**Fundamentals of Protein Chemistry**
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**Module - 04**
**Protein isolation and characterization**
**Lecture - 16**
**Protein Isolation Methods**

We start module 4 that is going to be dealing with protein isolation and characterization methods. Initially in the first two lectures we will look at protein isolation including chromatographic methods, followed by three lectures in this module on biophysical methods that are used for protein characterization and for many other studies in protein chemistry.

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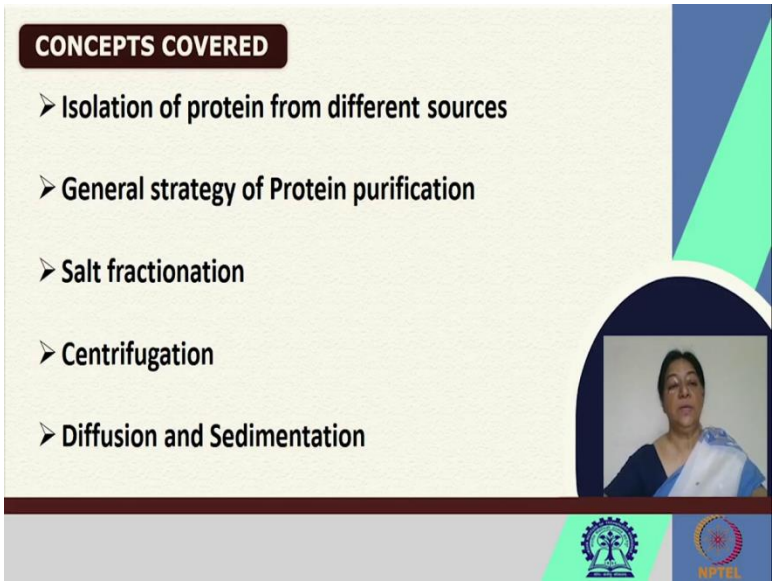


Our slides will continue with an isolation of protein from different sources. A general strategy of protein purification, salt fractionation, centrifugation, diffusion and sedimentation.

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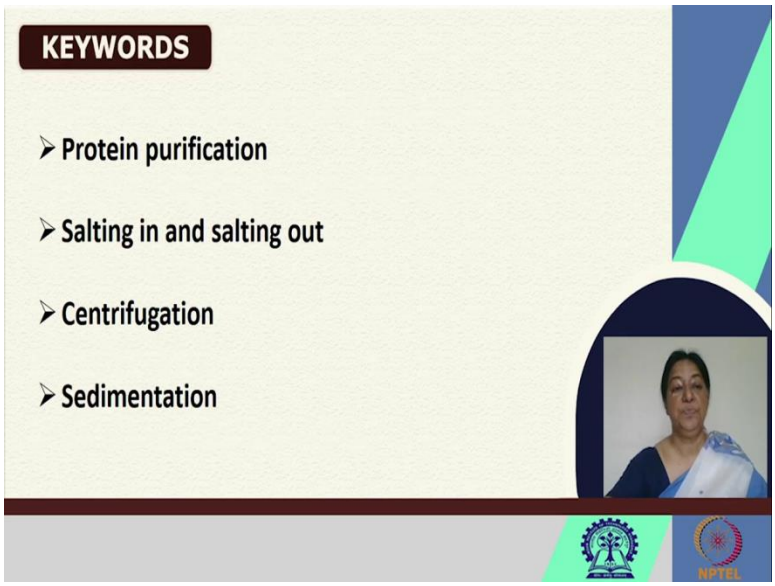


To understand what we mean by protein purification there are certain terminologies that we need to understand.

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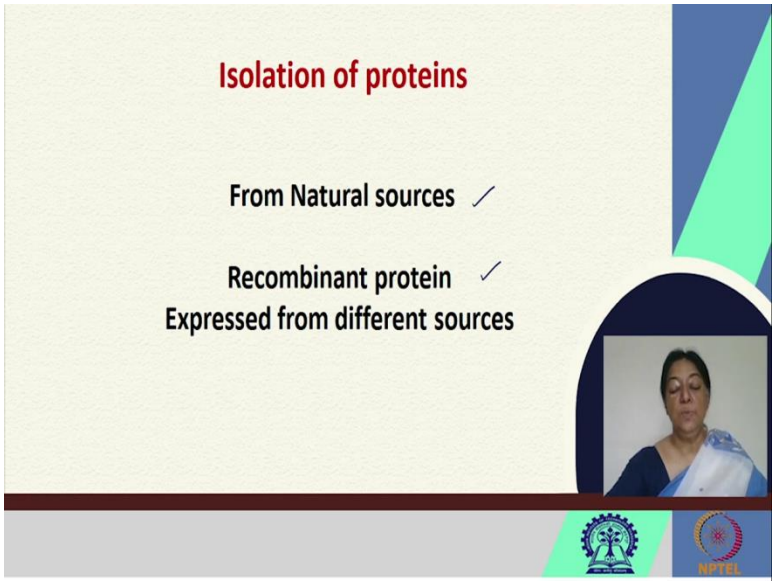


The first course of action in isolation of proteins, is the source from which the isolation is going to take place. So whether we are isolating it from natural sources or from recombinant protein sources, we would recombinant DNA as the technique is called protein expression. We will see what the basic methodologies imply.

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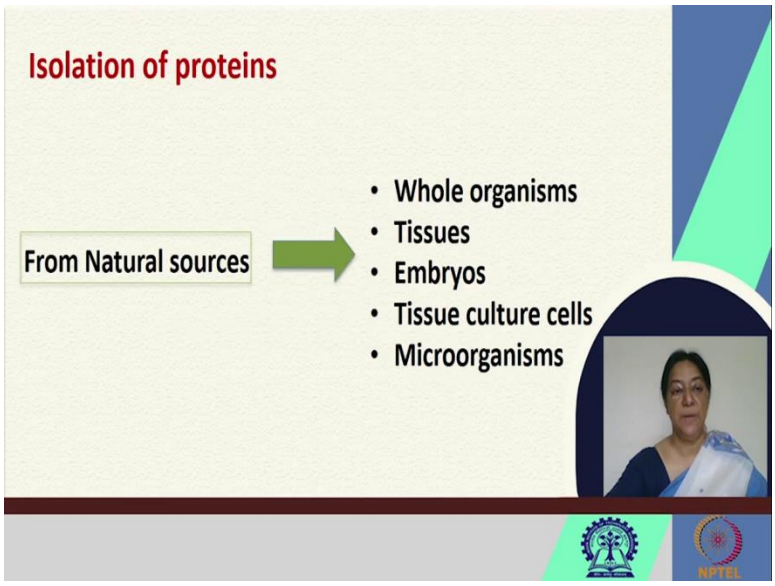


If we look at natural sources we can be isolating proteins from whole organisms, tissues, embryos, tissue culture cells or even microorganisms.

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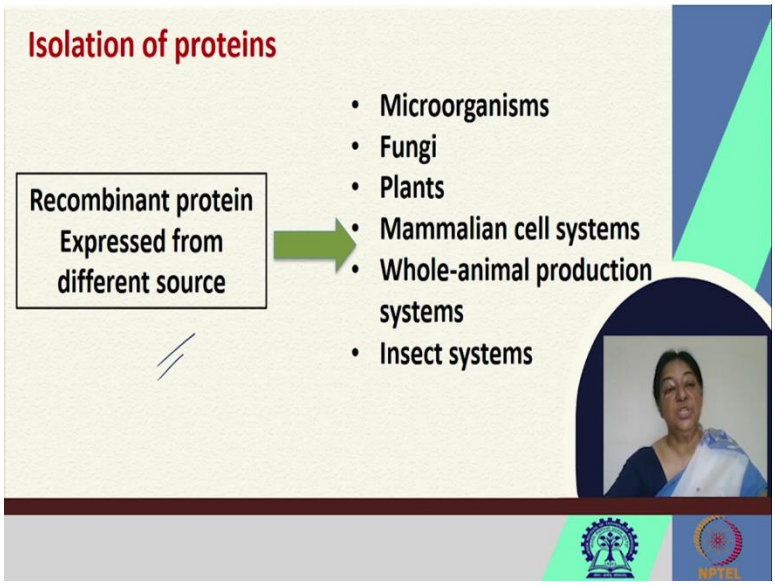


With it and with recombinant protein we have again the microorganisms, fungi, plants, specific cell systems and insect systems, in addition to whole animal production systems.

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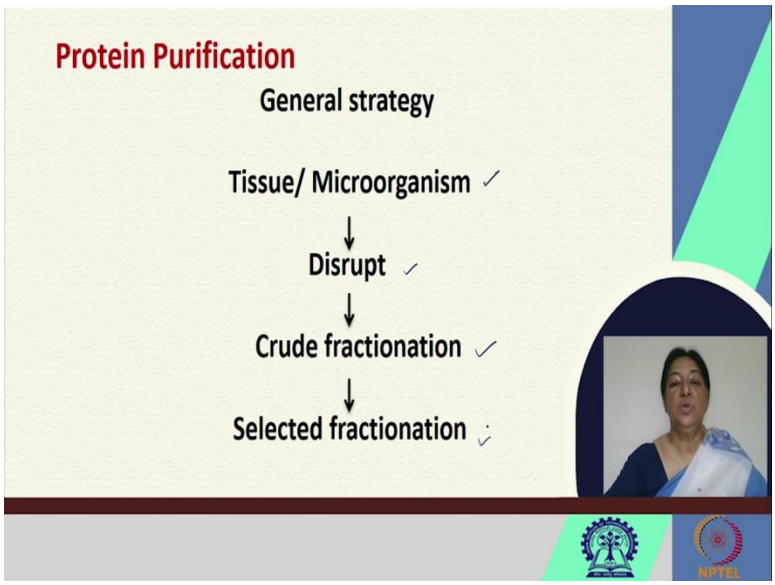


But the general strategy for protein purification means, we have a tissue or a microorganism, there is a disrupt so that we get the smaller cell fractions or the smaller fractions from whereby we can isolate the macromolecule of choice. We go for a crude fractionation followed by selected fractionation to get our protein of interest.

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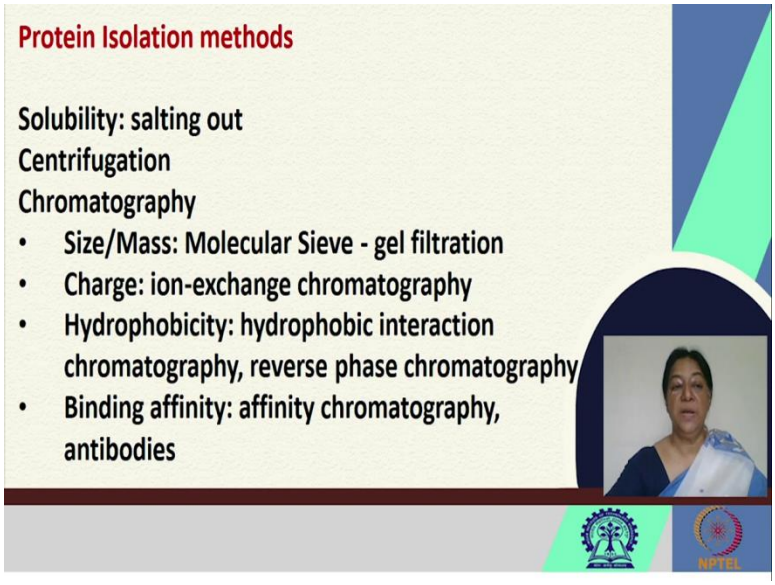


In protein isolation methods there is salting out, centrifugation, chromatography. In chromatography there are different types that we will be doing in the subsequent lecture, where we will be looking at separation based on gel filtration, based on charge, based on hydrophobicity and based on binding affinity.

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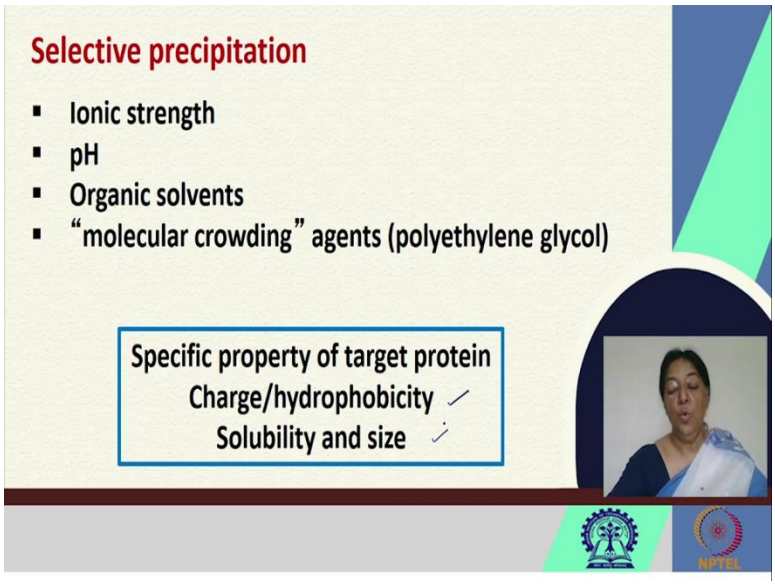


When we looked at selective precipitation, we have to remember that while we are doing this precipitation or while we are isolating or purifying our protein, we are exploiting certain properties of the protein. So, whether it be charge or hydrophobicity or the solubility or the size of the protein, we are exploiting a specific property to get to our protein of interest.

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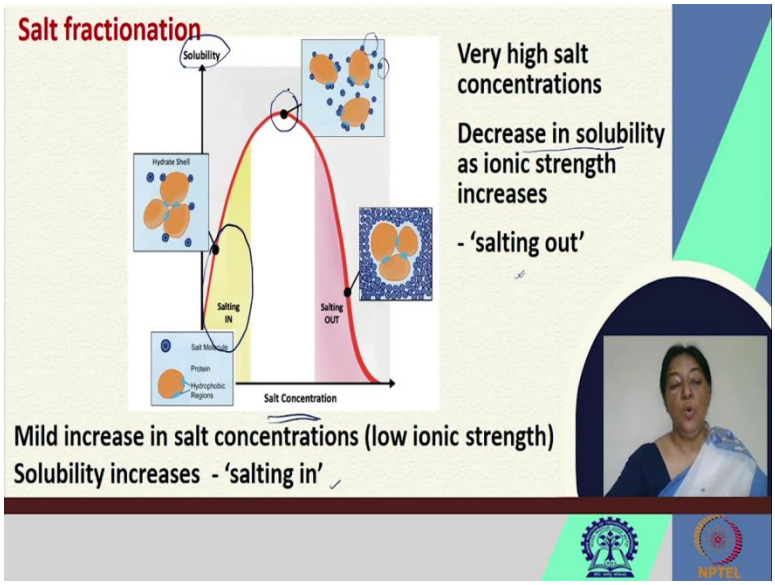


In salt fractionation, [refer to slide] as we can see from this curve, the solubility versus the salt concentration has been plotted. We see that at low concentration what we call salting in, results from a mild increase in the salt concentration. That means low ionic strength, from which we have an increase in solubility as we increase the salt concentration. However, at higher concentrations we have a decrease in solubility, as the ionic strength increases, resulting in what is called a salting out procedure.

This salting out means that we can have a precipitation of our salt because of the increase in ionic strength. The increase in ionic strength results in this disruption and we have the precipitation of our protein, because the additional salt molecules that we have in here will be preferably dissolving in water. We will see what that means in a moment. But we have an optimum salt concentration where we will have the highest solubility.

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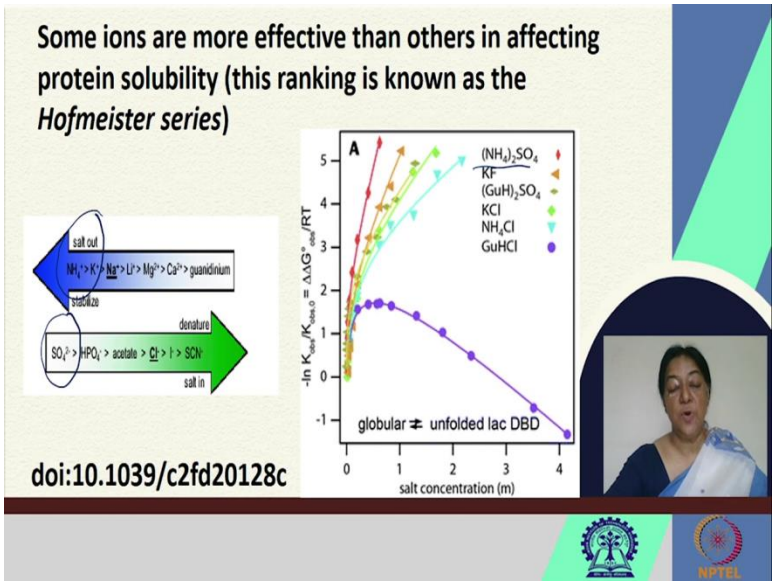


In the salting out method there are some ions that are more effective than others in bringing about this protein solubility difference. When we think about the salting out, we see that ammonium and sulfate are the most effective in salting out a protein, which is very commonly used for the salting out of proteins from a mixture of proteins.

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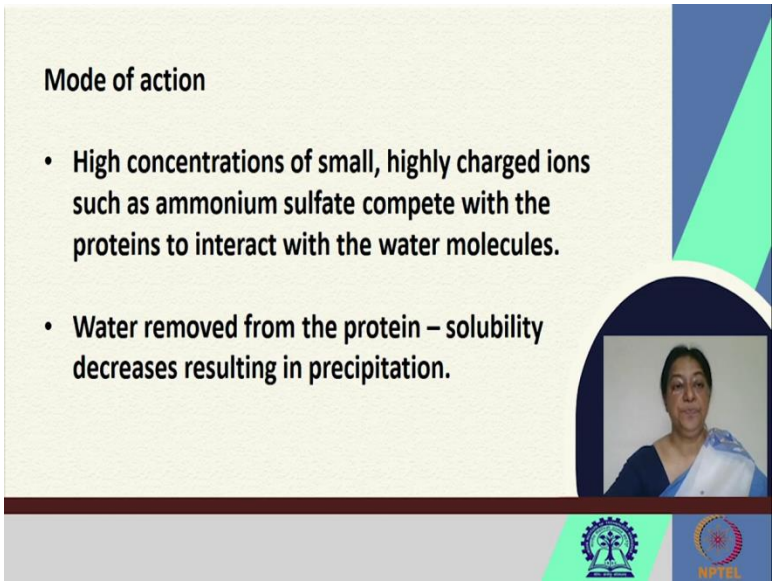

The mode of action is such that the high concentrations of small highly charged ions, for example as ammonium sulfate, they compete with the proteins to interact with the water molecules because of their higher solubility. As a result water is removed from the protein resulting in a reduction in the solubility, which in turn leads to precipitation.

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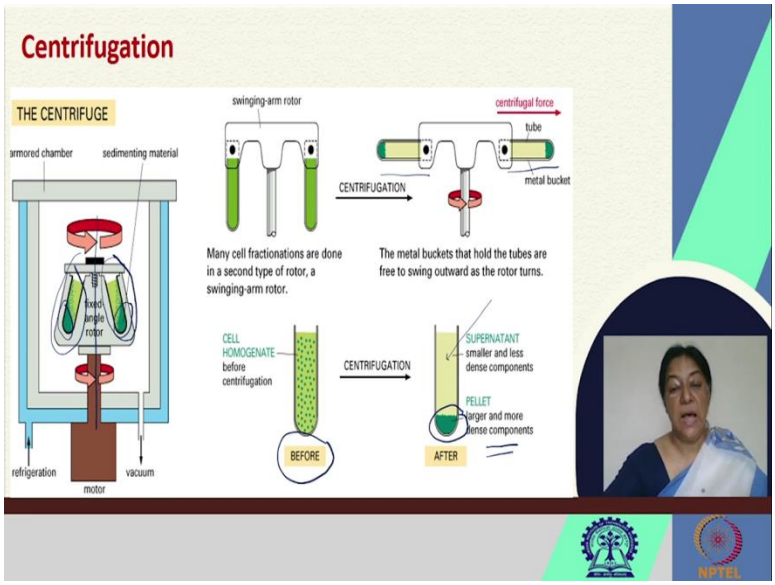


Once we have our particular system, we can then go for other methodologies to isolate our protein of interest. A common method is the use of a centrifuge. On the left hand side [refer to slide] we see a fixed angle rotor, where the test tube is fixed in the specific angle with respect to the axis of motion. We see the pellet here, where we have the sedimenting material and on top we have the supernatant, so we can pour out the supernatant.

Some centrifugation machines centrifuges have a swinging arm rotor, where on rotation the metal buckets that hold the tubes swing out due to the centrifugal force. As a result of which what we have; if we have a cell homogenate (a homogeneous solution) before centrifugation; we have a pellet that contains the larger and the denser components at the bottom of the test tube and the supernatant, with the smaller and less dense components at the top.

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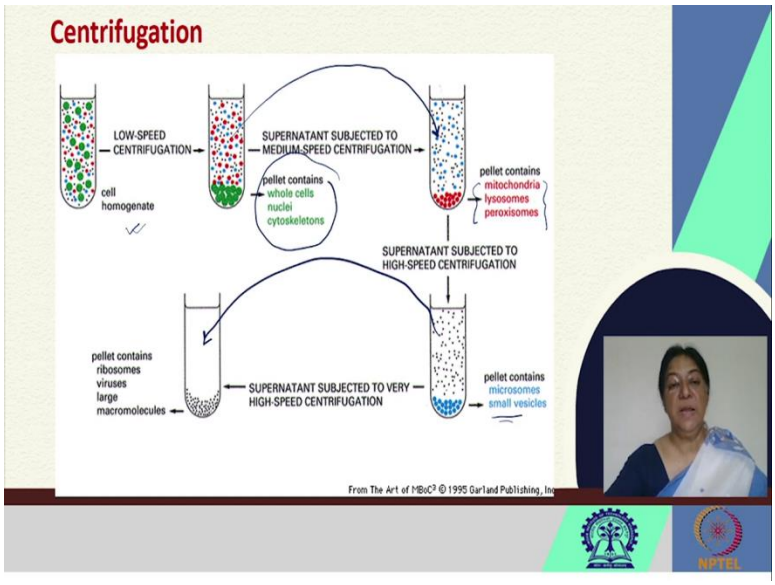


Now what is usually done is, we have initially low speed centrifugation, so that from the cell homogenate the pellet containing whole cells, nuclei and cytoskeletons can be removed. And from the supernatant this is taken for another centrifugation process at medium speed, where in this case the pellet will contain the mitochondria, the lysosome and the peroxisomes. So, depending upon our system of interest, we can go to selective centrifugation and collect the material of interest.

If we go to another high speed centrifugation, we get the pellet which have microsomes and small vesicles, this supernatant subjected to even higher speeds can give us large macromolecules and viruses. Again we choose the supernatant or the pellet depending upon our interest.

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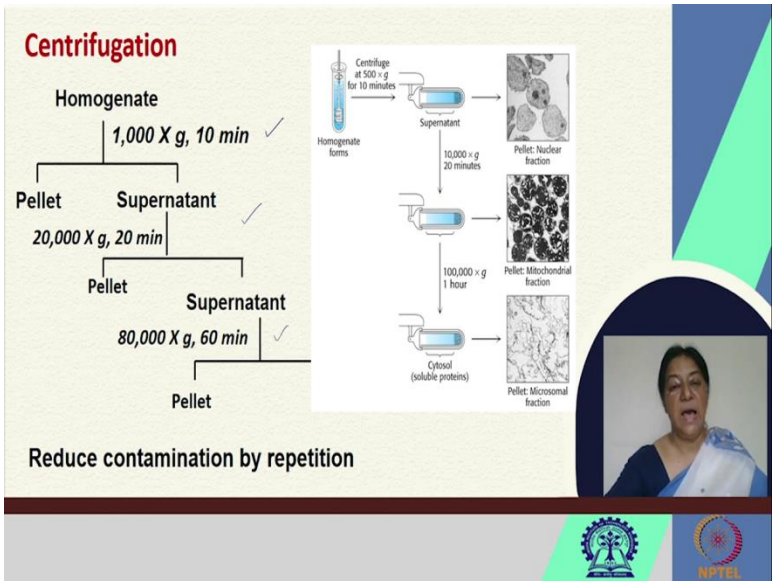

So the method is that we have a homogenate, low speed, medium speed, high speed to get to our specific compound or specific molecule of interest. The contamination is also reduced by repeating the centrifugation processes at the different speeds.

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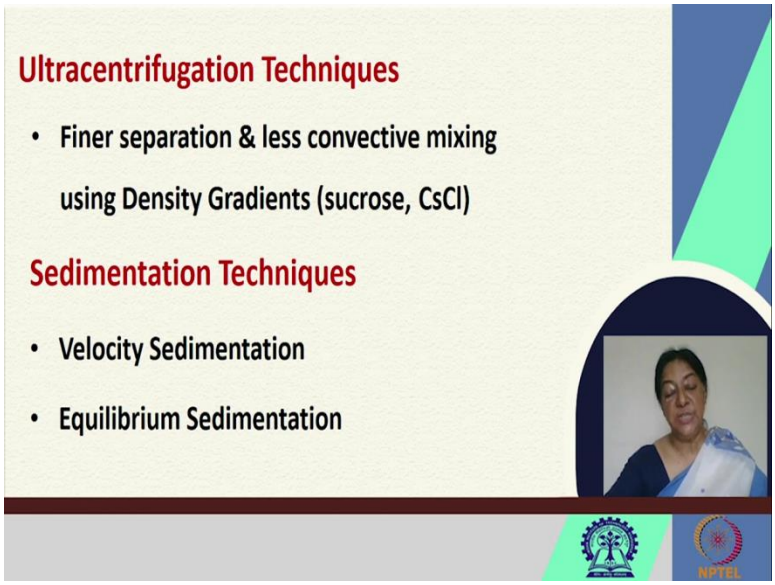


We can go for ultracentrifugation techniques that results in finer separation and less mixing of the pellets and the supernatants. And sedimentation techniques which involve velocity sedimentation and equilibrium sedimentation.

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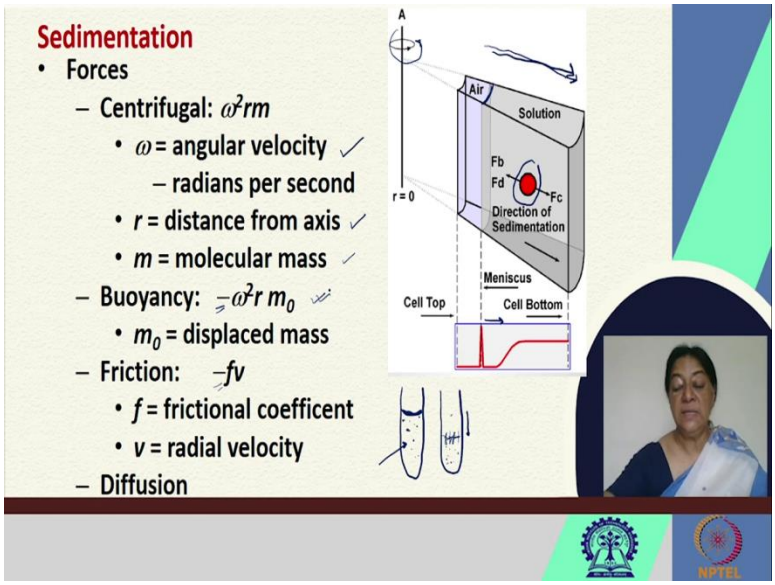

In the sedimentation technique what we look at is a motion. As rotation takes place around the axis, the solution that is present has a meniscus at the top of the tube. We would have a meniscus of the so called homogeneous solution that we start off with.

Now as the centrifugation proceeds, we have a clearer solution. On top the meniscus is here, but the pellets or the sediments begin to form here. So we have a meniscus that gradually moves. This is called a moving meniscus method and this is our direction of sedimentation.

As we move along this, there are several forces acting on our specific molecule. There is the centrifugal force. The centrifugal force, that is given by the angular velocity in radians per second, the distance from the axis and the molecular mass which is important depending upon our system of interest.

Followed by this we have two opposing forces. One is the buoyant force of the molecule that looks at the displaced mass and is negative. And the viscous force, that is due to the friction of the liquid that is present, which is also acting against the centrifugal force. However, these forces balance each other. The molecules settle down and the diffusion possibility between the pellet or the sedimenting material and the supernatant takes place.

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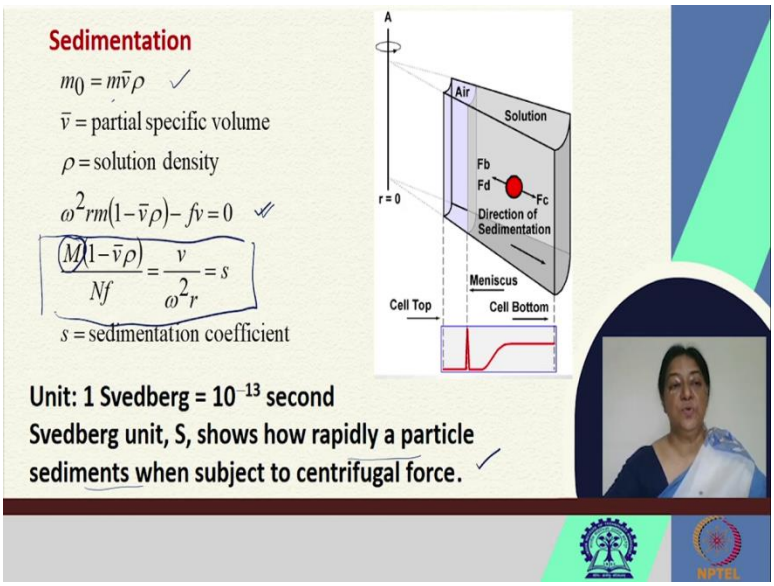


If we look at the sedimentation process of the moving meniscus, we see the specific forces. In the previous slide, we saw the buoyancy the specific displaced mass that we are looking at, which can be replaced by the partial specific volume and the solution density with the product of the mass, the partial specific volume given in cc per gram and the density.

And the balance of the forces gives us this equation here [refer to slide]; that is important in finding out the molecular weight of our protein of interest. This is given by units of Svedberg; that are given by $10^{-13}$ second.

In our experiment, the Svedberg unit, shows how rapidly a particle sediments when it is subject to this centrifugal force. What we see is we see a balance, a centrifugal force acting in the direction of sedimentation and the buoyant force and a friction, a viscous force that acts against the force; against the centrifugal force.

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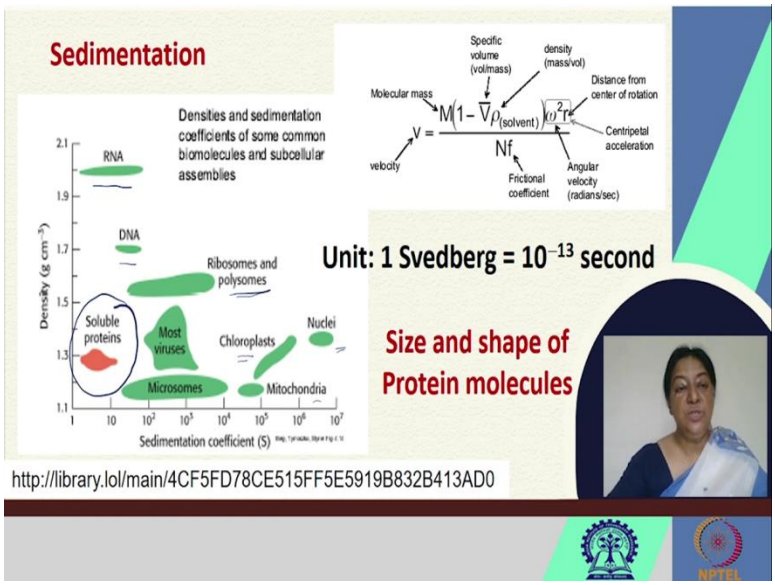


So, in this sedimentation method what we do have is, we have the molecular mass, we have the velocity, the specific volume; as we saw from the different aspects that we looked at. In the densities and the sedimentation coefficients of some common biomolecules.

We see that we have soluble proteins around this region giving us a low density and a relatively low sedimentation coefficient. We have RNA, DNA, larger and larger organelles, chloroplasts, nuclei, mitochondria, all in this specific graph that gives us the densities and the sedimentation coefficients of common biomolecules and subcellular assemblies. From here, we get an identification or an indication of the size and the shape of the protein molecules.

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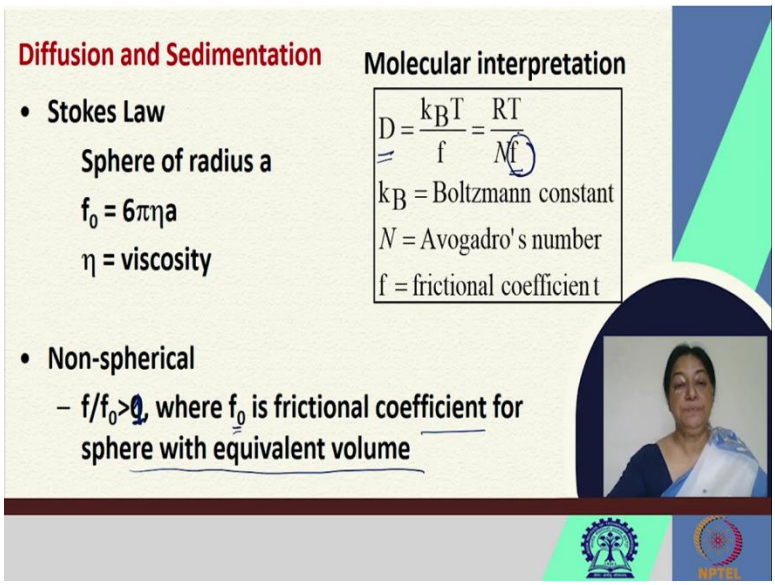


This is also given by our diffusion and sedimentation process, where in Stokes law we look at the viscous force that is acting on the liquid; we have $f_0 = 6\pi\eta a$, where $\eta$ is the viscosity and a is the the radius of the sphere, considering that the molecule is a sphere.

However, we can have non-spherical molecules as well. In this case we will have a frictional coefficient $f/f_0 > 1$, where the $f_0$ is a frictional coefficient for sphere with equivalent volume. The molecular interpretation here is given by $D = k_B T$ where we have $RT = Nf$; that gives us a value of the diffusion coefficient giving us the frictional coefficient.

This value should be 1 here [refer to slide] where we are indicating f by $f_0$, where $f_0$ is the frictional coefficient for a sphere with equivalent volume and f is given by the diffusion coefficient and the frictional coefficient that we have here.

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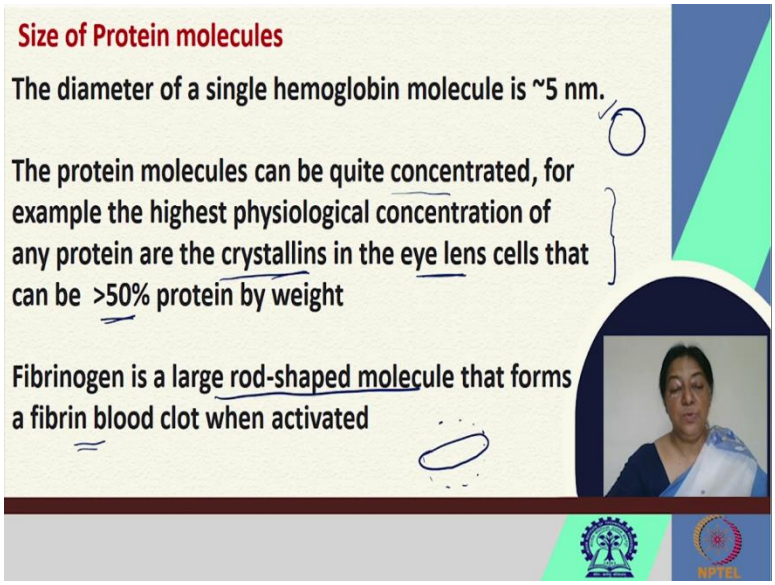

When we look at the size of the protein molecules, the general size of a protein molecule, say the diameter of a single hemoglobin molecule is around 5nm.

If we look now at the concentration of the protein molecules, to say how much of the protein molecules are actually present, the highest concentration that we see is the crystalline proteins in the eye lens cells. There we have over 50% protein by weight.

So, we can have a diameter of a single hemoglobin molecule that we can consider roughly globular in shape. The protein molecules can be concentrated and the concentrations that we can see are the highest for the crystalline proteins that are present in the eye lens and this can be >50% protein by weight.

Another possibility is looking at cells where we have rod shaped molecules. For example like fibrinogen. So, in this case we look at the sphere of a similar radius for calculating the frictional coefficient.

The size and the shape of the molecules is important in an understanding of how this molecule is going to sediment, as well as how this molecule can be separated, which we will see later.

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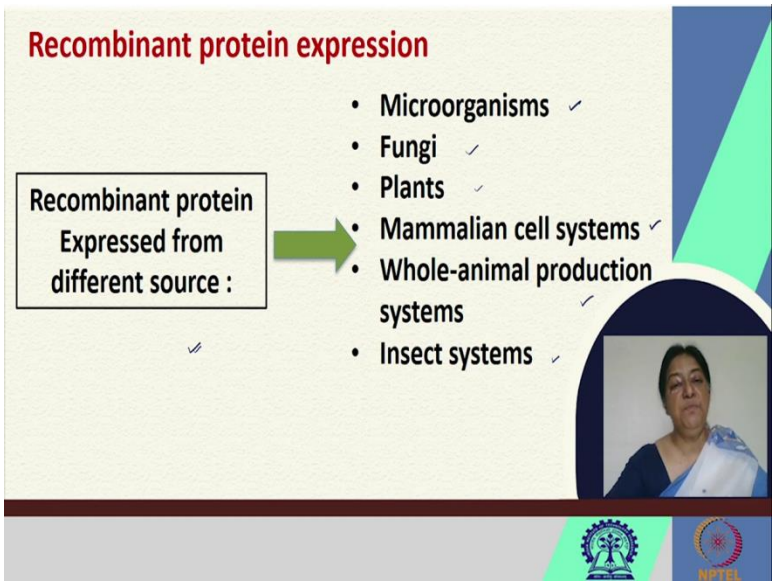


In recombinant protein expressions, an interesting point is, that we try to exploit our bacterial machinery to express the protein of interest. When we are looking at protein recombinant protein that is expressed from a different source, say we are looking at microorganisms, fungi plants, mammalian cell systems, whole animal production systems or even insect systems, then what happens is we can look at the ways in which we can actually bring about the protein expression.

We will see briefly how this is done.

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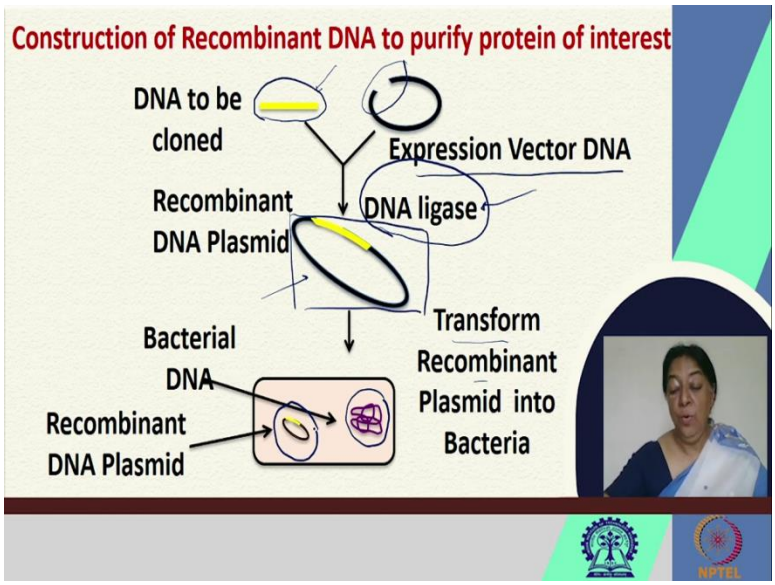


In our expression here [refer to slide] we have the DNA that is to be cloned. This is our DNA that is to be cloned and this is our expression vector DNA. Our expression vector DNA then is used to create what is called a recombinant DNA plasmid. How is this done? This is done by the use of an enzyme called DNA ligase.

We will see what this ligase means when we come to our topics related to enzymes. This is our DNA to be cloned, this is our expression vector where we have linked our DNA, using DNA ligase, to create a recombinant DNA plasmid. This is then transformed into bacteria. So here is bacterial DNA and here is the recombinant DNA plasmid.

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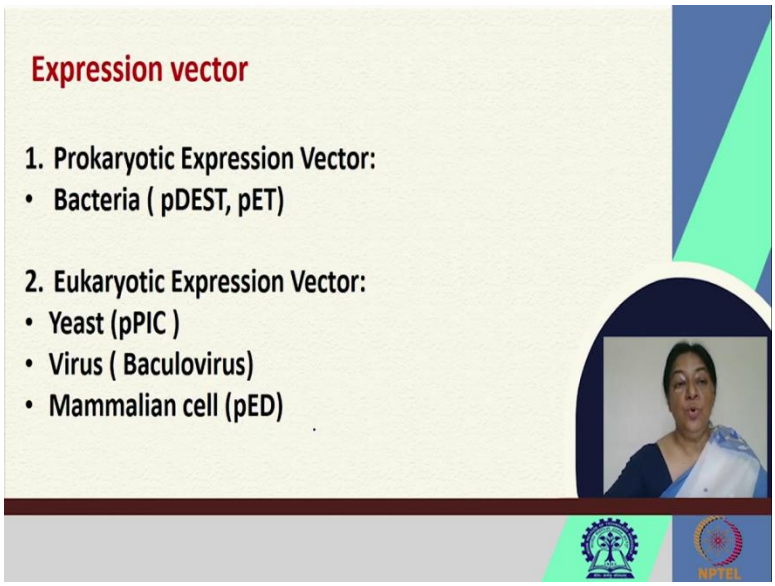

Now we do know about the expression vectors. When we look at the expression vectors, the expression vector DNAs can be prokaryotic expression vectors or eukaryotic expression vectors, such as yeast, virus and even mammalian cells that can be used.

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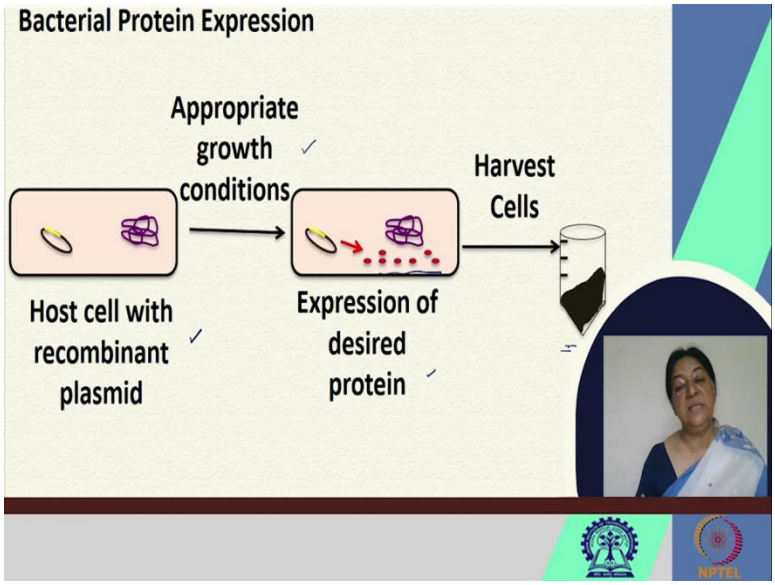


What happens in our bacterial protein expression is, here [refer to slide] we have the host cell that has the recombinant plasmid, under appropriate growth conditions we have the expression of the desired protein. And then we can harvest these cells and get our protein of interest.

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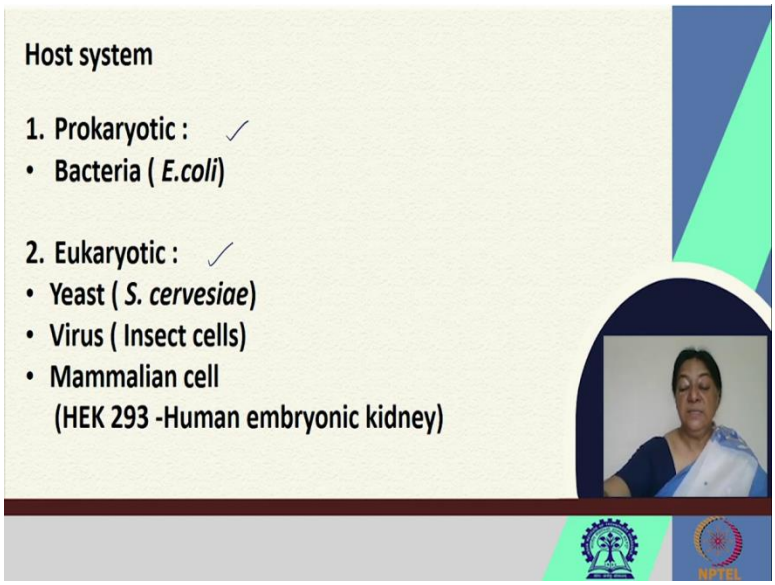

The host system here can also be prokaryotic or eukaryotic, where again we can have bacteria from the prokaryotic host system and for the eukaryotic host system we can have yeast, virus and several types of mammalian cells.

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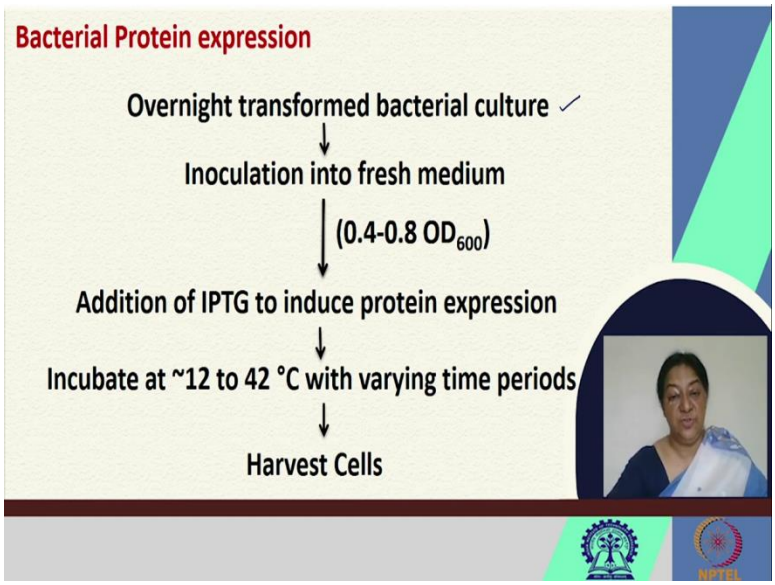


If we look at bacterial protein expression, we have an overnight transformation of the bacterial culture, the inoculation into the fresh medium and the addition of IPTG to induce protein expression; we will see how that is performed. And this incubation then is taken place from 12 to 42°C with varying time periods, following which we can harvest the cells that now have our protein of interest.

But we have to remember that this protein of interest is present in these cells which we have to isolate in the manner that we just saw in terms of using a centrifuge and then using specific chromatographic methods, which we will be discussing in the next lecture.

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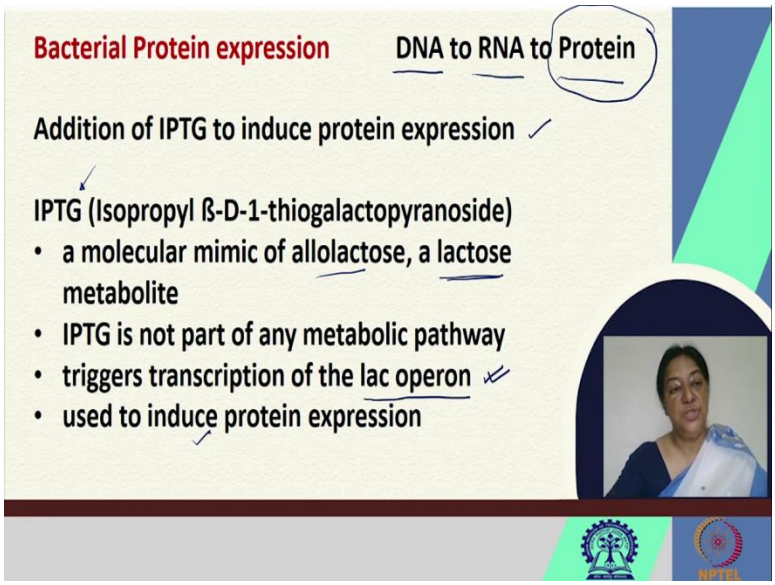


When we now look at bacterial protein expression, our idea here is that we have added or we have put in a DNA. A recombinant DNA that has been used to put into an expression vector, using DNA ligase. When we look at our specific protein, we know that we can go from DNA to RNA to protein from our central dogma of biology; given our DNA the specific DNA that we want for our protein of interest.

So now, we have to add IPTG to induce protein expression. IPTG is isopropyl β-D-1 thiogalactopyranoside. This is a molecular biology agent that is actually a molecular mimic of allolactose that is a lactose metabolite. IPTG is not part of any metabolic pathway, but it is induced, it helps in to trigger the transcription process of the lac operon, which is required for the process of protein expression. This induces protein expression giving us our protein of interest.

In a sense what we are doing is, we are mimicking the lactose for the triggering of the lac operon, using IPTG, resulting in the formation of the protein to be expressed. Now that we have our protein expressed in this harvest cells, we now take it over for centrifugation followed by chromatographic methods.

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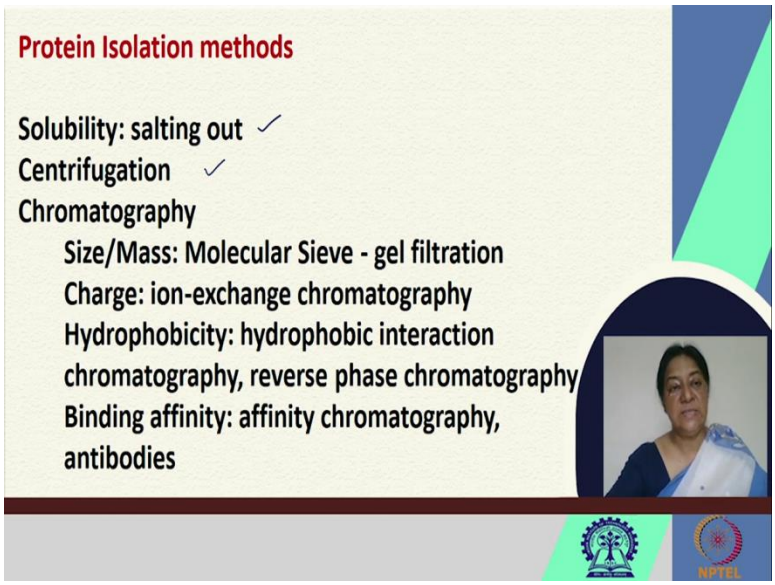


So we looked at the salting out of proteins, we looked at the centrifugation of proteins and methodologies to see isolations from natural sources and isolation from a recombinant DNA source. We will look at chromatographic methods in our next lecture.

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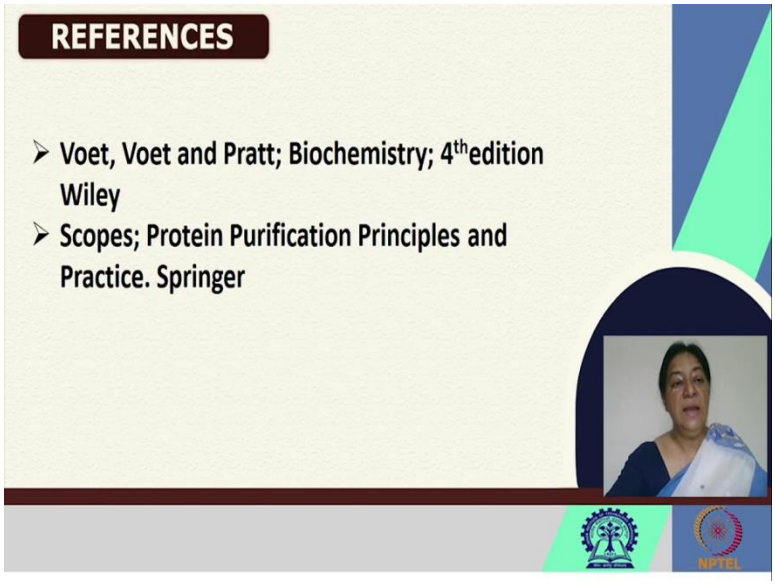


These [refer to slide] are the references, apart from the references that have been included in the specific slides.

Thank you.