Principles of downstream techniques in Bioprocess – a short course

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Lecture-13
Precipitation and Crystallisation

This class we are going to talk about precipitation and crystallization. Both the processes involve the removal of solids from a supersaturated solutions but precipitation is a sort of an uncontrolled process where the solids settles down or comes out of the solution as agglomerate. Whereas crystallization the solids comes out in a very well arranged manner and that is what is called crystallization.

Both are very important processes, the precipitation is used if I want to get proteins out from the mother liquor and crystallization is generally used that as the last step in the downstream process, where you want to purify your product, the product is generally in the solid form. So we are going to cover these two topics today.

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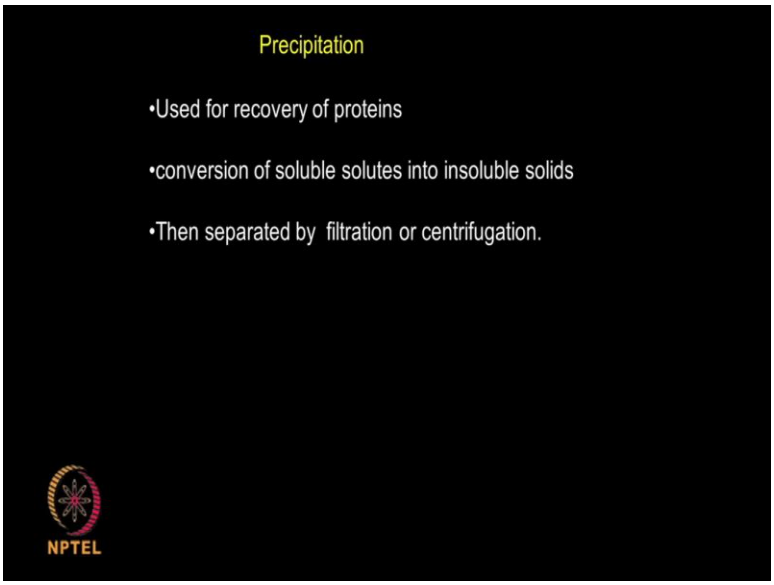

Precipitation is used for recovery of proteins, it is used for conversion of soluble solutes into insoluble and then you can separate without using filtration or centrifugation.

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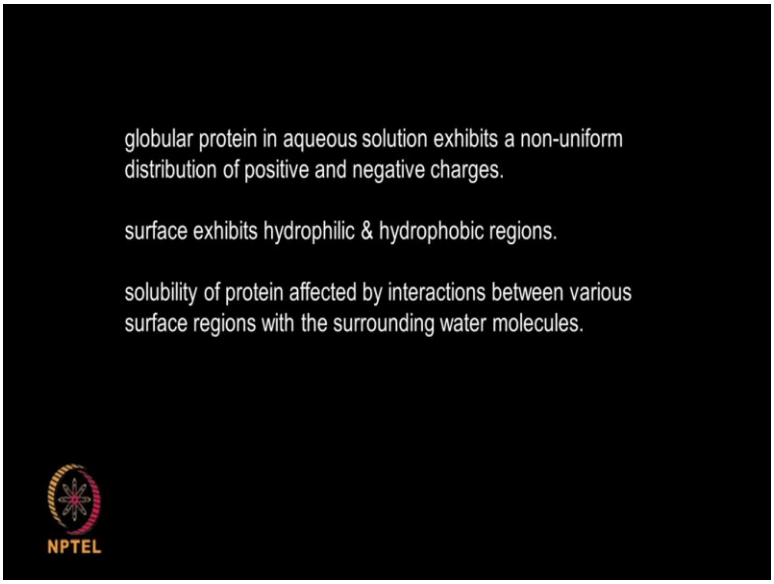

So the globular protein in an aqueous solution, they have a non-uniform distribution of positive and negative charges. Why do they get the charges? Because of the nitrogen that is present in the amide bond right. We will spend more time on that. So the surface exhibits hydrophilic and hydrophobic regions and the solubility of the protein is affected by all these types of the interactions and also because of the presence of the water that is surrounding these proteins.

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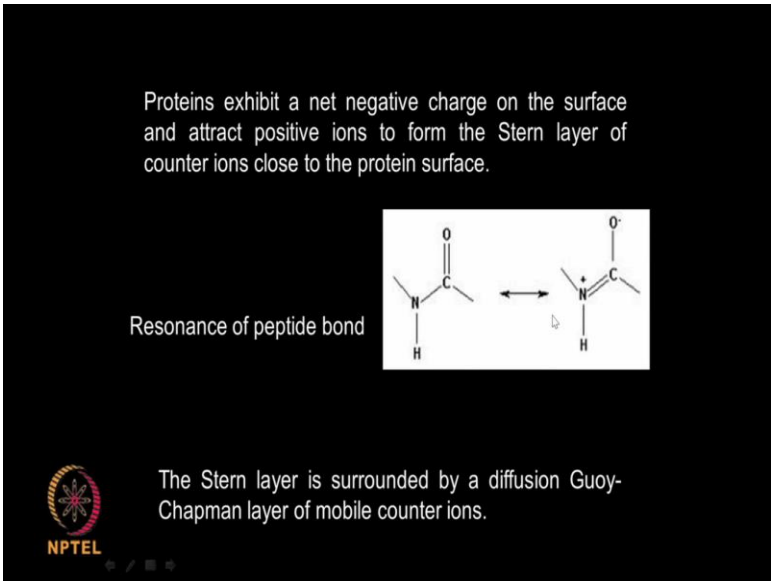

So proteins generally exhibit a net negative charge on the surface, okay on the surface and attracts positive ions because it forms a stern layer. So how do they get the negative charge? If there is a resonance in the peptide bond, although the peptide bond if you look at it, it is neutral. When the bond shifts here, the nitrogen gets a positive charge and there oxygen gets a negative charge

And because of this charge, positive ions are attracted and that is formed like something called the stern layer. Now this stern layer is surrounded by another layer which is called the Gouy-Chapman layer which is made up of counter ions.

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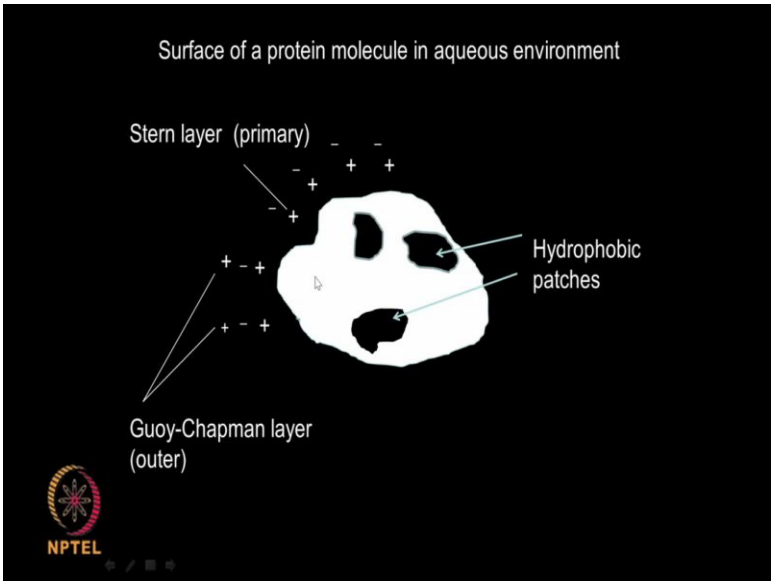

So what you have is, suppose you have this protein okay because of the resonance of the peptide bond, you get O - so all the positive ions are attracted. That is called the stern layer and on top of that okay we have the Gouy-Chapman layer which comes on top of that actually and these are the hydrophobic patches inside that.

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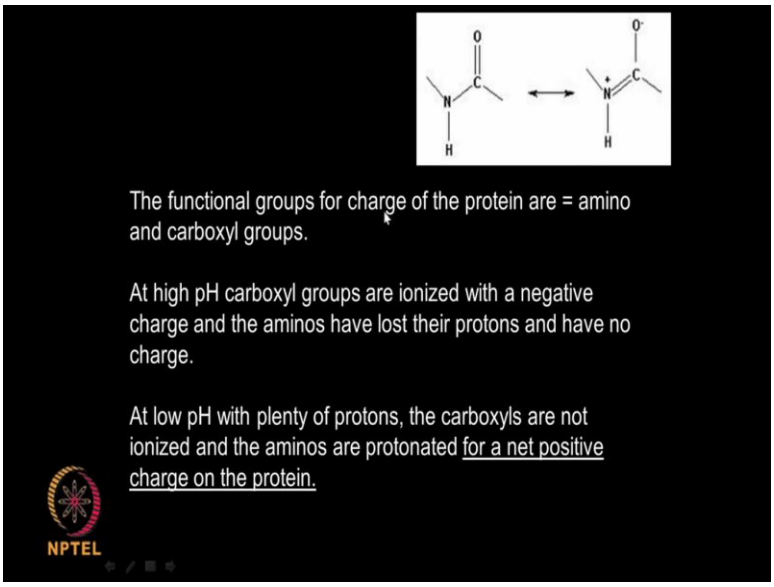

So the functional groups for the charge of the protein depends upon the amino and the carboxylic groups that are present in the protein. So at high pH these carboxyl groups are ionized with the negative charge and the aminos do not have any charge. Okay. There is no charge. But at low pH that means acidic pH, there will plenty of proteoms protons, so the carboxyls are not ionized but

the aminos are protonated for a net positive charge on the protein, okay like this. So you get something like this.

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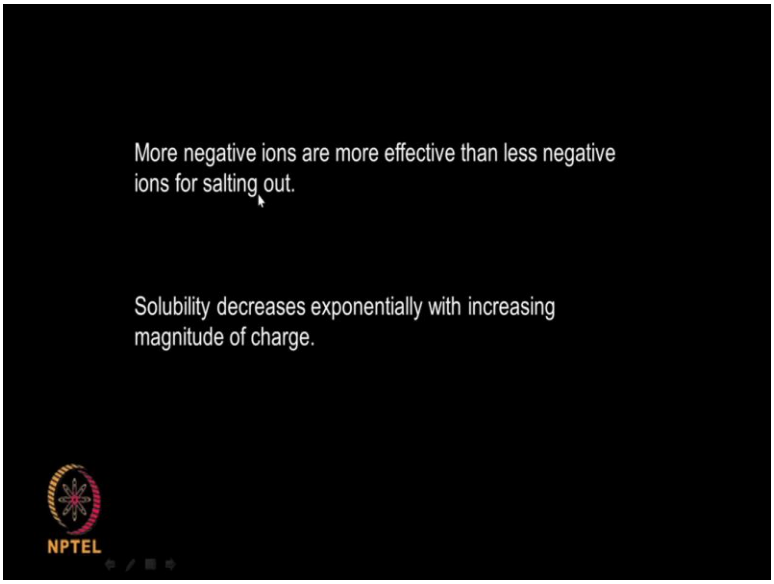

More negative ions are more effective than less negative ions for salting out. So when we want to remove the protein, when we are adding salt that is called the salting out. The solubility decreases exponentially with increasing magnitude of charge okay, so the solubility also changes depending upon the type of charge or the magnitude of charge that is present.

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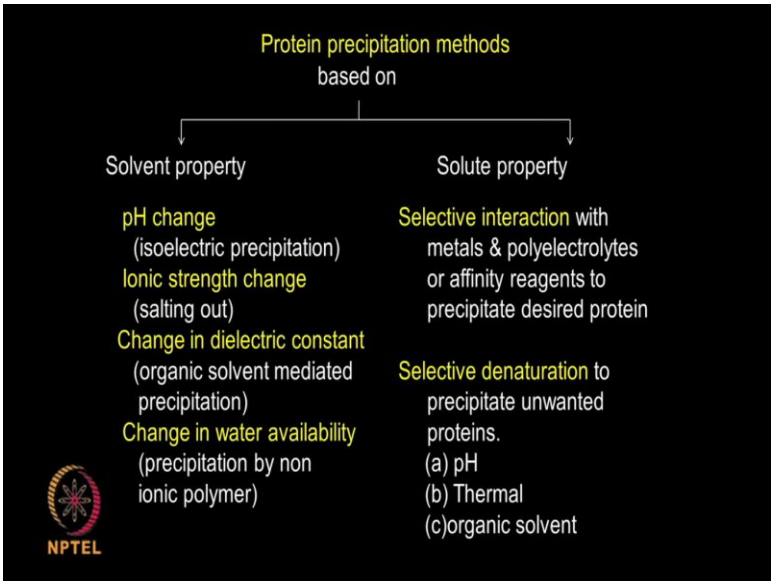

So there are different approaches by which we can achieve this protein precipitation. We can use different strategies to do that. The most important ones, things like solvent property that means I can change the solvent that is present or I can change the solute property, okay both are possible. So how do I do the solvent property? Like I can change the pH like isoelectric precipitation, I can change the ionic strength that is called the salting out; I can change the dielectric constant

That means I can add an organic solvent which will change the dielectric constant of the solvent. Change the water availability that means I can precipitation by non ionic polymer. So if you look at solute property, I can do selective interaction with materials or poly electrolytes, lot of materials as you know magnesium, ion, all those are available. Similarly polyelectrolytes, I can add affinity reagents so that your protein gets precipitated.

Another approach is called the selective denaturation. This is bit tough that means, I am interested in some proteins whereas I am not interested in some other proteins present in my broth. So those proteins I am trying to denature either through pH or thermal or organic solvent. So this is a bit tricky type of process but generally these two are generally followed changing the solvent property as well as the solute property.

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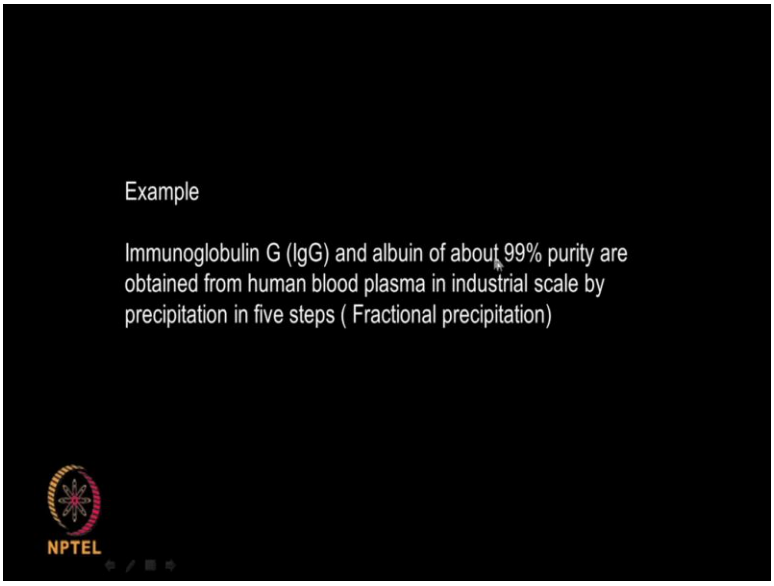

So for example if you take immunoglobulin g and albumin that is present about 99% purity are obtained from human blood plasma in industrial scale, using precipitation five steps that is called fractional precipitation.

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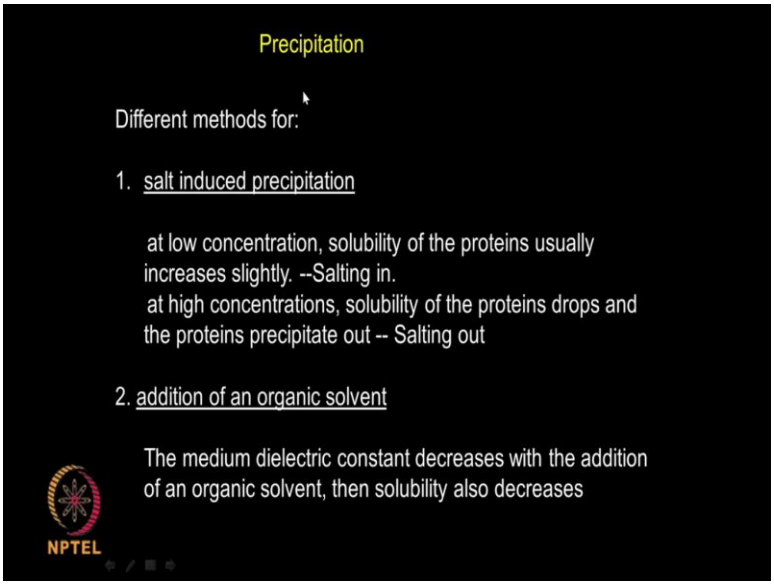

So there are different methods for precipitation like salt induced precipitation. So at low concentration of the salt, solubility of the proteins usually increases slightly. That is called salting in. Whereas at high concentration, solubility of the protein drops out drops and the proteins precipitates, that is called salting out. So you can sometimes have salting in, you can

sometimes have salting out. Addition of an organic solvent, so when you do that you are changing the dielectric constant of the solution, okay that can lead into the protein precipitation.

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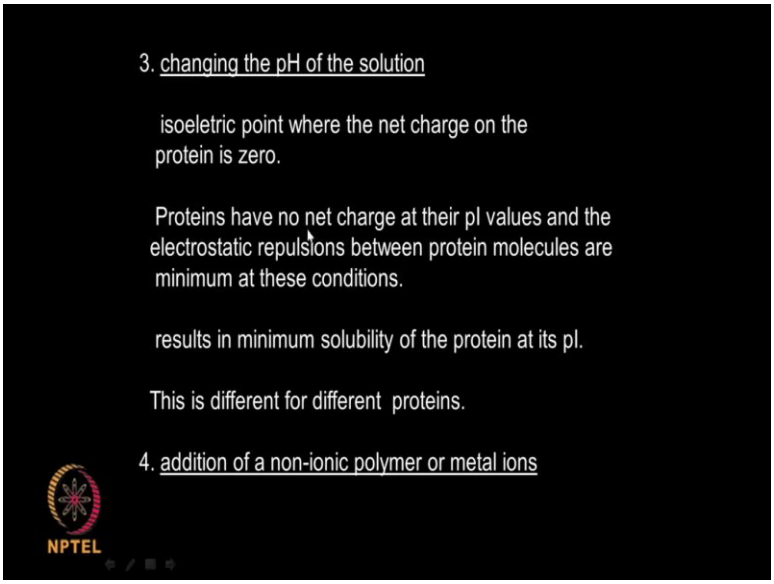

Changing the pH of the solution, so as you know at the isoelectric point, the pH of the protein is zero. So the proteins will have no net charge at their pI values, so the proteins will have minimum repulsion with each other. So they will come together and then they will precipitate out. So this is different for each of the proteins. That means the isoelectric pH can be very different for each protein.

So I could try to separate 2 proteins or 3 proteins very selectively by slowly playing with the pH. The 4th approach is adding non-ionic polymers or metal ions, we will spend some time on each one of these strategies okay.

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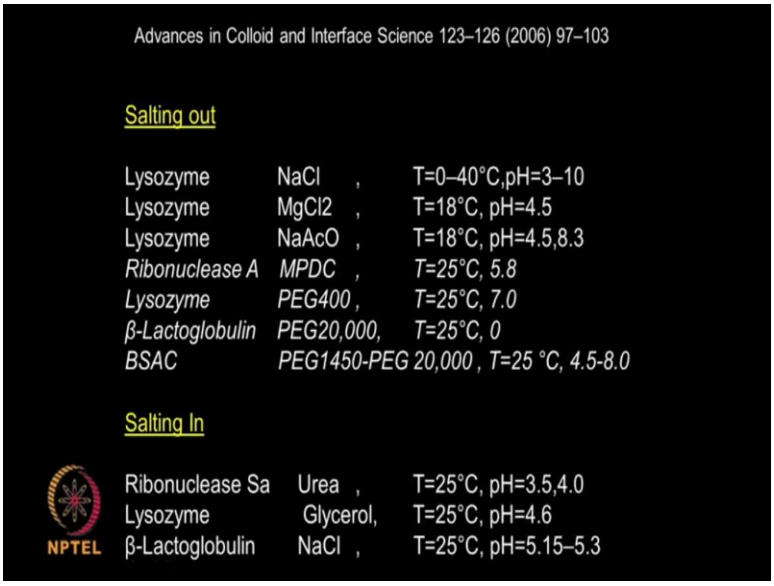

So the salting out, for example if I want to collect lysozyme, I can use different types of salt sodium chloride, magnesium chloride, sodium acetate or even peg, that is a polymer with molecular weight of 400 and so on actually. Okay. Look at the temperatures generally it is varying between 10 degrees to almost 40 degrees and the pH also varies in the acidic range. Salting in, we can add urea, glycerol, sodium chloride, so that the proteins gets taken into the solution, that means solid protein becomes part of the solution. Generally I do not know whether there are any industrial examples of salting in but yes there are a lot of examples for slating out.

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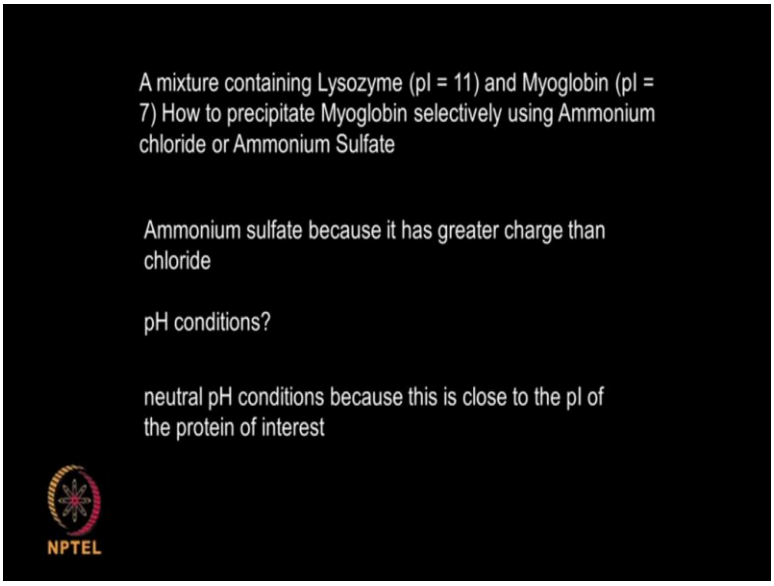

So let us look at it. For example a mixture containing lysozyme, the isoelectric pH is 11 and myoglobin, isoelectric pH is 7. How to precipitate this selectively using ammonium chloride or ammonium sulfate, now suppose you have, you are asked such a question. What do you do? Okay, you generally take ammonium sulfate because it has got a greater charge than chloride. What should be the pH? As you can see, at neutral pH, myoglobin has it is isoelectric pH. So you can work around that pH. Right. So neutrals pH is the best for this because myoglobin will have net zero charge at a pH of 7.

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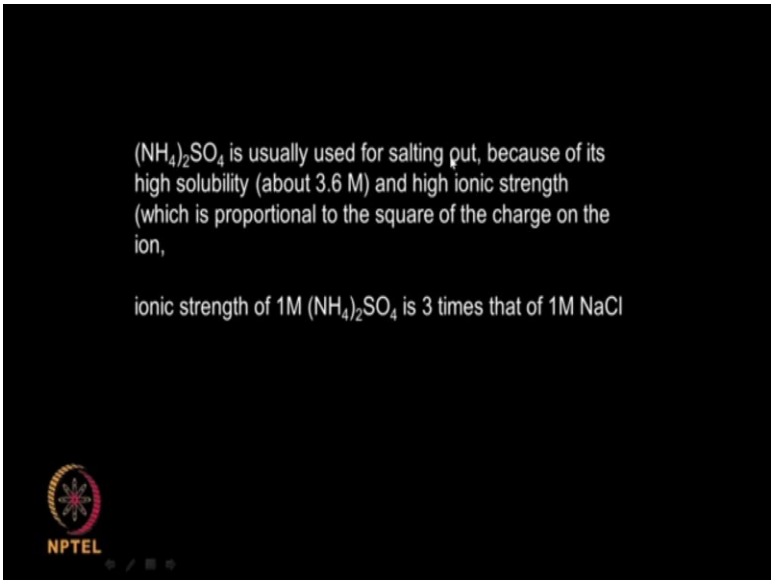

Generally ammonium sulfate is usually used for salting out because of it is high solubility about 3.6 molar and high ionic strength which is proportional to the square of the charge of the ion. So ionic strength of the one mole of ammonium sulfate, if you take is three times that of one mole of sodium chloride. That is why generally in any salting out studies, you will you always come across ammonium sulfate nothing else actually okay.

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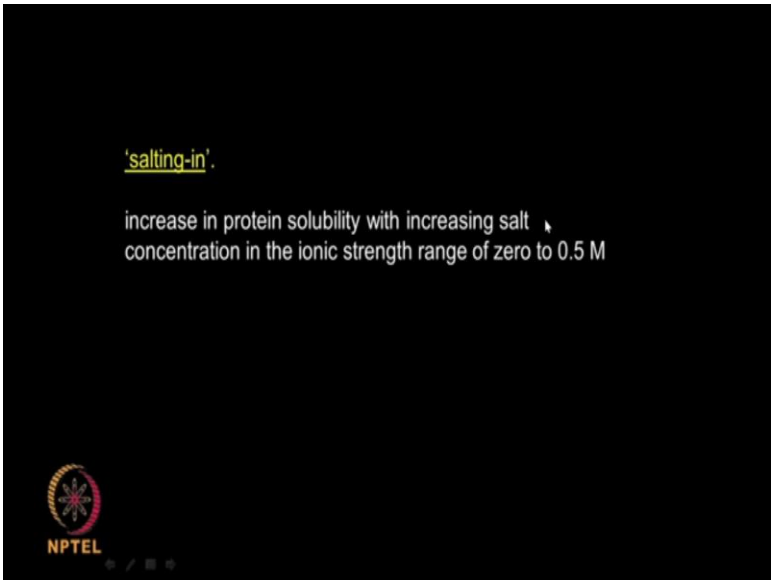

Salting in, so what does salting in mean? Increase in protein solubility with increasing salt. Generally this is seen only at very low concentration of salt but high concentrations we generally end up having salting out okay.

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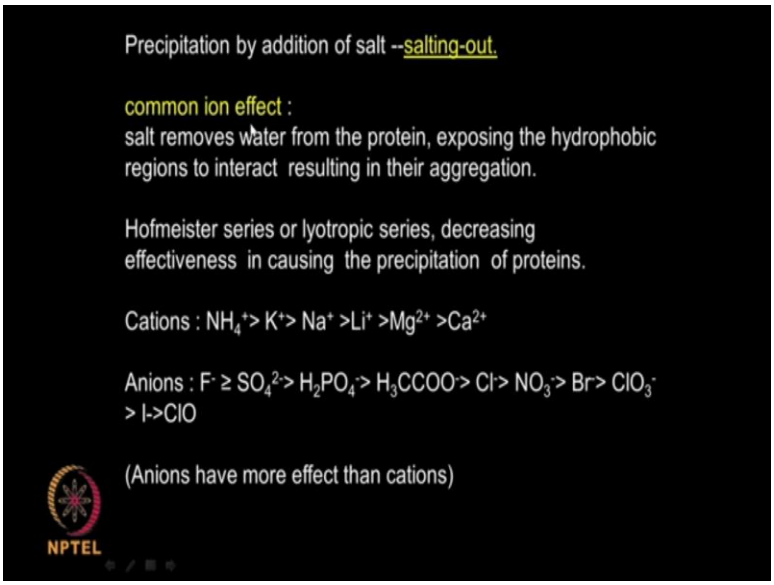

So salting out is called common ion effect. Salt removes water from the protein, so when the salt removes water, the hydrophobic regions start interacting and they start aggregating. So the proteins precipitate. There is something called the Hofmeister series or the lyotropic series. That is, they are, these salts, the cations and the anions are listed in this fashion showing the most

effective to the least effective. Okay. So that is why ammonium comes right at the top and sulfate also comes right at the top.

There could be some changes depending upon the type of protein. So you have to remember that. This order is not valid for all the proteins but it can change depending upon the protein. So sometimes magnesium could be more effective than calcium and for some proteins calcium could be more effective for magnesium. So that changes can be there. But generally if you look at the Hofmeister series for cations, this is the order and for anions, this is the order and anions are more effective than cations. So the sulfates are more effective than the plus ions. So you have to remember that.

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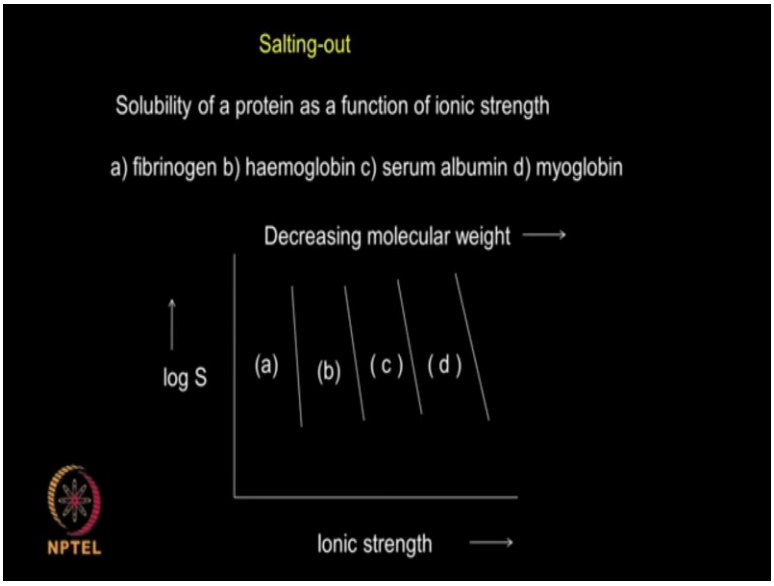

So the salting out, suppose I have 4 proteins, fibrinogen, haemoglobin, serum albumin, myoglobin. Okay. S is the solubility, log of solubility. So as the ionic strength goes up the molecular weight goes down, that means proteins with high molecular weight will precipitate fast, then comes next one, next one and finally the protein with the smallest molecular weight. So I can use this type of precipitation if I am very smart to selectively precipitate proteins and also that is called that is what is called fractional precipitation okay.

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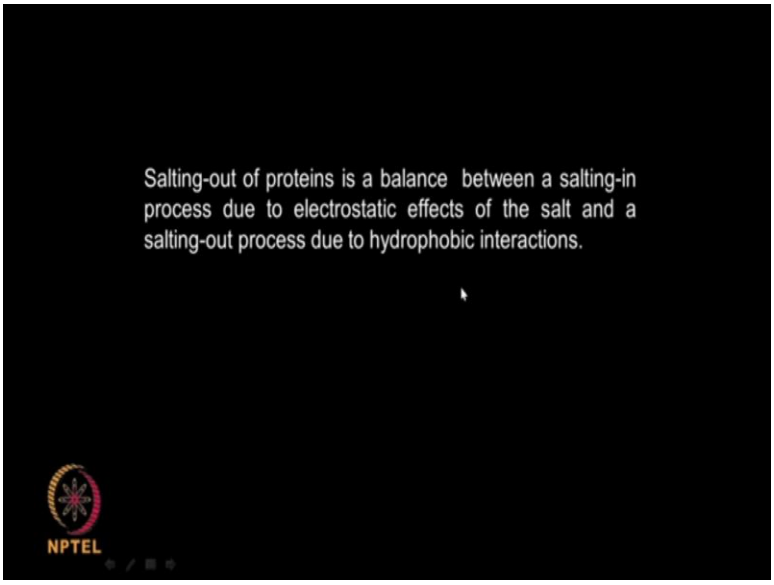

Salting out of proteins is a balance between salting in process due to electrostatic effect of the salt and salting-out process due to hydrophobic. So you have electrostatic, you have hydrophobic interactions, electrostatic comes because of the salt and the proteins charge. Hydrophobic comes because of the hydrophobic groups like alkanes, carboxyls and benzene rings and so on actually.

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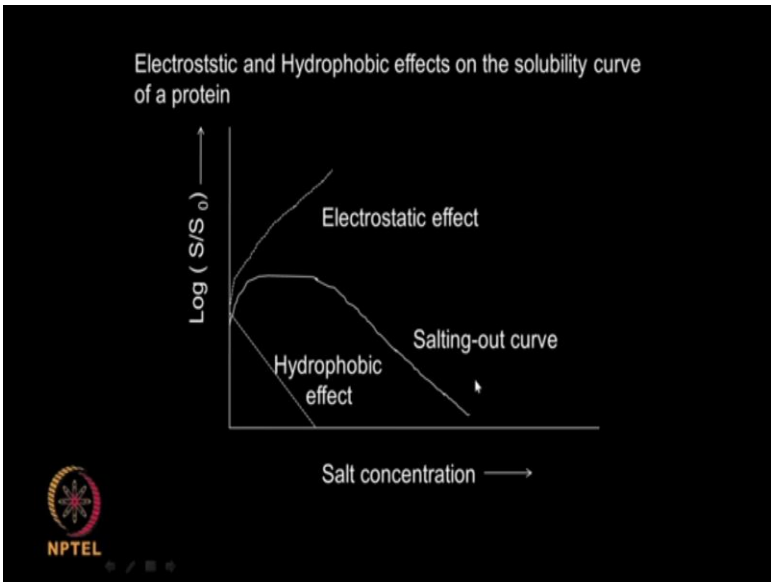

So you have the electrostatic effect, we have the hydrophobic effect, so in between you can have the salting out happening. So one side you have the salt concentration, other side you have the logarithm of solubility in the presence of salt/S0 that means pure solvent S0 is pure solvent. So 2

forces electrostatic effect, hydrophobic effect, hydrophobic comes during salting out, electrostatic comes during salting in okay.

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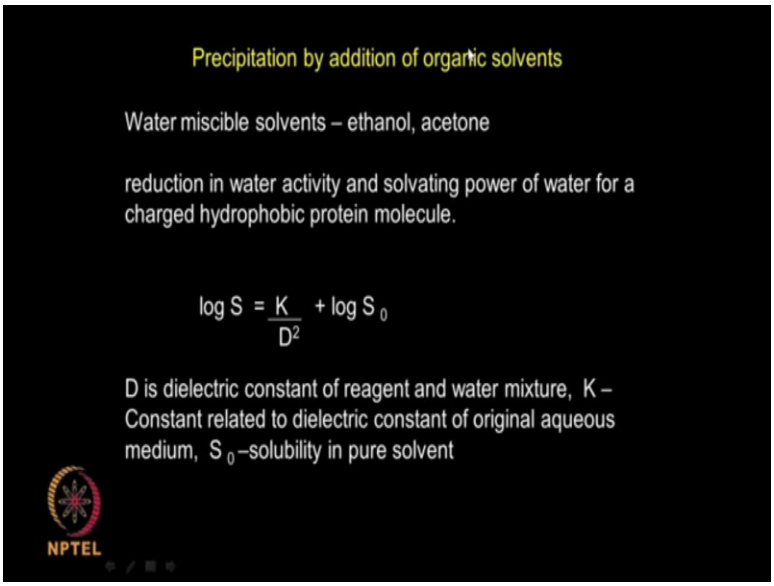

So you can do a precipitation by adding organic solvents here, you are changing the dielectric constant of the solution, that is what it is. We can add ethanol, acetone, so the water activity is reduced and then the hydrophobic molecules start interacting each other and they can salt out. So this is an equation where log S that is the solubility in the presence of solvent, S0 is the solubility of the pure solvent, K is some constant, D is the dielectric constant. So this is an equation which connects the dielectric constant with the solubility of the protein okay.

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So organic solvent mediated precipitation depends on the molecular size of the protein, larger the protein you need less concentration. As I have shown even in salting out, larger the protein you need less salt, smaller the protein it require more salt. So something happens here larger the protein you require less solvent okay.

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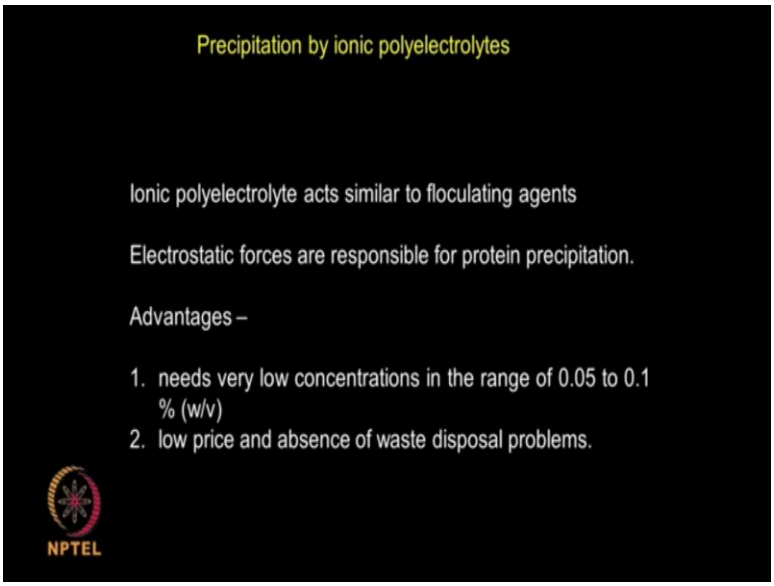

So we can add polyelectrolytes. By adding polyelectrolytes also we can achieve protein precipitation. This is also similar to flocculation, if you remember in our previous class, we talked about flocculating agents, process of flocculating where it basically there, we are looking

at the charges, electrostatic forces which you are trying to neutralize. So you can use this type of approach also.

So you need very low concentrations, generally 0.05 to 0.1%, low price, absence of waste disposal problems, all these if because you are using a solvent or if you are using the salt, we need large concentrations waste disposal is a big problem.

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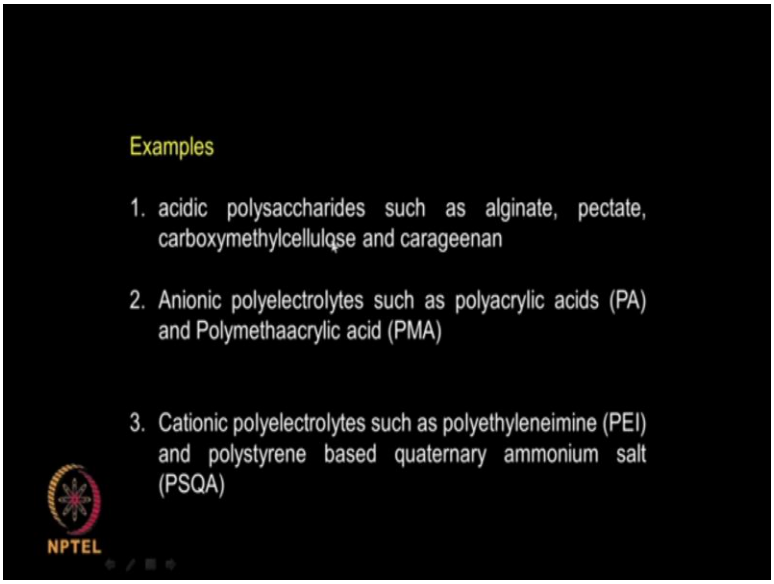

So acidic polysaccharides such as alginate, pectate, carboxymethylcellulose, cmc, carageenan. Anionic polyelectrolytes such as polyacryclic acid, polymethaacrylic acid. Cationic polyelectrolytes such as polyethyleneamin eimine, polystyrene based quaternary ammonium salt, so all these are examples of compounds which can help in the precipitation of the protein agglomeration of the protein basically they neutralize the charge on the surface of the protein.

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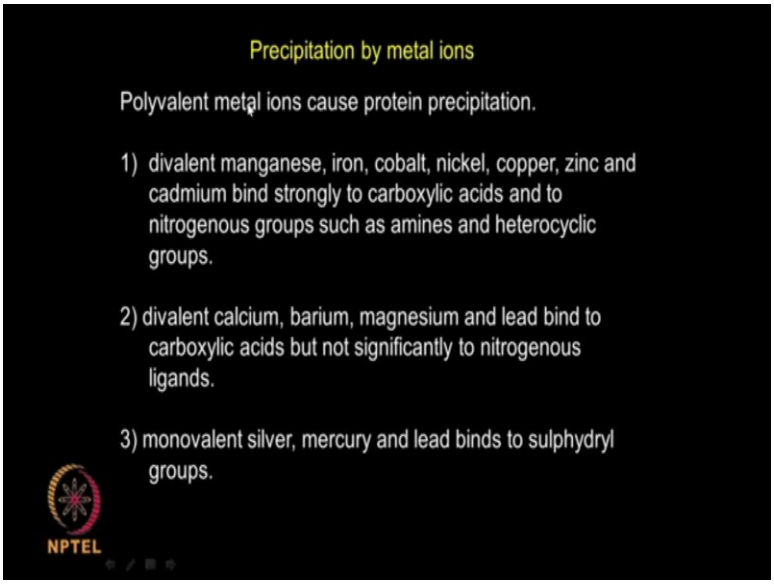

We can also add metal ions like polyvalent metal ions, divalent magnesium, iron, cobalt, nickel, copper, zinc, cadmium, bind strongly to carboxylic acids into nitrogenous groups such as amines and heterocycles. Divalent calcium, barium, magnesium okay and they can bind to carboxylic acids. Monovalent silver, mercury, they can bind to sulphydryl groups. So we can also think of adding metal ions which help in chelating with the protein and allow the protein to precipitate.

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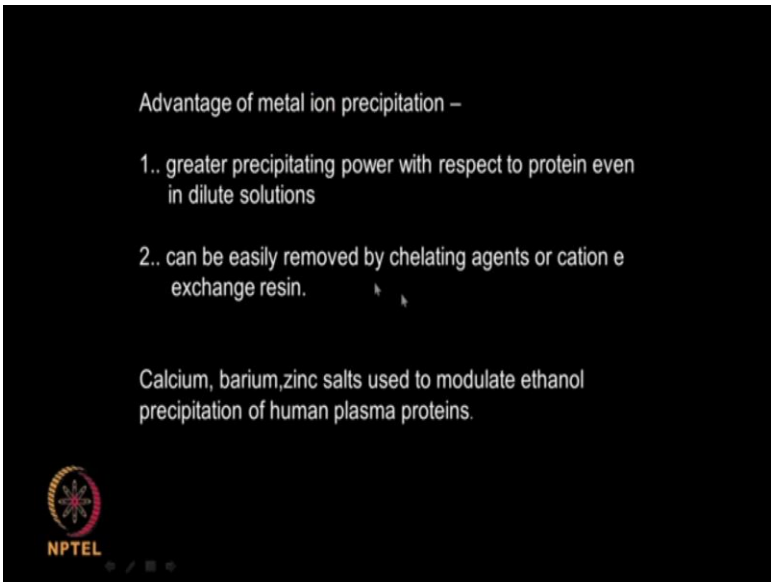

So what are the advantages? They have a very good precipitating power, can be easily removed by chelating agents, so we can add some of these metals. Sometimes we can also add some solvents for precipitation also, so we can even think of combinations of these two methods.

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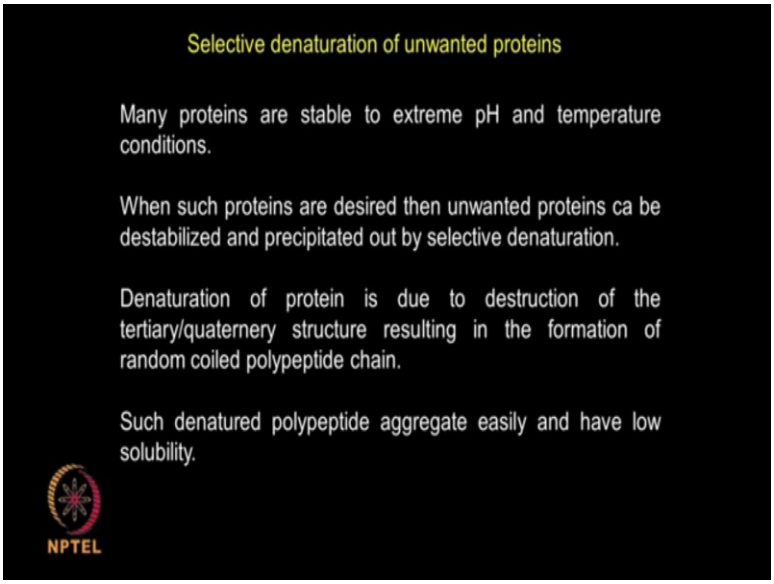

Selective denaturation of unwanted proteins, this is a bit tricky as I said you should not denature the protein of your interest. Generally you use pH or temperature extreme conditions, so that the proteins which are of no use or denatured, left behind with good protein, okay such denatured poly proteins generally aggregate and settle down.

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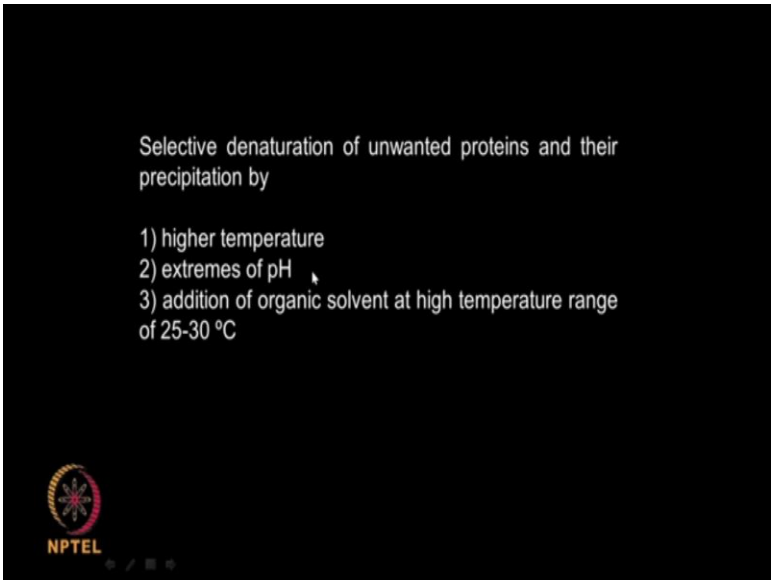

So higher temperature, extreme pH, addition of organic solvents at high temperature, all these are techniques adopted for selective denaturation.

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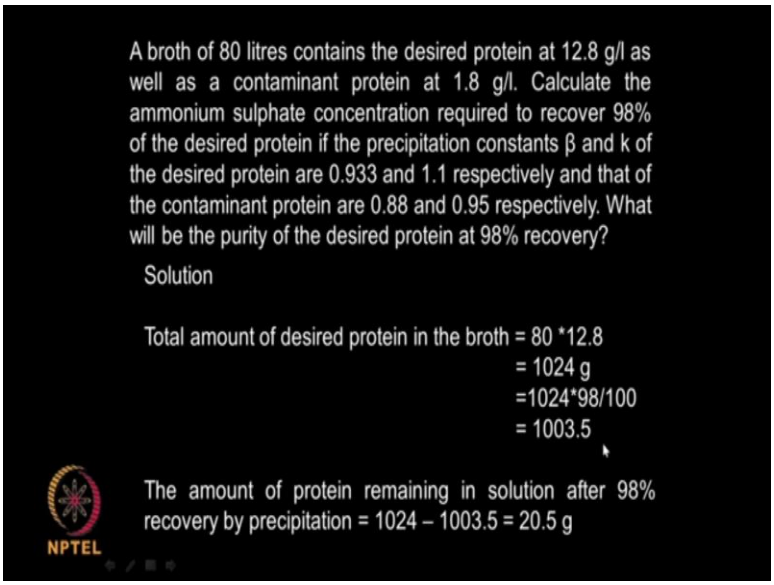

Okay now we can look at a small problem, you have a broth 80 litres containing your proteins 12.8 grams per litre, there is a contaminant 1.8 grams per litre. Now am adding ammonium sulphate 3 moles okay, okay you want to know how much ammonium sulphate you want to add, if I want to recover 98 % of this. Now the beta and k, that determines the solubility of the protein depending upon the ionic strength of the salt you are adding, that is called Cohn's equation.

We will look at the Cohn's equation. So there is a relationship which combines the solubility of protein with the ionic strength of the salt that is being added. So the there are two constants that is called beta and k. Beta and k for your protein is given here and beta and k for your contaminant is given here and I want to recover 98 % of my protein. So how do I find out how much salt required.

So 80*12.8 gives you total amount of protein present, I want to recover 98 % that means 1003.5 grams of protein, I want to recover. So how much is left behind? 20.5 grams that means that is your amount of protein present okay.

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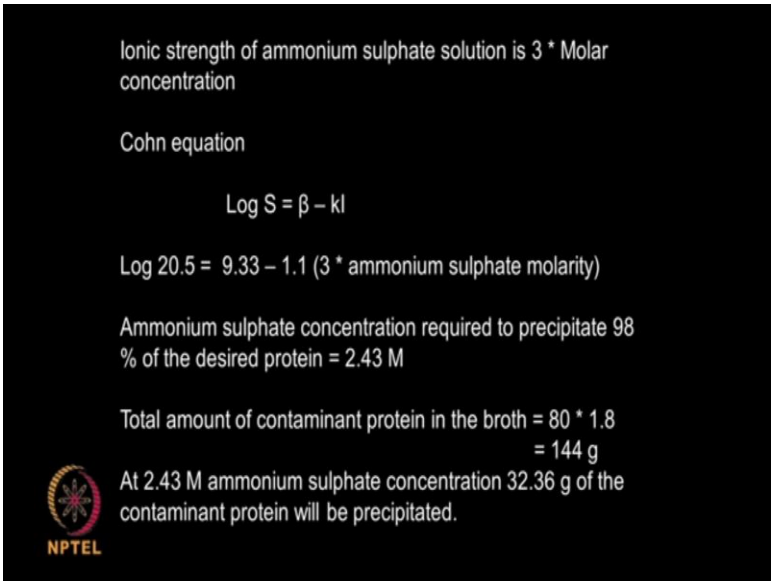

So this Cohn's equation log S, S is the amount of protein present, beta and k are 2 constant, I is the ionic strength okay. So in the problem, 20.5 is what is left behind, beta is 9.33, k is 1.1 okay. 3 moles of ammonium sulphate, molarity of 3 so I can 3 * ammonium sulphate molarity, so if I do that, I will get 2.43 moles of ammonium sulphate okay. I is the ammonium sulphate which I want to get. Now total amount of contaminant protein in the broth, as you know is 80*1.8 that is 144 grams.

So at 2.43 moles, I use this equation, I can calculate what is S part of it okay understood. So we can calculate what will be the amount of contaminant that is left behind in the solution. So this much amount of contaminant is precipitated out and we have some amount of contaminant present, 144, so the remaining is left behind.

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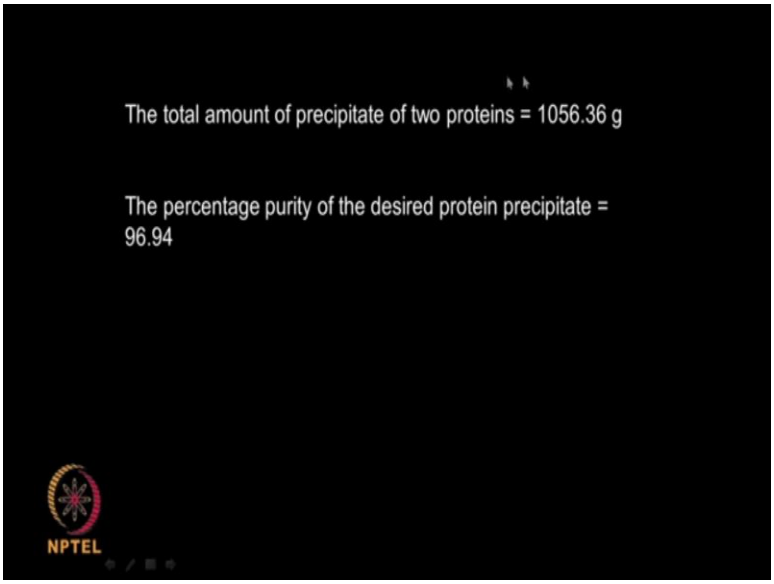

So the total amount of protein precipitated is given here, total amount of impurities precipitated is given here, so that will give you an idea about the purity of the protein that is precipitating out.

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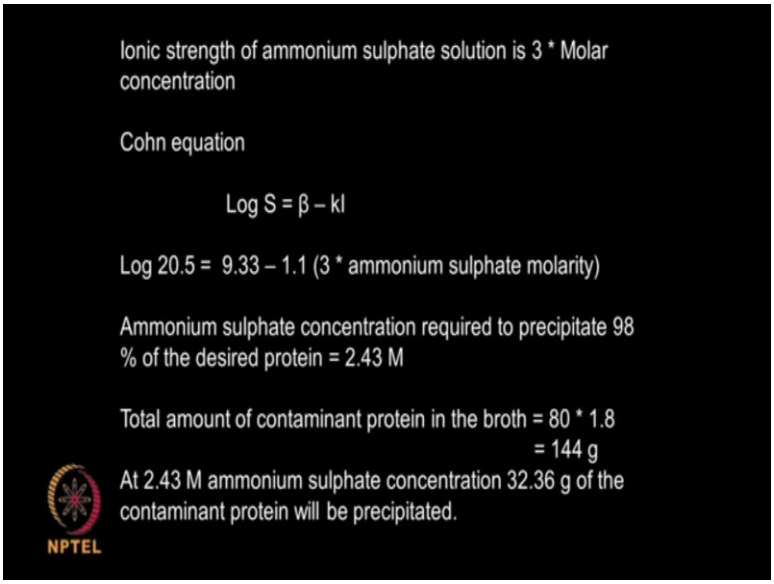

So this is a simple problem and here we make use of something called the Cohn's equation. So Cohn's equation please look at it, S is the solubility that is the amount of protein present and beta and k are constants and I is my ammonium sulphate concentration which I am going which I need to put in so that we get a salting out effect okay. So it connects the concentration of ammonium sulphate or the required with the solubility of the protein that will be present in the solution that is in equilibrium in the salt.

Quite simple. So far we looked at precipitation, where we are talking about protein coming out of the solution because of the addition of either salt or because of which is changing your hydrophobic electrostatic balance or adding a solvent which changes the dielectric constant okay or sometimes adding a polyelectrolyte or adding a metal the divalent metal or sometimes even the monovalent metal for chelating purposes or selective denaturation.

Let us look at crystallization this is a very controlled process, so that you get solid coming out of the solution, generally supersaturated solution in certain exact size and shape. That is what is called crystallization and these solid that is coming out is called the crystal. They are arranged in the uniform order and the crystallization is resorted to as the last step in the downstream.

Especially if your product can come out in the form of crystal, then it is very good because if can be used for removal of the impurities.

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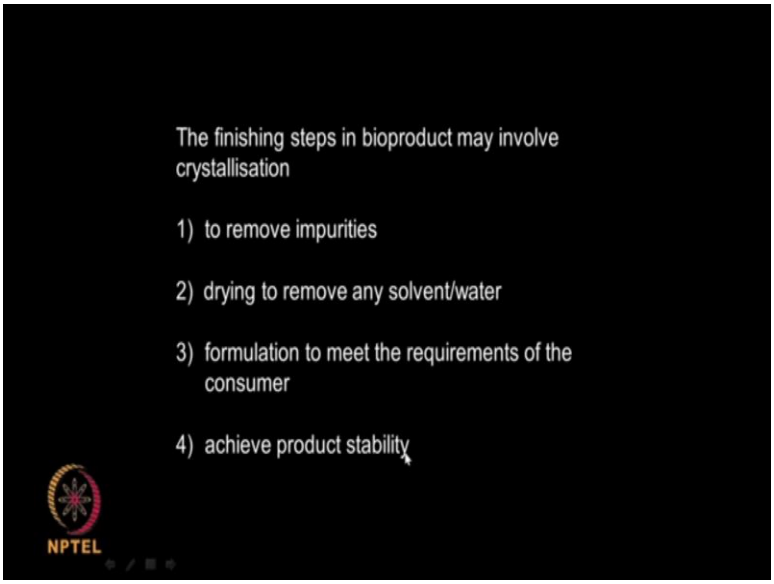

So for removing impurities drying, formulation to meet the requirement of the custom consumer and also achieve products stability because if the solid is present, then generally the products are much more stable than in the liquid form.

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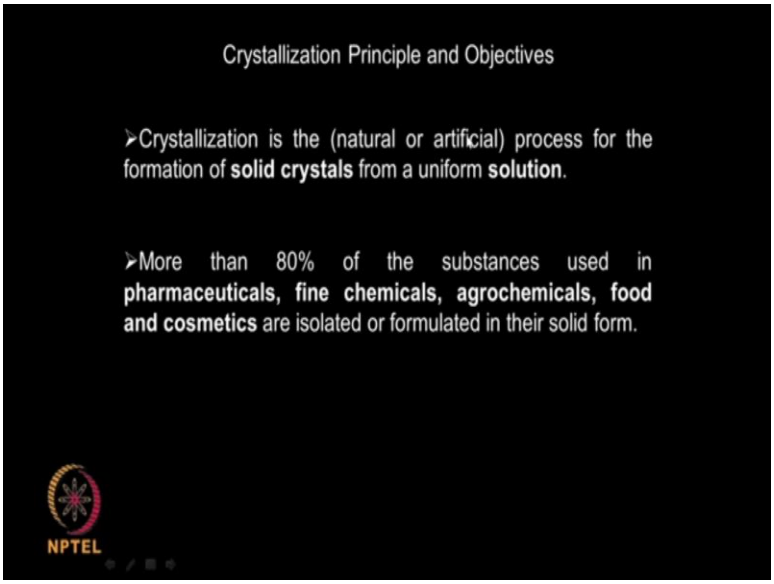

This is, you can approach through either natural or an artificial process okay from the solution 80% of all the substances used in pharmaceutical industries, fine chemicals, agrochemicals foods, cosmetics are always in the crystal form or solid form okay because it is easy to operate manage and take it further down for formulation purposes actually.

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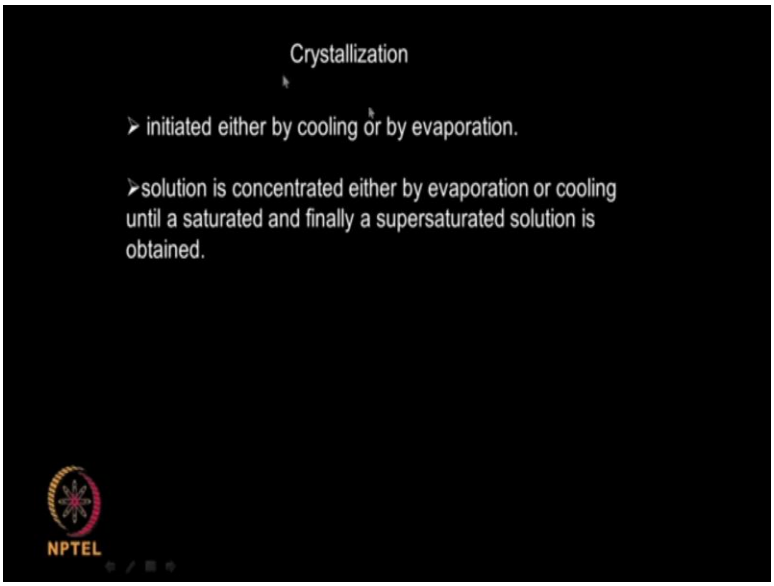

So how do you initiate this process? It is done through cooling or by evaporation. So that means you remove the solvent so that your solution becomes supersaturated or you cool it, so that your solution becomes supersaturated. So solution gets concentrated either by evaporation or cooling

until the saturated and finally it becomes a supersaturated solution and when it becomes supersaturated solids start crystallizing out.

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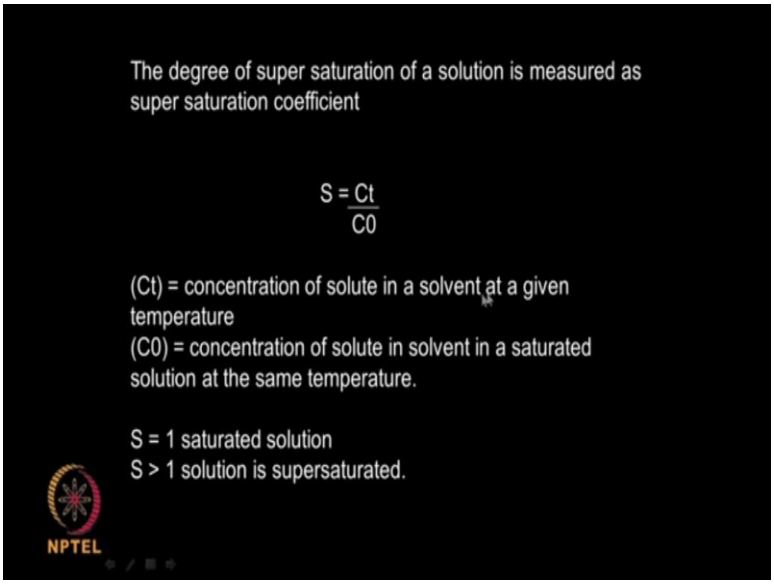

So the degree of super saturation is given by this formula S=Ct concentration of solute in a solvent at a given temperature, c0 concentration of the solute in a saturated solution, okay if S=1 then we call it saturated, if S is greater than 1, it is called supersaturated. So you need to arrive at a supersaturated condition, so that your product will crystallize out. So you need to arrive at that condition.

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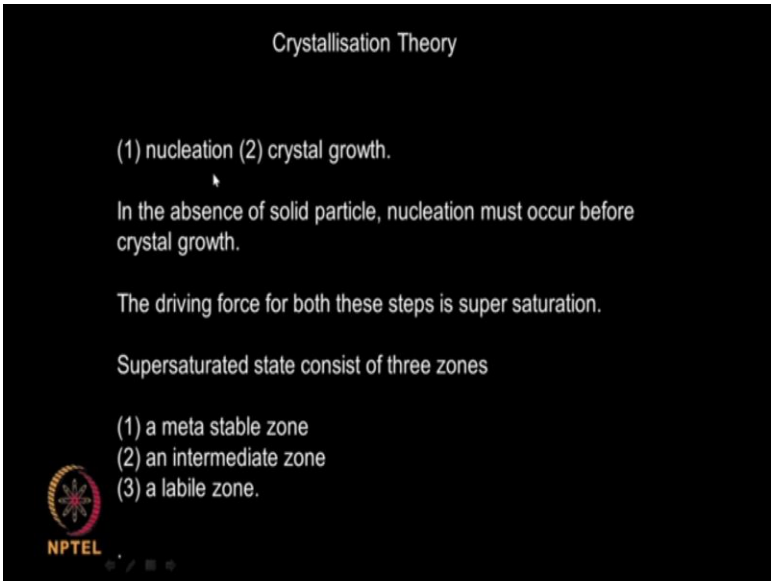

The theory states there is a nucleation, that means small very minuet solids are formed micron sized solids are formed initially, that is called nucleation once that is formed, it starts growing. The crystal starts growing, that is called crystal growth. If you do not have any solid particles then nucleation may occur so and then the solids will start growing. That is called crystal growth. Sometimes they had small amount of the same product itself.

So that the crystal starts growing around that, so that you get much better product quality even if you add small amount of impurity, that acts as a nucleation site. So the driving force for this is super saturation. So this supersaturated condition consists of meta stable zone, intermediate zone, labile zone, meta stable, intermediate and labile zone okay.

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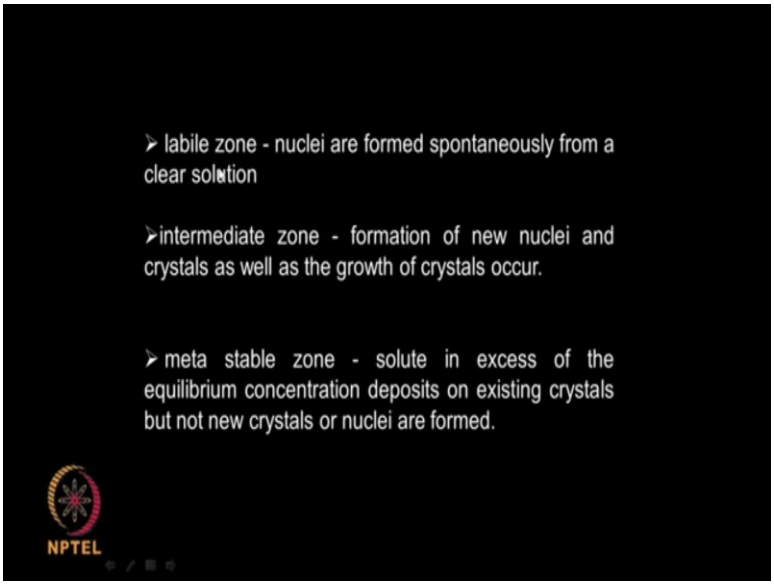

So if you look at labile, nuclei are formed spontaneously from a solution because it is highly supersaturated, it forms little small very small micron sized particles that is spontaneous. Intermediate zone formation of new nuclei and crystals as well as the growth of the crystals. So it is forming new crystals as well as existing crystals starts growing. Meta stable zone, so solute in excess of the equilibrium concentration deposits on existing crystal.

That means crystal is growing that means no new crystal is formed, okay, whereas in intermediate zone we are forming new nucleus as well as we are forming the growth of the crystal whereas in the meta stable zone, there is no new nuclei are formed but the existing crystals are grown okay. So three different zones actually whereas in labile zone we have only new nucleus that are getting formed.

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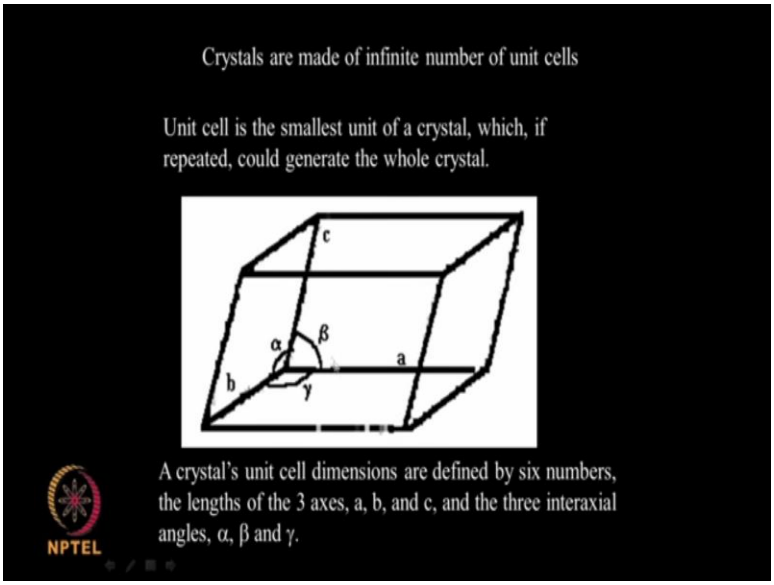

Typically crystals are, it is like this, they are shown like this. So it is got 3 sides a, b, c and it is got 3 angles alpha, beta, gamma, so sometimes a can be =b can be = c angles can be equal, sometimes these angles can be 90 degrees. So you can have different permutations combinations that will lead to different types of crystals actually okay. This is called an unit cell so if you have a crystal, it will have multiple unit cells attached to each other.

So there is an uniformity, so if you cut the crystal you will see some uniform dimension. If you cut it still further smaller, you will keep seeing that same dimension that is why it is very very uniform in whatever be the size, larger size, when you cut it, when you cut it further and further. So as I said you can have different combinations of a, b, c, you can have different alpha, beta, gamma, so you will get large number of types of crystals.

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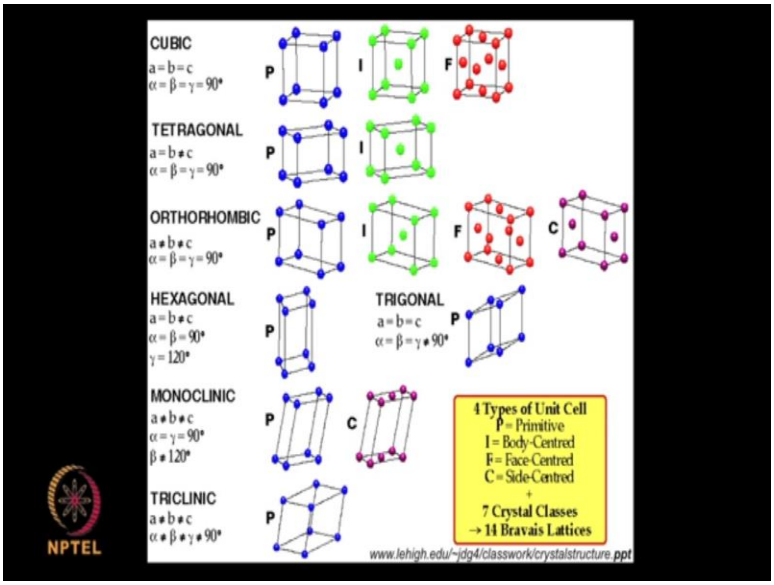

For example, cubic it will have a = b = c alpha = beta = gamma so you will have the atoms at corners of this cube okay and you can also have the atom in the middle okay that is called a body-centered cubic, you can also have a atoms on the faces that is faces that means there will be six faces for the cube. So that will be face-centered cubic so you can have just plain primitive form where you have the atoms at the corners, you can have extra one more atom in the center, that is body-centered.

You can have all along the faces so that is face-centered, this is called cubic when a = b = c alpha beta eq gamma all = 90. Then you can have tetragonal a = b but b is not = c alpha beta gamma = 90 then you can have orthorhombic where a b c are different but angles are all 90 degrees. You can have hexagonal where a = b not = c alpha beta = 90 but gamma = 120, similarly you can have trigonal a = b = c alpha = beta = gamma but they are not = 90 degrees.

Then you can have monoclinic where a, b, c are not equal alpha and gamma = 90 beta not = 120 then you have triclinic where a, b, c are not equal alpha, beta, gamma, are not = 90 as you can see there are 7 class, cubic, tetragonal, orthorhombic, hexagonal, monoclinic, triclinic and trigonal, 7 classes and there are 14 types, if you add up all these because you will have a primitive form p you have a body-centered form, you have a face-centered form.

So you have 14 bravis lattices it is called and you have 7 crystal types okay this is how all the solids come out in one of these forms actually, you can x-ray crystallography, you can use powder x-ray to find out the crystal structure, crystal dimensions and so on actually.

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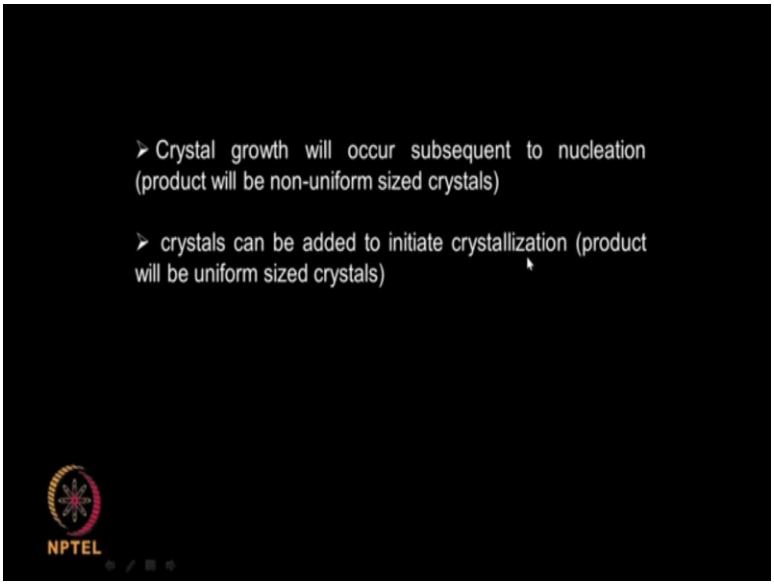

So crystal growth will occur subsequently to nucleation crystals can be added to initiate crystallization, so as I said we can add small crystals to initiate that crystallization.

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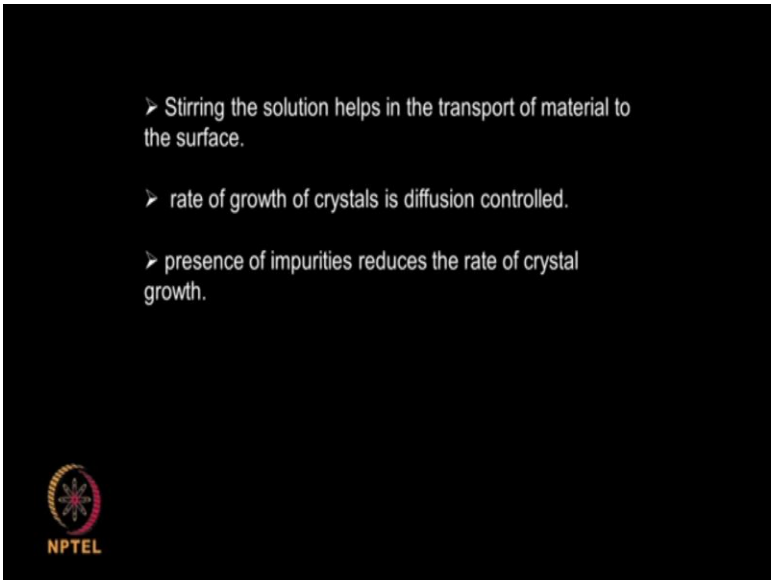

So stirring the solution helps in the transport of the material to the surface rate of growth of crystal is diffusion control because this from the solution the solute has to come to the crystal phase, get attached to it, so that the crystal starts growing. Presence of impurities will reduce the rate of crystal growth, so generally you should have very pure solution if you want good crystal product.

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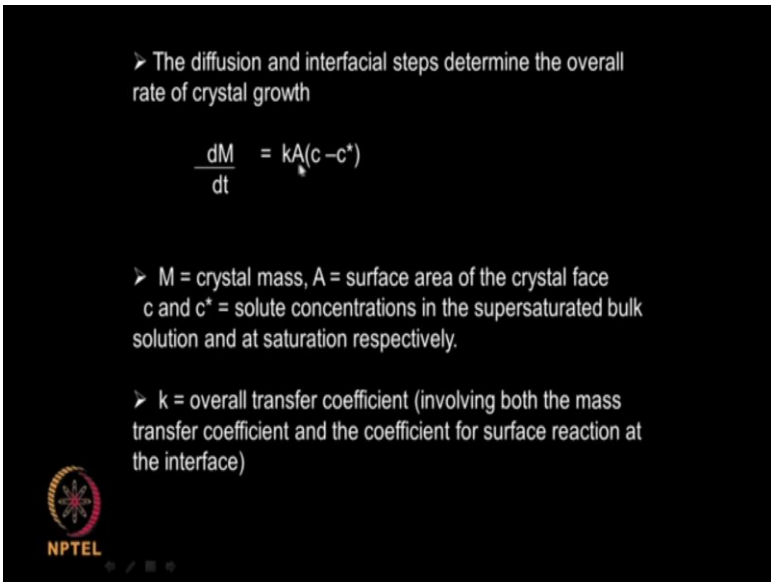

So dM/dt that is crystal mass how the crystal mass grows as a function of time t k is the overall transfer coefficient A is the area surface area of the crystal phase c - c* is the driving force c is the solute concentration in the supersaturated c* at a saturation concentration. So when c reaches c* the growth of the crystal will stop okay because this is the driving force when c reaches c* it will stop.

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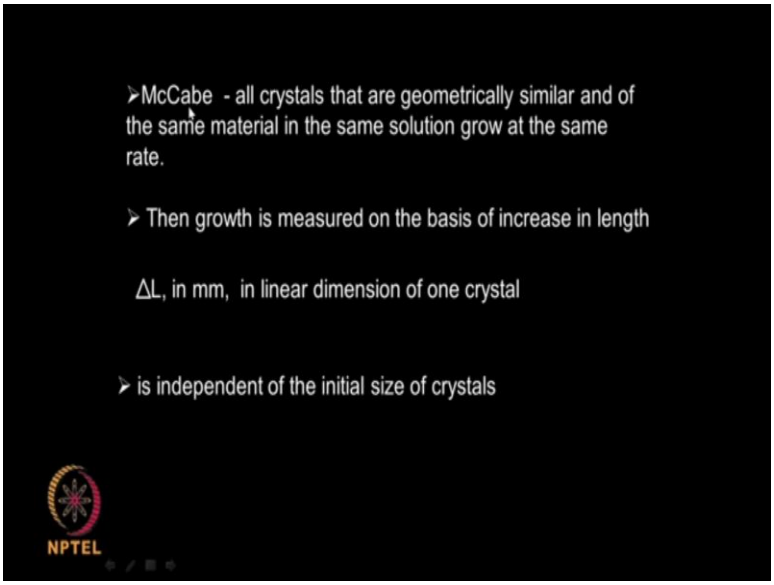

McCabe, according to McCabe, all crystals that are geometrically similar and of the same material in the same solution grow at the same rate okay, so the growth is measured on the basis of increase in length, delta l, it could be in mm, so linear dimension of one crystal and is independent of the initial size of the crystal. So according to McCabe he said it will all depend upon only the delta l, that is the change in the length and it will have no effect on the size of the crystal okay and that is called delta l law.

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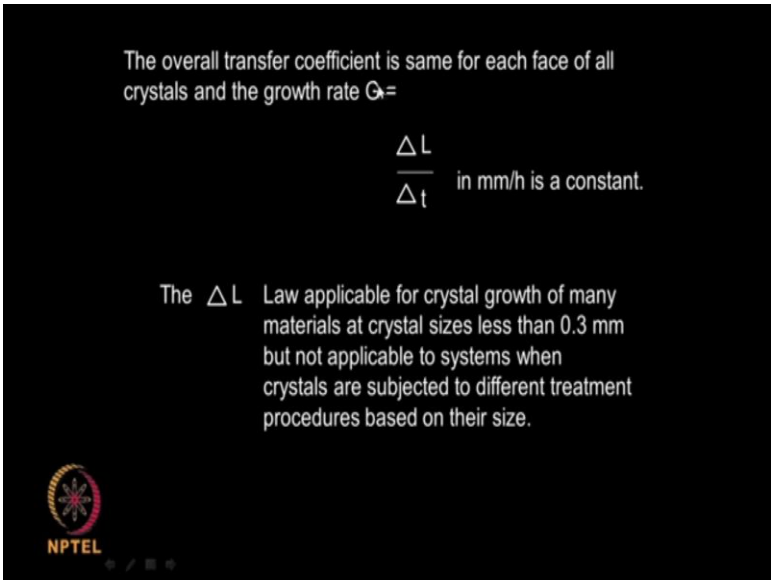

So the overall transfer coefficient is same for each phase of the crystal and the growth rate g, growth rate g is given by delta l / delta t. Delta l is the increase in the size that is in mm, delta t is a change in time, so this is growth rate mm per hour and he said it is always a constant, okay, this is called delta l law. So if you use this law it becomes easy for us to monitor rate of crystal growth but generally this law holds good only for crystals of small size no less than 0.3 mm okay.

The crystals are very large above 0.3 or if you are subjecting it for different treatment procedures then this delta l law doesn't hold good but the advantage of delta l law is it does not depend upon the actual crystal size but the rate of growth is constant. It depends on change in the length as a with respect to change in time delta l / delta t. So that is the advantage of this delta l law.

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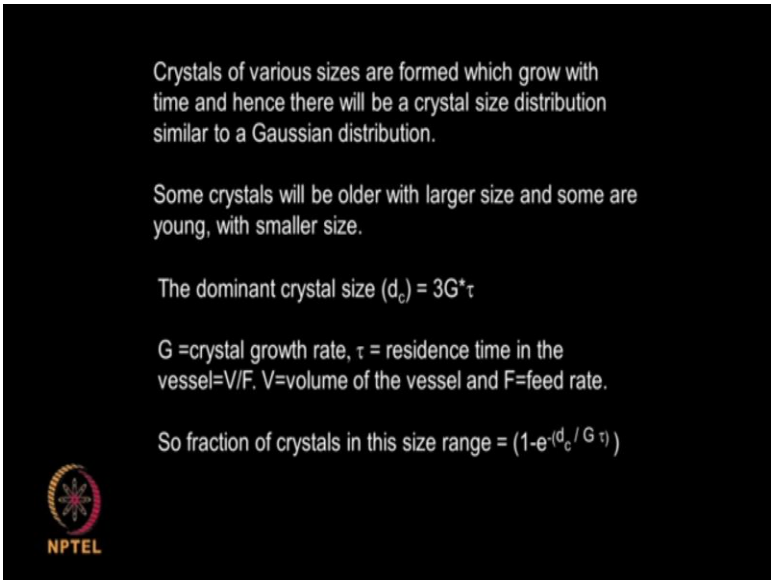

So crystals of various sizes are formed, as you know some crystals may smaller, some crystals may be larger and there will be a crystal size distribution, generally we can call it a Gaussian distribution okay and there will be some average crystal size, okay because some crystals will be older with larger crystal size some crystals will be younger with smaller size okay. So the dominant crystal size dc is given by 3 into G* tow G is the crystal growth rate and if you follow delta l law it will be = delta l / delta t.

Tow is residence time in the vessel. So how long it is going to be there okay that is given by volume by flow rate v /flow. So this is the dominant crystal size, okay the crystals that will be predominantly present there. So fraction of crystals in this crystal will be 1-e - dc by G tow okay this is the formula for this, so the fraction of the crystals that will be present in that in that actually okay.

So advantages of these equations are, we can use this for finding out crystal growth, we can use this for finding out the draw dominant crystal size also, right, that is the advantage of this type of relationship. So we saw 2 important downstream here, one is the precipitation where it is widely used for recovering proteins from mother liquor. It is very very common where proteins are

recovered collected using either adding salts or changing the dielectric constant of the solution by adding some solvent.

The other one is precipitation, where you use a technique where the solids come out in the form of crystals in the uniform size and shape and it starts growing, especially pharmaceutical industries, food industries, use this type of crystallization process quite a lot.

Thank you very much for your time.