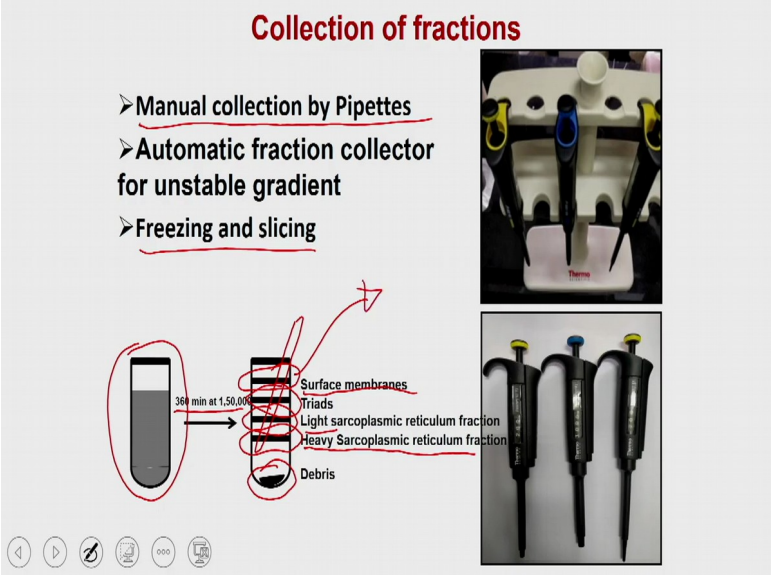

You have the 2 options. So you can imagine, we have started like this and we run it for 1,50,000 for 3 hours and that has given you the different bands corresponding to the different types of fractions. For example, in this case this fraction belongs to the Sarcoplasmic reticulum fractions, then this fraction belongs to the light sarcoplasmic fractions, this fraction belongs to the triads and this fraction belongs to the plasma membranes or the surface membrane.

Now what you earn at the bottom, what you have is the cellular debris. So what you have, you can actually use the different way of collecting these fractions. One of the way you can do is you can manually put the pipettes. So these are the typical pipettes which you can use. As you can see, these are the different pipettes and what you can do is, you can put a pipette tip in the media and you can easily suck your band of interest.

The other way is that you freeze these samples and then to cut this tube in different sizes and then individual slice, you can take out and then isolate the material which is present in that particular individual band. So this is what we have discussed so far.

What we have discussed? We have discussed about the presence of different organelles in the case of eukaryotic cell and we have also discussed about the different regions which are present in the prokaryotic samples, we have also discussed what are the instruments which are available for you to isolate these organelles from the eukaryotic as well as the prokaryotic cells and then very briefly, we have also discussed the how to isolate the periplasmic fraction or cytosolic fraction from the prokaryotes.

And in detail we have discussed the different approaches what you can use to isolate the organelles from the eukaryotic cells, either you use the different centrifugations or the density gradient certifications. Thank you.