Principles of downstream techniques in Bioprocess – a short course
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Lecture – 10
Reverse Micellar And Aqueous Two Phase Extraction

In this class, we are going to talk about reverse micellar extraction and aqueous two phase extraction. Both involves water as you know biomolecules like proteins or enzymes they can be easily denatured if you are using a solvent. When you are using a solvent type of extraction, so if you are using water, then those biomolecules will retain its activity and that is where these types of reverse micellar and aqueous two phase extraction techniques are very very useful.

That means that phases are only water, so there is no chance of those biomolecules getting denatured or degraded. so let us first understand what is a surfactant okay.

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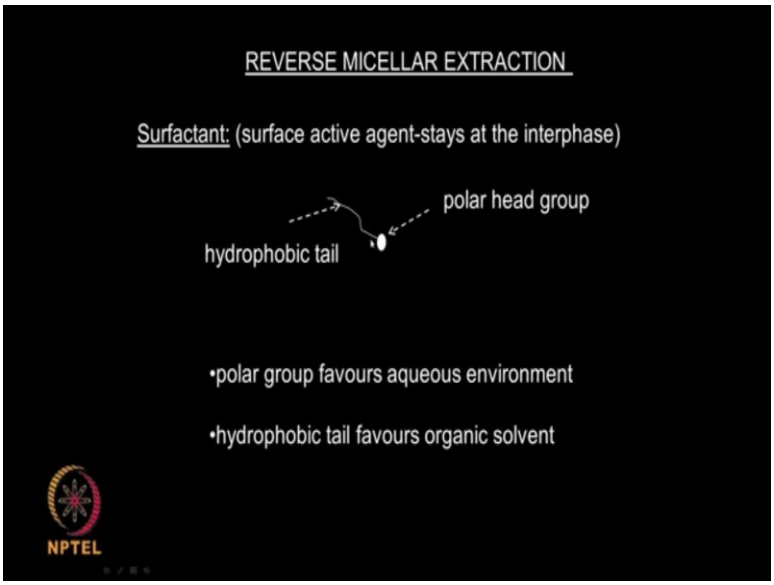

Surfactant is a surface active agents and it contains hydrophobic tail okay and it could be long-chain hydrocarbons or sometimes it could be an automatic group and it will have a polar head group. It may have oxygen nitrogen and so on so this portion will always like water and this portion will like organic solvent. So these surfactants or amphiphillic molecules, they are always found in the interface between water and any solvent.

So that is the advantage of these type of compounds, which have a polar group and a long hydrophobic tails. So there are large number of surfactants. There are surfactants that are neutral. There are anionic surfactants, cationic surfactants. Surfactants have become part of our life, even your washing up liquid, your dishwasher. So is soap everything contains this type of surface active agents, so they can in interface between an oil layer and water layer.

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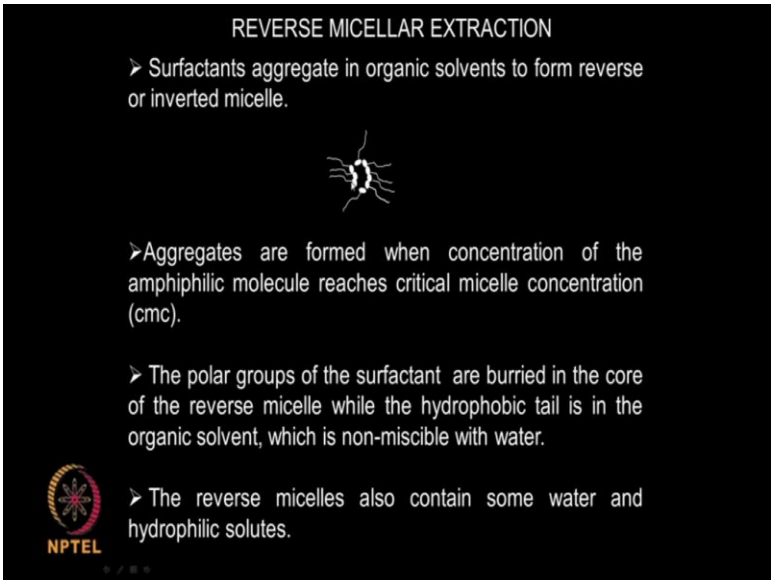

So surfactants are used in reverse micellar type of extraction, so how does it work? So surfactants aggregate in organic solvents, so suppose I put these in organic solvents, they will aggregate. So all the polar groups will come together and form sort of a cavity okay and the all the tails which are hydrophobic will be outside and this happens especially at a concentration which is called critical micellar concentration CMC.

So at lower concentration, it is not enough to form aggregate. But around that concentration, critical micellar concentration, they form an aggregate. So what happens is you get polar pockets and the tails which are hydrophobic point outside. So proteins or enzymes could be captured in these polar pockets because water also will be present inside this polar pockets actually. So the reverse micelle systems will contain some water inside as well as some hydrophilic solutes.

Like that can contain even hydro biomolecules and so on so that way we can transport biomolecules without getting worried that they will get denatured in a hydrocarbon or a hydrophobic environment this is what is called a reverse micelle.

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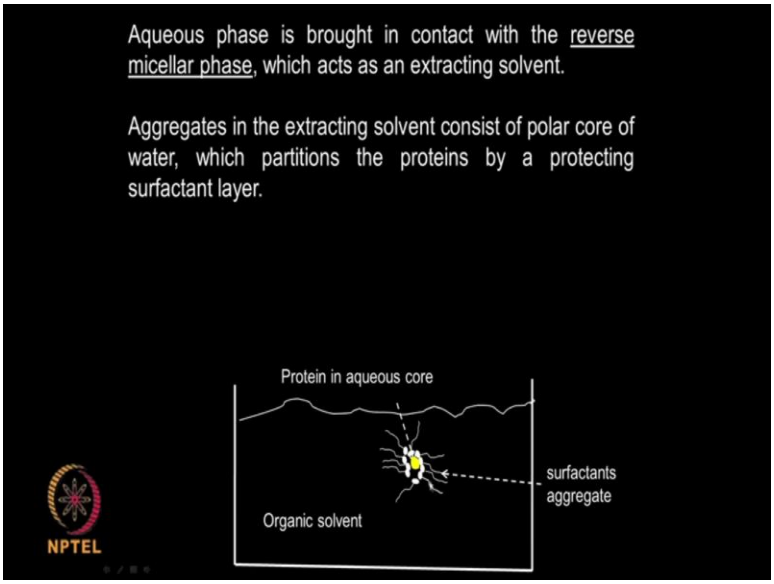

So what do we do? We have the organic solvent all these surfactant aggregate, the polar groups all pointing inwards. So inside you have a polar cavity, so the protein and the water will be captured inside actually and this is what is called reverse micellar phase. And this can act as a extracting solvent, so it will have a polar code that is what it is actually a polar code. So I can use different types of surfactants, surfactants which will form big hydrophilic cores, surfactants which will form small polar cores and so on actually.

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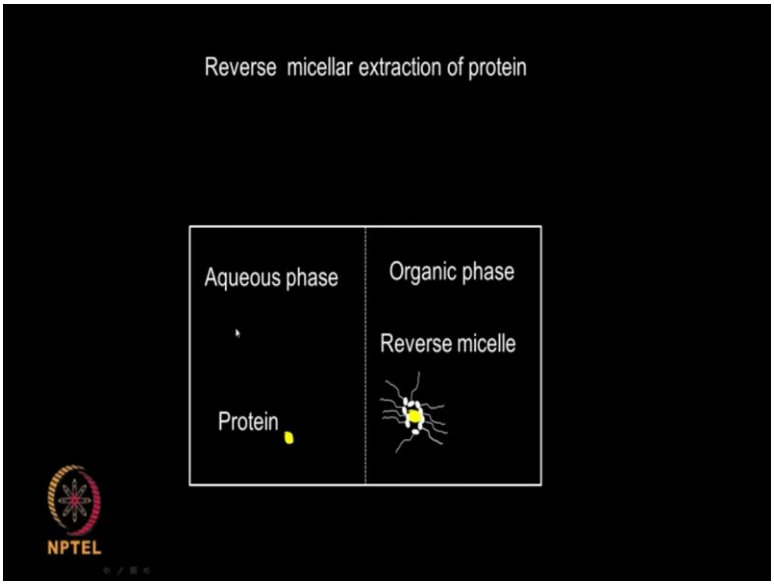

So what do I do? I have the aqueous phase, I have the protein here, then I have the organic phase. So I can have in the organic phase reverse micelle of surfactants and the proteins are inside the reverse micelle.

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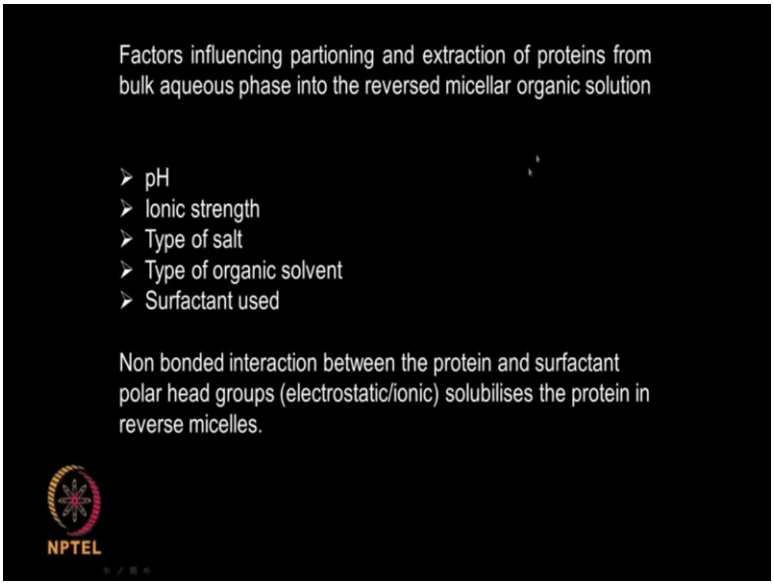

So what are the factors that influences partitioning and extraction of proteins from bulk aqueous phase into the reverse micellar organic solution? So basically you need to know that the protein originally are in aqueous phase and you have taken these proteins inside the organic phase. If I do not do it in a reverse micellar form the proteins will get denatured because they have come

into the organic phase. But having this type of reverse micelle, I am maintaining their aqueous environment inside the polar cavity.

So what are the factors that affect this? the pH, the ionic strength, type of salt I use, type of organic solvent I have, type of surfactant I use and so on actually. So there will be lot of non-bonded interaction between the proteins and the surfactant polar head groups could be electrostatic ionic. If the surfactant is ionic it could be ionic forces and so on actually these are the different type of forces that act,.

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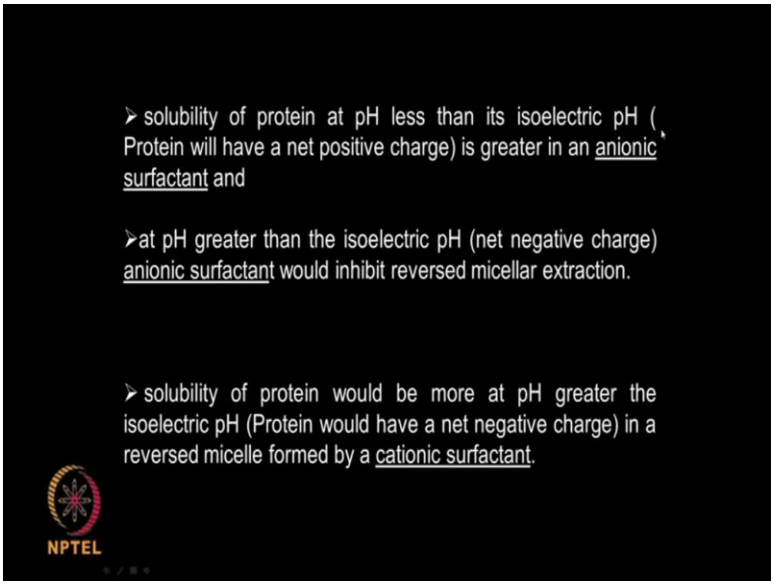

So the solubility of protein at pH less than its isoelectric pH. So you all you all know what is isoelectric pH right? That is the pH where the protein will not have any charge okay. That is the isoelectric pH but when the pH is less than the isoelectric pH, then the protein will have the positive charge. Now then it is greater in an anionic surfactant. So if you are working with the pH less than the isoelectric pH of the protein, protein will have a positive charge.

So it is better to have a anionic surfactant, if the pH is greater than the isoelectric pH okay. So then it will have a negative charge and then anionic surface will inhibit reverse micellar. Solubility of protein would be more at pH greater than isoelectric pH, so if it is greater than isoelectric pH, then what happens protein will have a negative charge okay, then it is good to have cationic surfactant.

So depending upon the pH, whether I am operating below the isoelectric pH or whether I am operating above the isoelectric pH. So if I am operating below the isoelectric pH, it is good to have anionic surfactant. If I am operating above then it is better to use cationic surfactant so pH effect is essential.

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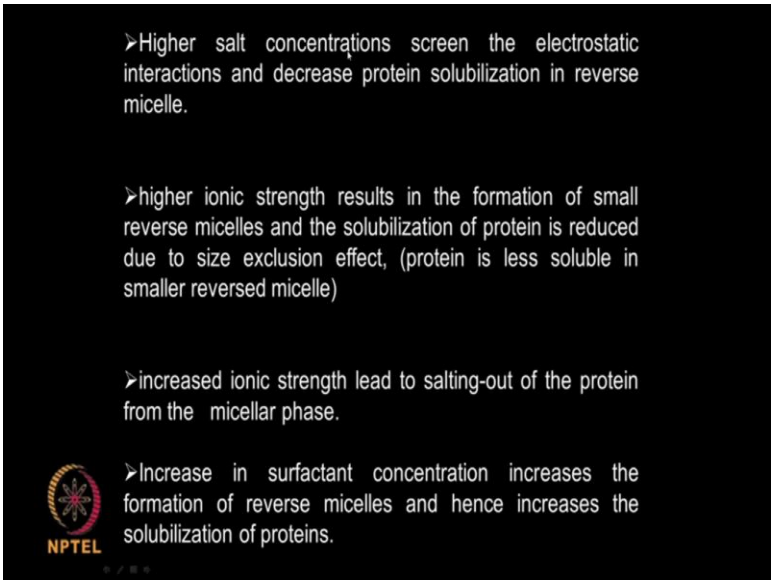

Higher salt concentration, if I am using very high salt concentration, they will screen the electrostatic interaction, so it will decrease protein solubilization. Higher ionic strengths results in small reverse micelle and protein solubilization is reduced because of the size. Size of these the polar pockets are very very small because protein will not go because inside because the size effect. So increased ionic strength leads to salting out of the protein also from the micellar phase, increase in surfactant concentration increases the formation of reverse micelle.

Hence it also increases the solubilization of protein. So I can have higher surfactant concentration, I can solublize more protein. If I use higher salt obviously, I am going to end up having the salt sort of screening the interaction between the protein and the surfactant molecule okay. But I have too much ionic strength, there could be salting out of the protein. So I can sort of balance between all these ionic strength, the salt concentration and the surfactant concentration.

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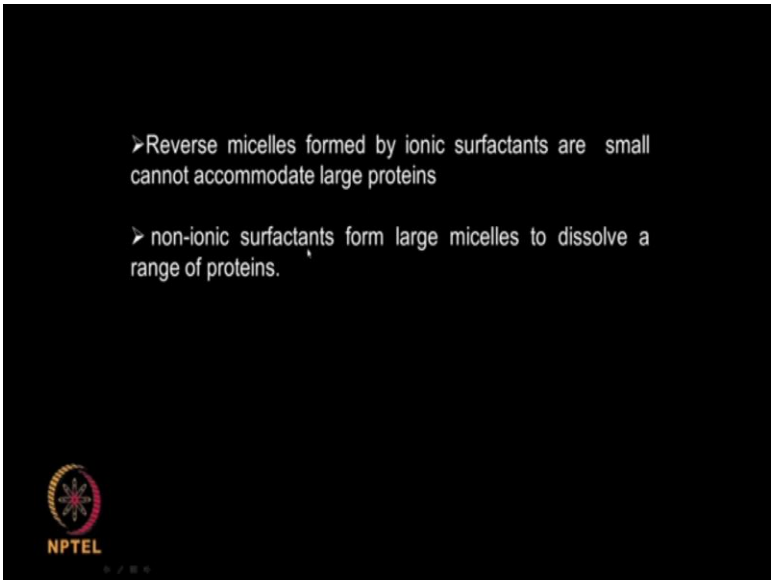

Reverse micelle formed by ionic surfactants are small, so it cannot accommodate larger proteins. Whereas nonionic surfactants can form large micelle, so we can have larger amount of proteins okay. So if I am nonionic surfactant, then I can have larger micelle but then I may select ionic surfactants based on, whether the proteins has net positive charge or negative charge, so that the capture of those proteins by these surfactant is also enhanced okay.

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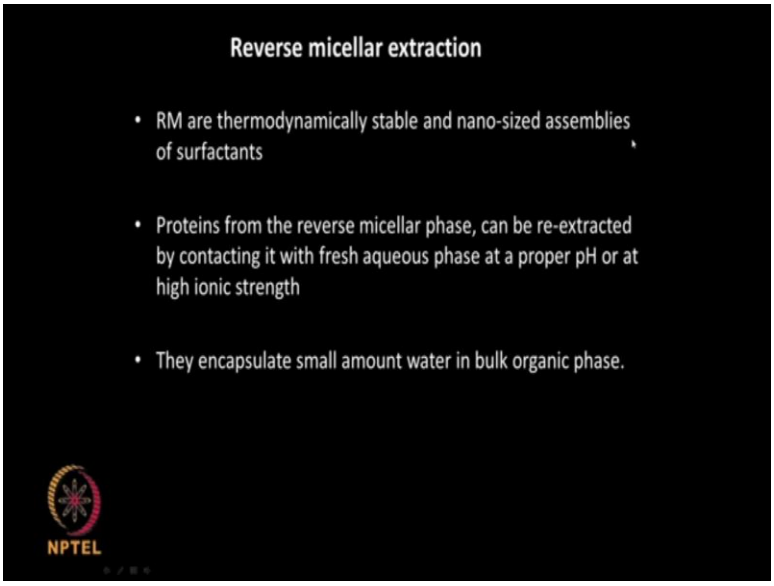

Then reverse micelle are thermodynamically stable and they are nano-sized assemblies of surfactants. How do you re-extract? I can re-extract by adding fresh aqueous at pH, at proper high ionic strength, okay. They encapsulate small amount of water in bulk organic phase okay so

because I need to again re-extract the entire protein which has been taken using a reverse micelle system. So

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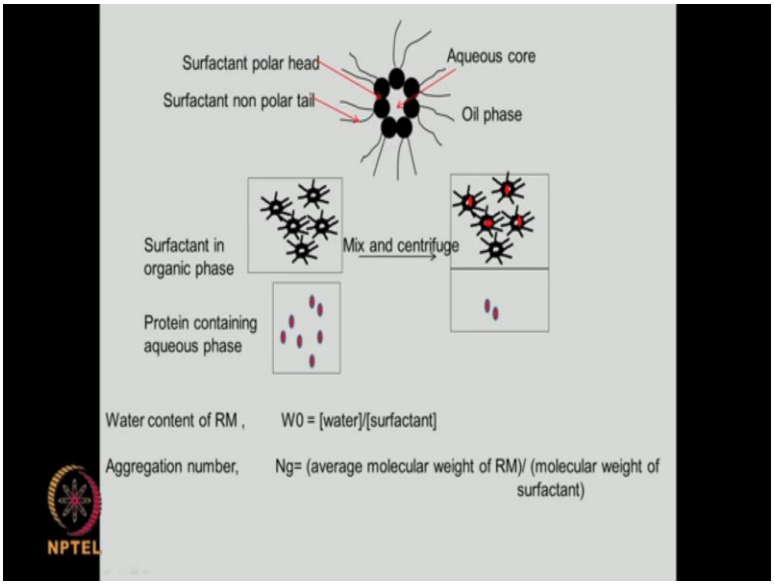

For example I have surfactants like this and then they are forming a core inside, they have the oil phase, the core is the polar core or aqueous core. So the surfactant in the organic phase so it gets taken in and then later on we use mixer and centrifuge and so on. So that as much of proteins is captured inside actually. So water content of reverse micelle, we will say water divided by amount of surfactant, there is some term called aggregation number. Okay.

So this aggregation number is average molecular weight of reverse micelle divided by molecular weight of the surfactant okay these are some numbers which you may have to remember.

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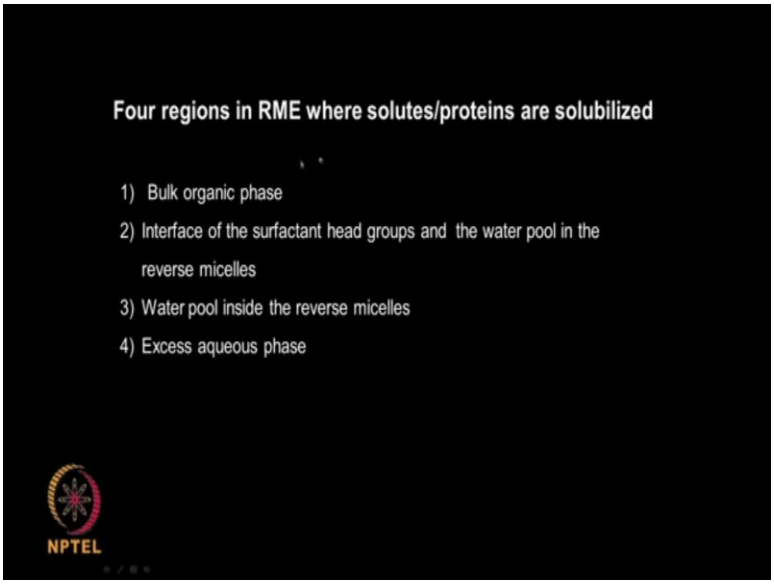

So four regions where reverse micelle will have the solutes of the protein either bulk organic phase interface of the surfactant head groups and the water pool in the reverse micelle water pool inside the reverse micelle excess aqueous phase. So all these places you may have the solute or the protein present.

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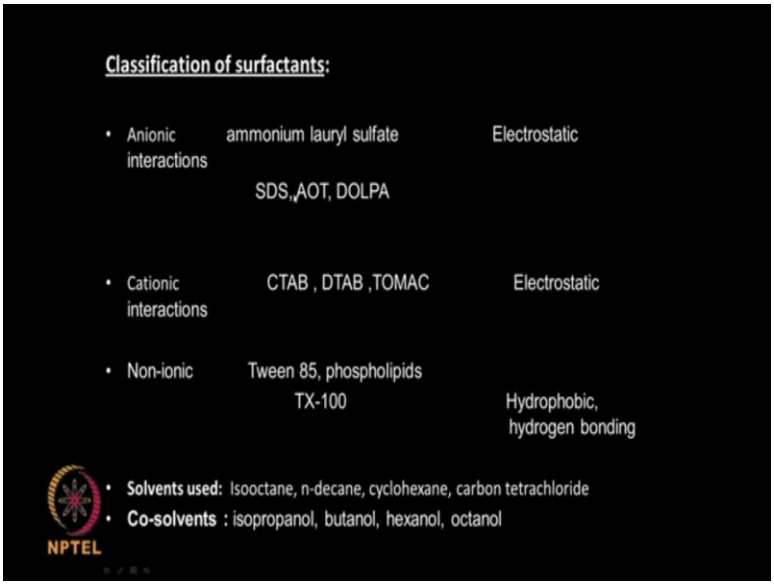

Okay so how do you classify these surfactants? Anionic, cationic, nonionic. So anionic, ammonium lauryl sulfate, SDS, sodium dodecyl, AOT, DOLPA. Cationic could be CTAB, DTAB, TOMAC. Nonionic. Tween 85, TX-100, phospholipids and so on. So we have three types and generally with the ionic or anionic or cationic. You have electrostatic forces whereas

nonionic there is no electric charge. So generally this is hydrophobic and hydrogen bond type of nonbonding. So what are the solvents we use?

We use isooctane, n-decane, cyclohexane, carbon tetrachloride, sometime you may use small co solvent like isopropanol, butanol, hexanol, octanol and so on actually. So you can see the type of surfactants that are used and the type of solvent and co solvent used actually.

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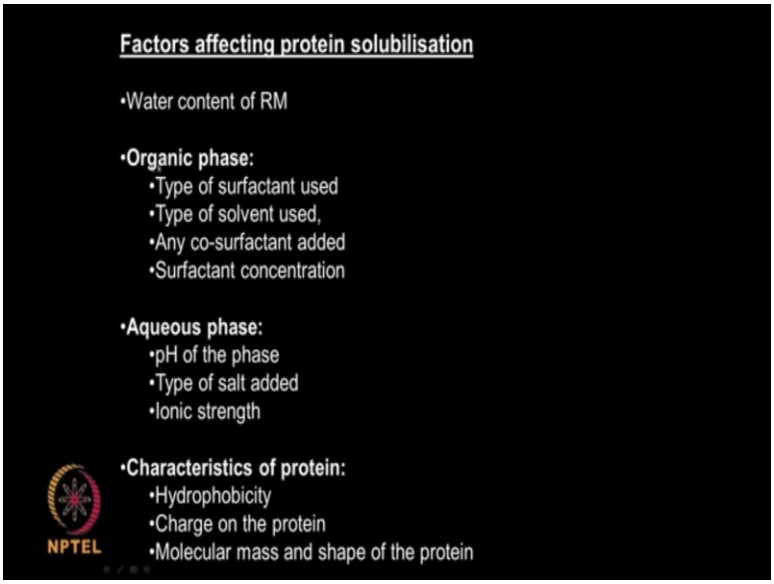

So what are the factors that affect the protein solubility? The water content in reverse micelle, the type of organic phase, the type of aqueous phase and characteristics of the protein will also come in picture. So the organic phase will say type of surfactant used, type of solvent used, any cosolvent used, surfactant concentration. If you take aqueous, what is the pH type of salt of used, what is the ionic strength, if you take the characteristics of protein, whether the protein is hydrophobic or hydrophilic charge on the protein molecular mass that means how bulky it is, shape of the protein so all these affect.

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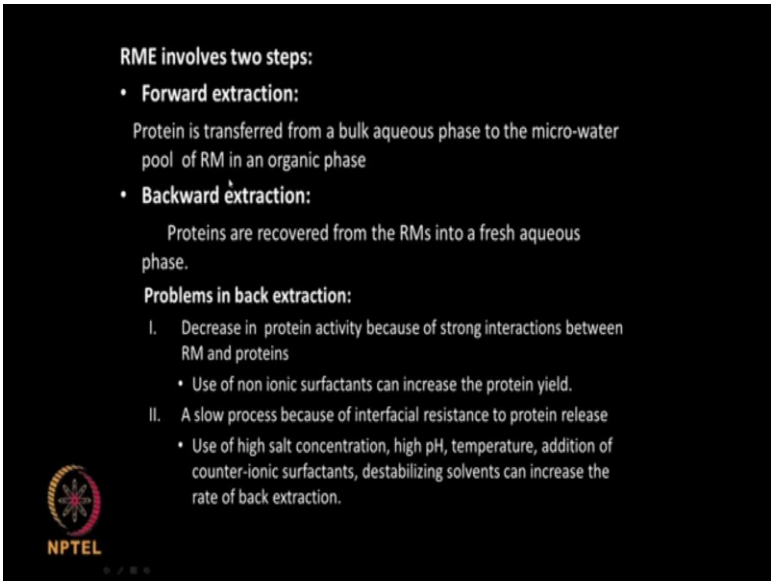

So it involves two steps one is the forward other one is the backward okay. Backward, forward backward, so protein is transferred from the bulk aqueous phase into the micro water pool in the organic phase and then protein is taken back into the fresh aqueous phase okay. So what are the problems in back extraction because decrease in protein activity because you have put in reverse micelle in surfactant, then again you are re-extracting in the aqueous phase so the activity of the protein will lose.

Use of non ionic surfactant can increase the protein yields, the slow process this is a very slow process, the backward reaction because of the interfacial resistance to protein to get released. So we can use high salt concentration high pH temperature, addition of counter ionic surfactants, destabilizing solvents, all these we can add. Here you have to be very careful that you do not reduce the activity of the protein which you have extracted originally okay. That is very important.

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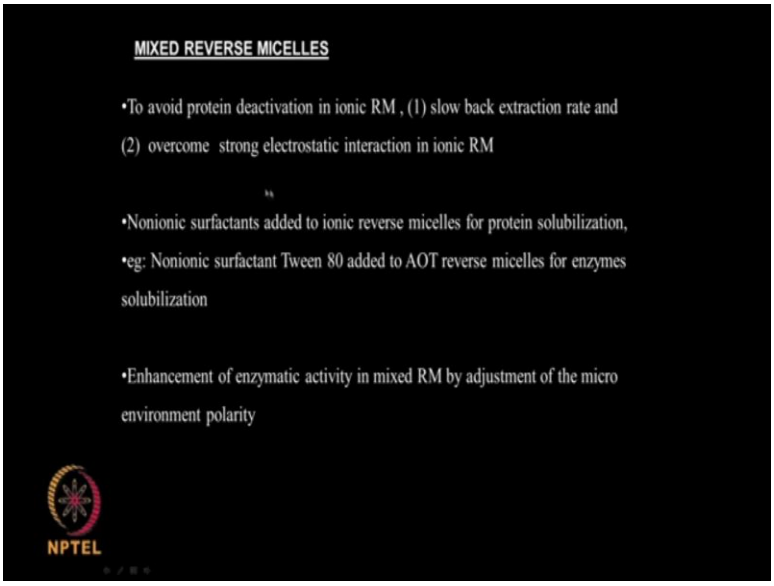

You can also have mixed reverse micelle. To avoid protein deactivation in ionic reverse micelle, you can have slow back extraction rate and we can have over strong especially to overcome strong electrostatic interaction in ionic reverse micelle okay. So we can use non ionic surfactants added to ionic reverse micelle for protein solubilization. This nonionic could be Tween 80 AOT and so on enhancement of enzymatic activity in mixed reverse micelle by adjustment of the micro environment polarity okay. So that also can be done by adding non ionic surfactants.

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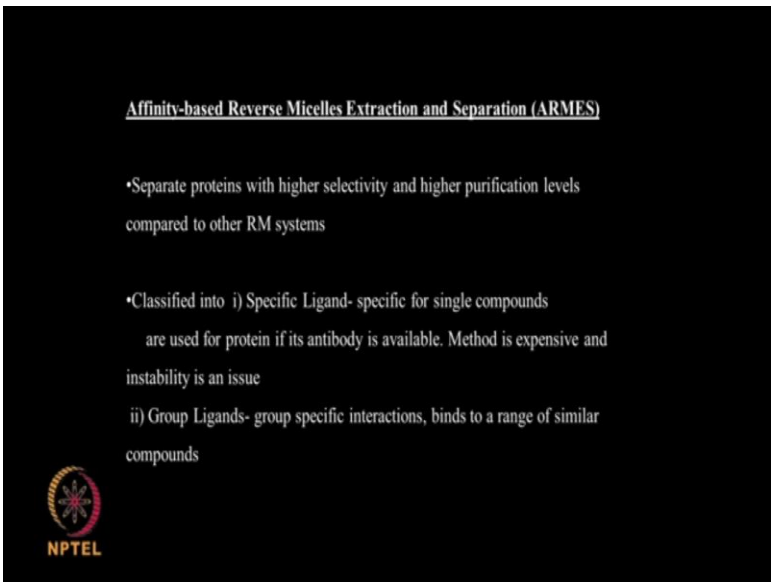

Then there is something called affinity based reverse micelle. So what do we do? We add a affinity ligand, so that the selectivity can be enhanced that means there will be an affinity ligand in the which will specifically capture only the protein of our interest. Okay. Ligand protein

interaction so we can separate proteins with higher selectivity and higher purification by adding an affinity ligand during the reverse micelle process actually.

So this is affinity based reverse micelle. So this can be specific ligand specific for simple single compound to select the proteins. If it is antibodies available, if it is very expensive group ligands that is group specific interactions binds to range of similar compounds okay.

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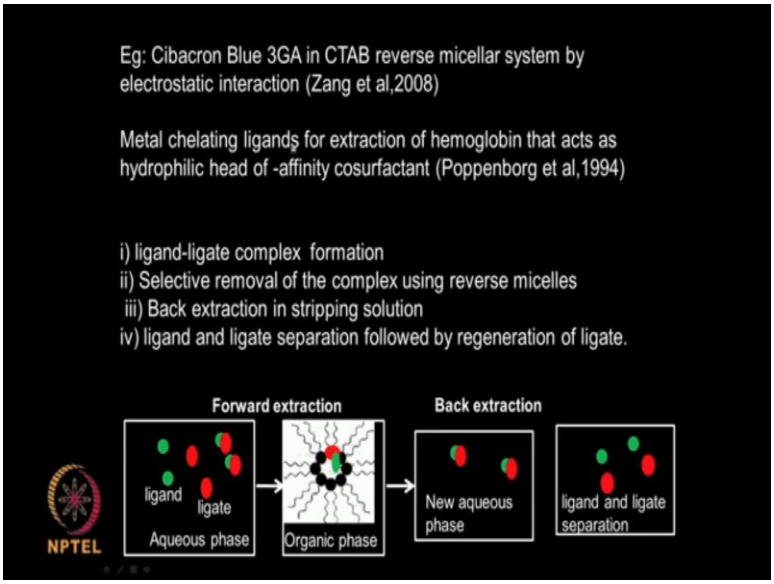

So that is what it is. For example metal chelating ligand for extraction of hemoglobin that acts as hydrophilic head of affinity cosurfactant. That is one idea okay so ligand-ligate complex formation okay then you have selective removal of the complex using reverse micelle okay then back extraction in stripping solution and then you have the ligand in the ligate. That is very important so initially, we have the ligand-ligate they form the complex

And then we go into the extraction process. This is the normal aqueous two phase extraction, then you have the back extraction. Okay then you have to separate the ligand and ligate. So four steps are there okay but then you can get very protein of your interest from mixture of proteins by having the proper ligand, which are very specific for that protein that is the advantage.

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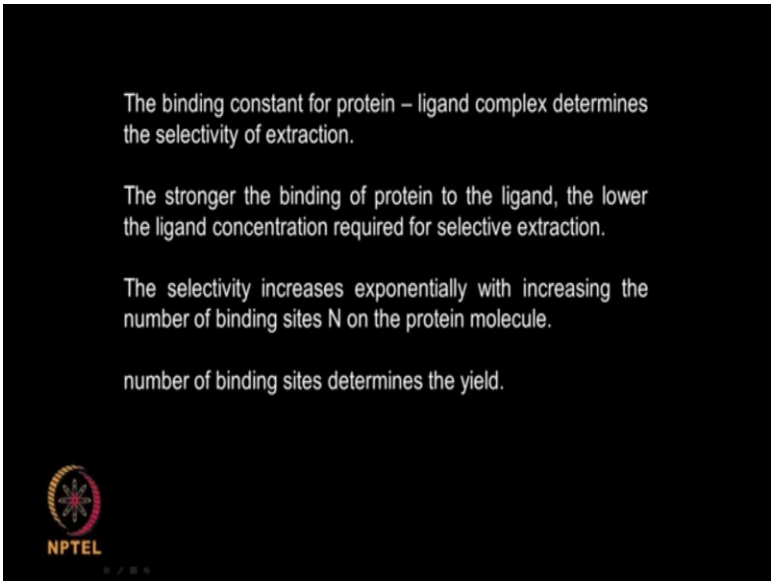

So the binding constant for protein-ligand complex for the selectivity of extraction, the stronger the binding of protein to the ligand, the lower the ligand concentration required for selective extraction. This is obvious the selectivity increases exponentially with increasing the number of binding size of the protein molecules. So number of binding sites determines the yield. So the same ligand if it can bind on n sites then your extraction efficiency also goes up by that factor okay.

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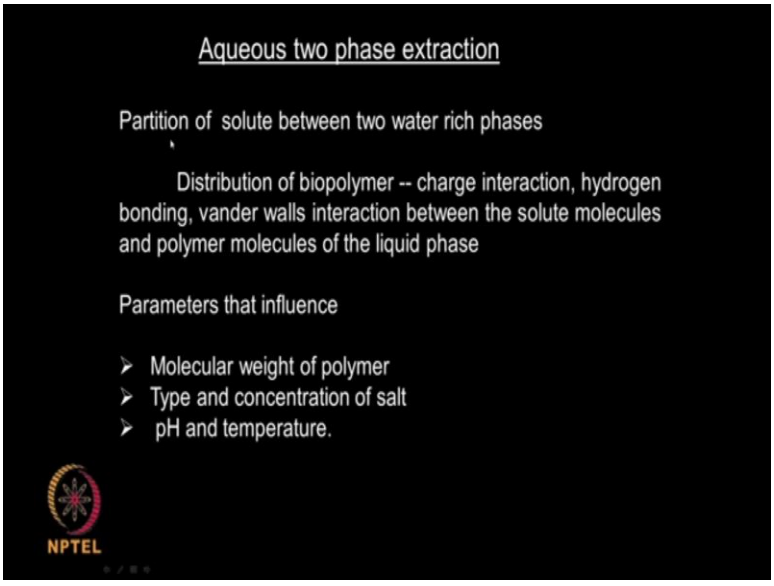

Then let us come to something called aqueous two phase extraction okay. So then we looked at the reverse micelle aqueous two phase, imagine we have a protein in one phase, I want to extract

with another aqueous phase. That is very challenging right. So how do you do that? We have a polymer solution added to one of the phases and we have the salt and we have the solute in one phase. So the addition of polymer affects the partitioning of the protein from one phase to another. So we have different types of biopolymers.

So the partition of solute between two water rich phases distribution of biopolymers, so it could be charge interaction hydrogen bond liquid phase, Vander Waal between the solute molecules and the polymer molecules. So what determines the separation? Molecular weight of the polymer type and concentration of salt pH and temperature okay so I can have different types of polymer like pg or dextran glucagon in one phase and in another phase where the solute is present contains salt. So the solute partitions into the polymer phase. That is what happens in the aqueous two phase extraction.

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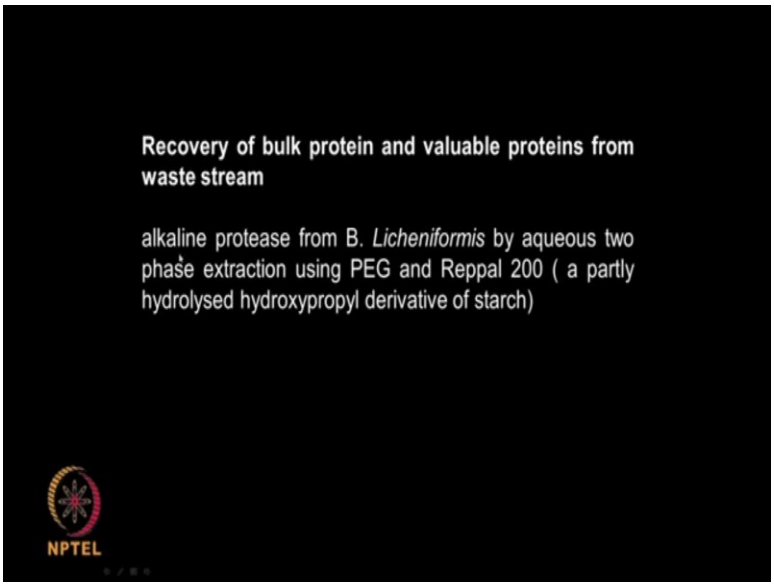

So recovery of bulk protein and valuable from waste stream for example alkaline, protease, from b.licheniformis, by aqueous two phase extraction, using PEG, so we are using PEG as your polymeric solution here.

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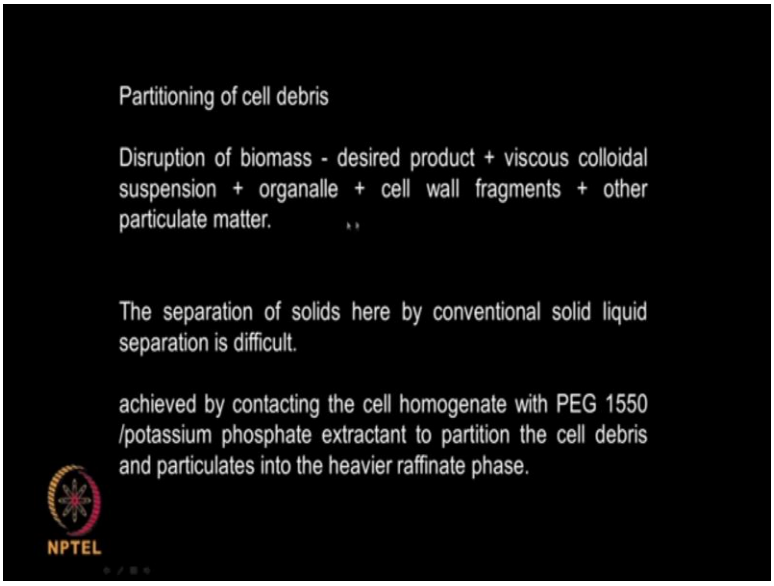

So partitioning of cell debris disruption of biomass then you have the desired product the viscous colloidal suspension organelles cell wall fragments and other. So the separation of solids by conventional solid-liquid separation is difficult whereas we can achieve by contacting the cell homogenates with PEG and potassium phosphate extractant to partition the cell debris and particulates into the heavier raffinate.

So normally we used to say that after when you have the cell debris and cell wall fragments, you can do a centrifugation or we can a do a to remove cell debris of the solids but sometimes, it may become very very difficult because of the viscosity. So then we can use this type of two phase aqueous two phase extraction using PEG so all the cell debris and other particulate matter can go into that phase leaving your desired product in the mother liquor okay.

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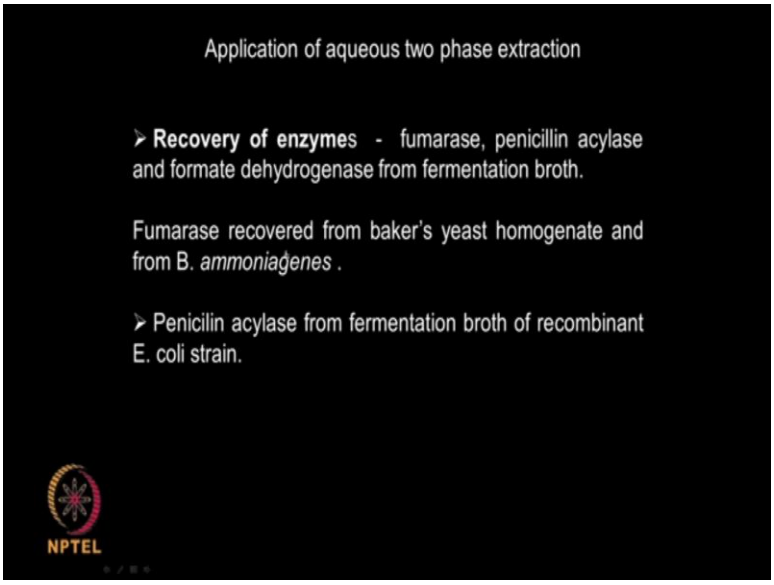

So application of aqueous two phase recovery of enzymes fumarase, penicillin, acylase, formate, dehydrogenase from fermentation broth. Fumarase recovered from baker's yeast, homogenate and penicillin isolates from fermentation broth of recombinant e.coli. So all these process are carried out in aqueous two phase extraction type of method.

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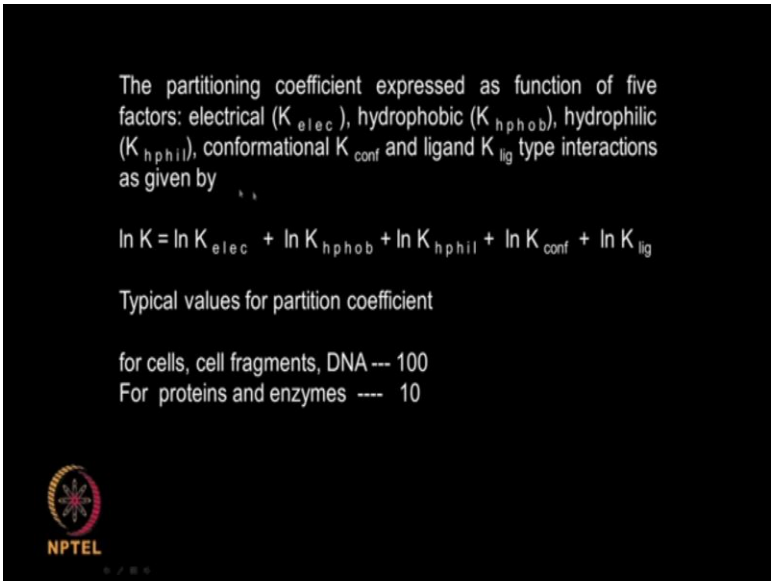

So the partition coefficient is expressed as function of 5 factors. You know electrical hydrophobic effect, hydrophilic effect, conformational effect and ligand type interaction. So that is how you calculate. So typical values for partition for cell, cell fragments DNA 100 for proteins

and enzymes 10. So what does that mean? So if I have a PEG solution, cells and debris will partition specific preferentially rather that the proteins and the enzymes

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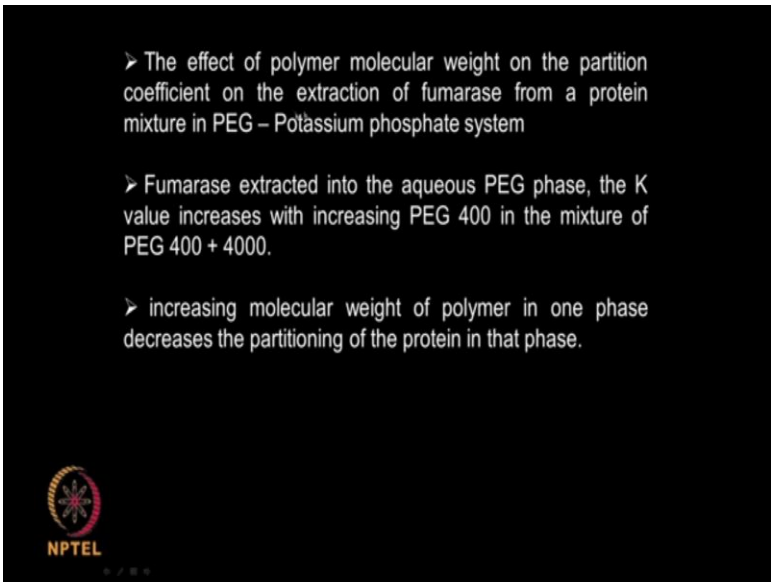

So the effect of polymer molecular weight of the partition coefficient is also there and if I am looking at extraction of fumarase from the protein mixture in PEG protection phosphate system fumarase extracted in the aqueous PEG phase the K value increases with the increasing PEG 400 okay. So if I put in more PEG 400 am able to increase the extraction of PEG. Whereas when I am increasing the molecular weight of the polymer it decreases the partitioning of the protein in that phase okay.

So if they can increase the molecular rate the partition coefficient decreases So if they keep lower molecular rate partition coefficient increases.

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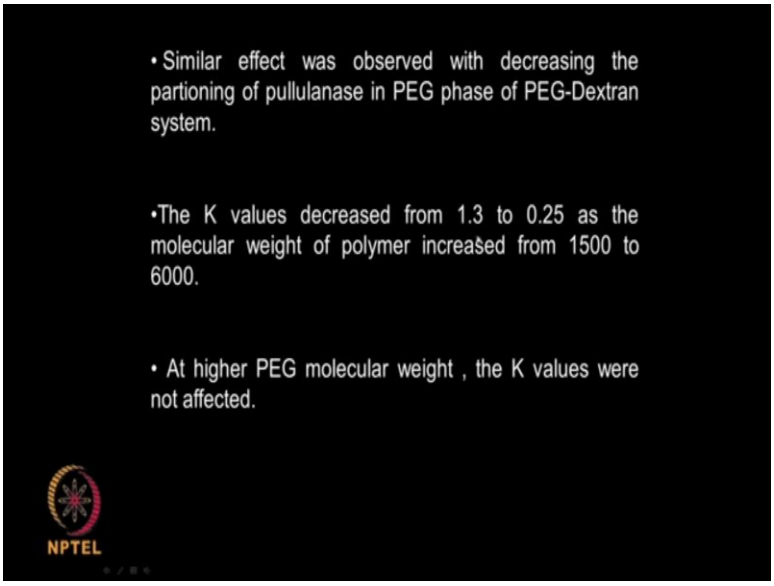

Similar effect was observed with decreasing the partitioning of pullalanase in PEG phase of PEG-dextran system the K value decrease from 1.3 to 0.25 as the molecular weight of the polymer is increased from 1500 to 6000. So at higher PEG molecular rate the K values are not affected so at higher but at certain range as I increase the molecular weight of PEG the K value keeps decreasing.

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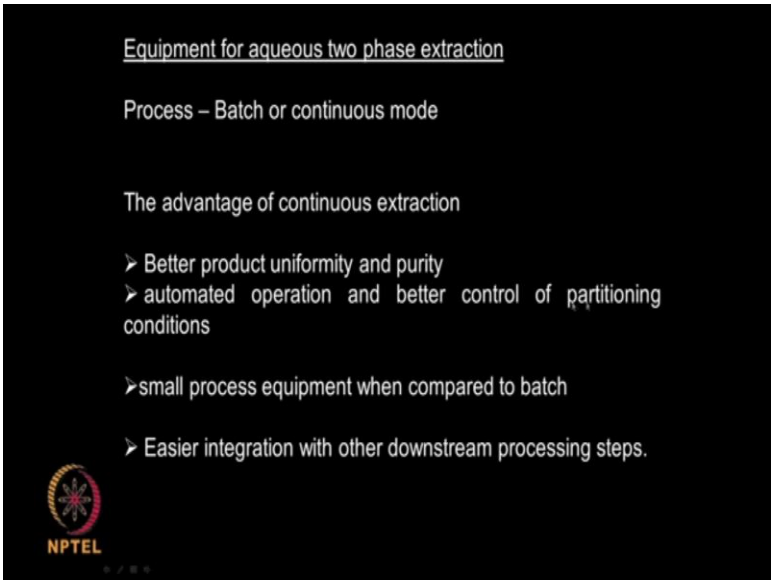

So what are the equipments for aqueous two phase? We can have batch or continuous mode, So continuous extraction better product uniformity and purity automated operation better control of partitioning condition can be a small process equipment when compared to batch. Batch I require very large expense, it is easier to integrate with other downstream process okay. So when I have

a continuous it is easy to integrate okay otherwise if it is a batch I need a storage vessel where I keep the product and then,

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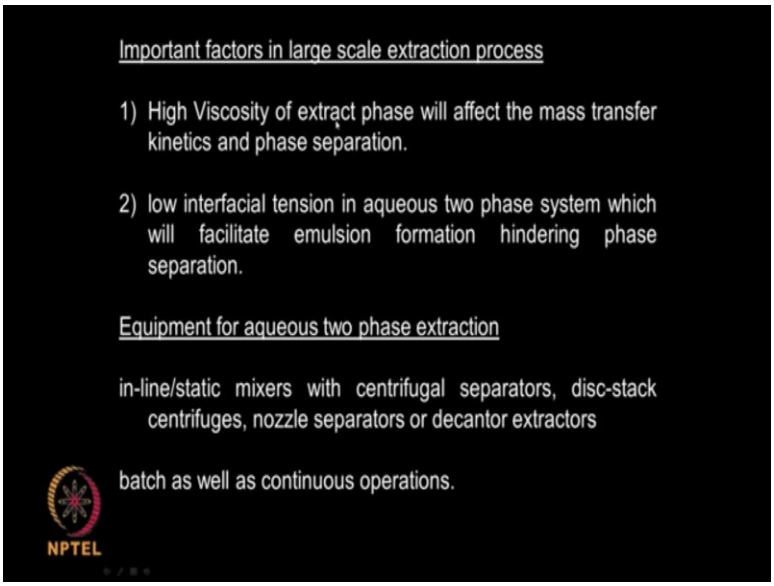

High viscosity of extract phase will affect the mass transfer kinetics and the phase separation as you know viscosity affects the diffusion coefficient hence the mass transfer coefficient. Low interfacial tension in aqueous two phase system which will facilitate emulsion formation hindering phase separation. So the equipments inline static mixtures with centrifugal separations disc stack centrifuges nozzle separators or decantor extractors batch well as well as continuous operations in all these actually.

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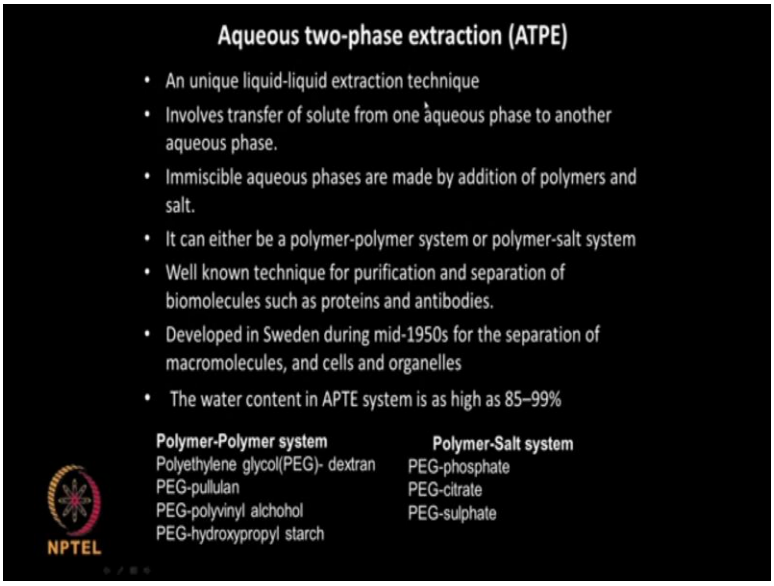

So it very good because it involves transfer of solute from one aqueous to another as I said invisible aqueous phases, as I said that is very important phases have to be invisible. So you may have polymer in one phase salt in front phase, it will be invisible h it can be either be a polymer-polymer system or a polymer-salt system. So as I said it is very useful for biomolecules. So it was very old developed very long time back but then it became popular only recently because of large biomolecules which needs to be removed without losing its activity.

The water activity content in aqueous systems is as high as 85 to 99, so type of polymer we used polyethylene, glycol, PEG and polyethylene glycol, pullulan, PEG-pva, PEG-hydroxypropyl, starch. If you look here for polymer-salt, PEG, phosphate PEG-citrate PEG-sulphate. So these are very common as you can see PEG is very very common that is widely used because it is very hydrophilic and does not affect the protein systems.

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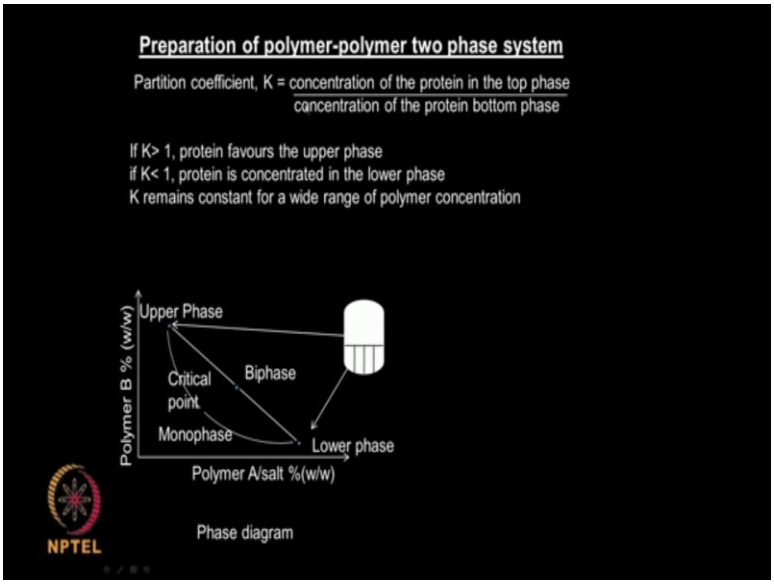

So there is something term called prep partition coefficient K that is the concentration of the protein in the top phase by concentration of protein in the bottom phase. So if K is protein favors the upper phase obviously right if K is 1 less than 1 protein favors the bottom phase. Now K remains constant over a wide range of polymer concentration and this is how the phase diagram will look like polymer and a and salt-polymer b okay. So we have polymer or salt here or polymer b here.

So this portion will remain biphasic this portion will remain monophasic so we want to work at biphasic region here for the upper phase and for the lower phase.. So our goal is to work at biphasic so that after the extraction we can separate the phases actually we do not want to work below this. So this is called the critical point okay so we need to remember that.

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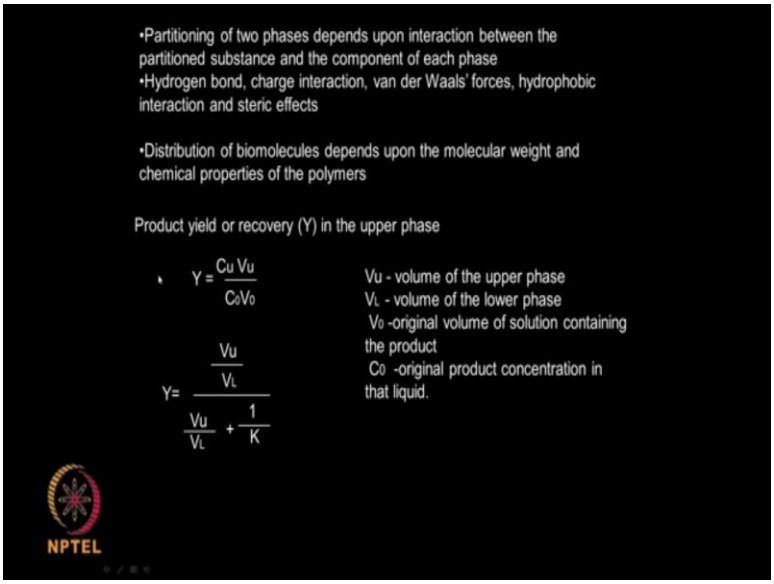

There are some terms which we need to remember one is the product yield or recovery in the upper phase so Y yield is equal to concentration in the upper phase volume of the upper phase divided by concentration total concentration containing original volume of V0. So I we modify this equation this become Y equal to Vu volume of the upper phase by volume of lower phase/volume of upper phase by volume of lower phase + 1/K. K is the partition coefficient so this is the useful relationship.

So if I know the partition coefficient, I can decide on what could be my ration Vu / Vl what should be the volume the upper phase to lower phase to achieve certain yield. I want 90 % yield, what should be the volume of upper and what should be the volume of lower phase. That sort of calculations I can do so that partitioning of two phases depends on the interaction between the portioned substance and the component of each phase.

So you have hydrogen bond charge interaction Vander Waals forces hydrophobic interactions steric effects, all these and the distribution of biomolecules depends on the molecular weight and chemical properties of the polymer. okay How much interaction it is offering towards the biomolecules.

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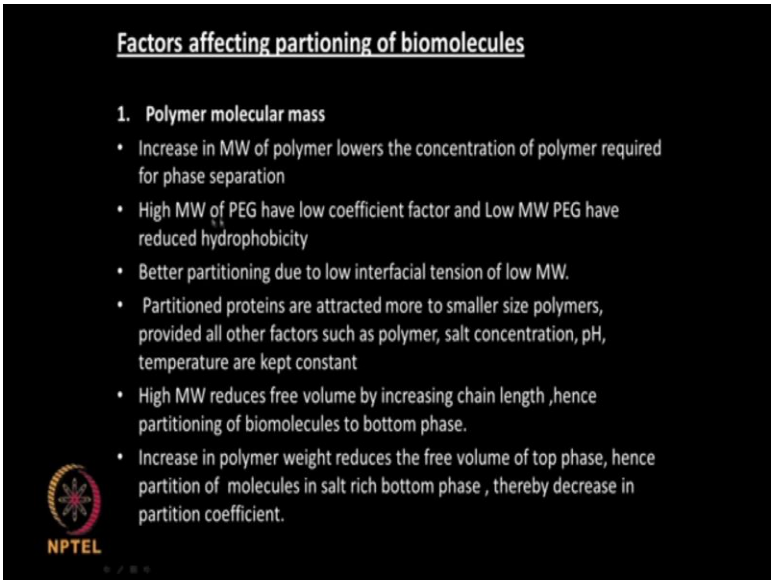

So what are the factors affecting portioning polymer molecular mass increasing molecule weight of polymers lowers the concentration. So high molecular weight of PEG have low coefficient factor, low molecular weight PEG reduces hydrophobicity. Better partitioning due to low inter facial tension of molecular weight partitioned proteins are attracted more to smaller size polymers high molecular weight protein reduces free volume by increasing the chain length hence partitioning of biomolecules to bottom phase.

Increase in polymer weight reduces the free volume of top phase hence partition of molecules in salt rich bottom phase okay.

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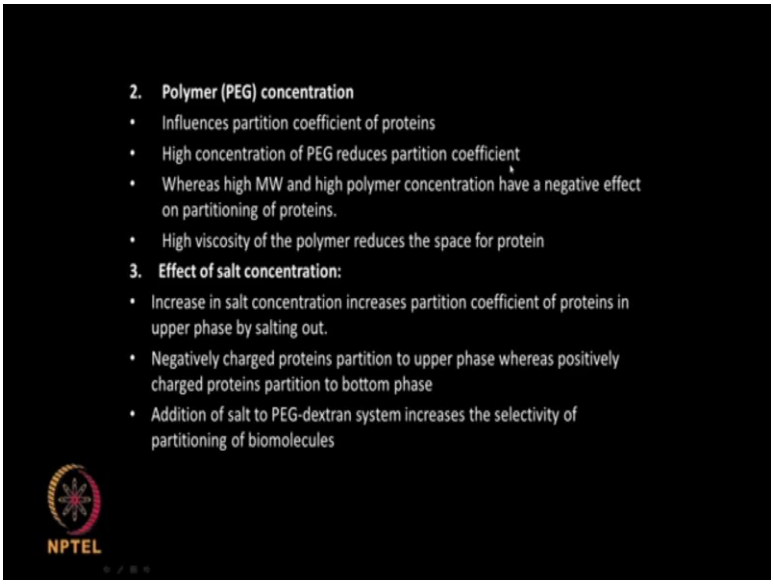

Polymer concentration influences partition coefficient of proteins high concentration reduces partition coefficient high molecular weight have a negative effect on partitioning. So if I use a high molecular weight protein and a high concentration then it will not do good. High viscosity of the polymer reduces the space for proteins, effect of salt, increase in salt concentration increases partition coefficient because it did it acts as a salting out.

Negatively charged proteins partition to upper phase whereas positively charged proteins to the bottom phase addition of salt PEG increases the selectivity of the partitioning okay.

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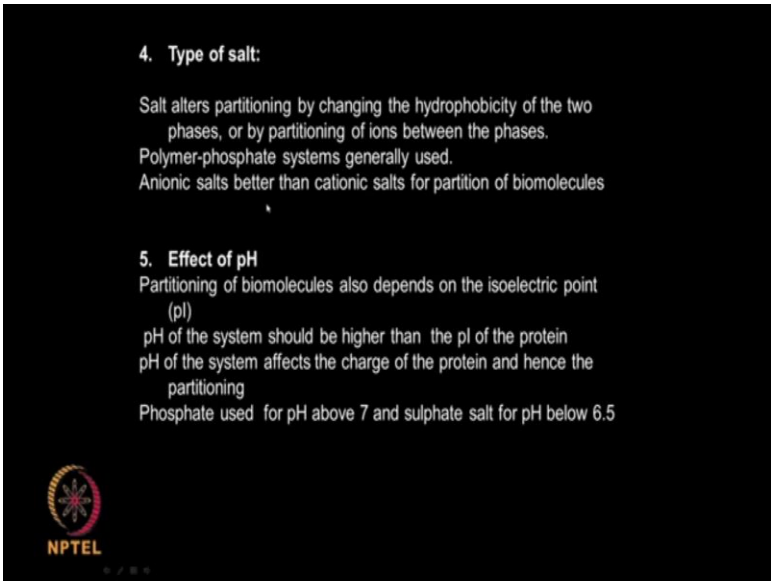

Types of salt, salt alters partitioning by changing the hydrophobicity of the two phases partitioning of ions right, so polymer phosphate systems are generally used sometimes anionic salts are better than cationic salts for partitioning biomolecules. Effect of pH partitioning of biomolecules also depend on the isoelectric point. It should be higher than the isoelectric point, the pH of the system affects the charge of the protein hence the partitioning the phosphates use for pH above 7 and sulphate salt for pH below 6.5 so if I am working at above 7 I will use phosphate and if it is below 6.5 I will use sulphate.

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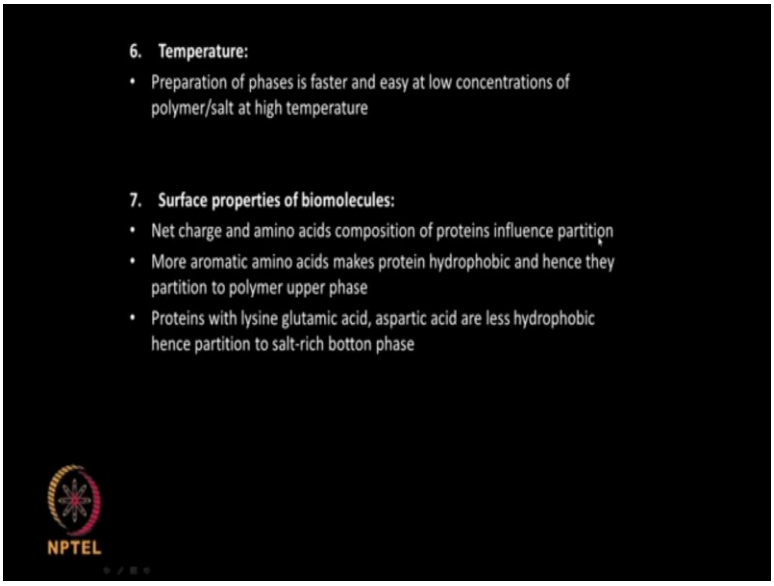

Temperature preparation of this is faster but then you have to be very careful that it does not degrade your denature your protein surface, properties of biomolecules the net charged amino acid composition of protein influence portioning. More aromatic amino acids make protein hydrophobic so they will partition to polymer phase. Protein with lysine, glutamic acid, aspartic acid are less hydrophobic.

So they will go to salt rich phase, so you will have to be careful okay what is the charge that is present on the protein surface properties.

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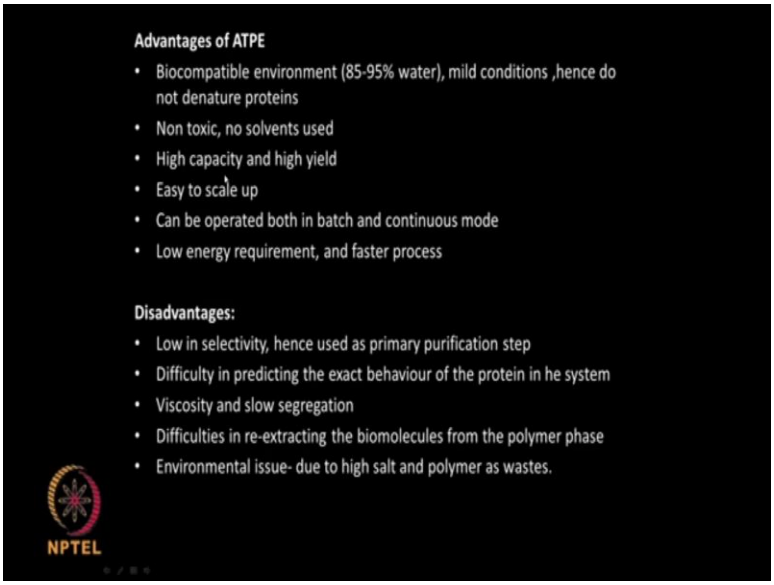

So it is biocompatible environment, non toxic, no solvents, high capacity, high yield, easy for scale up, can be operated in batch, low energy practically low energy. What are the disadvantages? Low in selectivity difficult in predicting the exact behavior viscosity and slow segregation because both are water, water difficulty in extracting. How do we re-extract then there are environmental issues because how do I dispose of this salt material?

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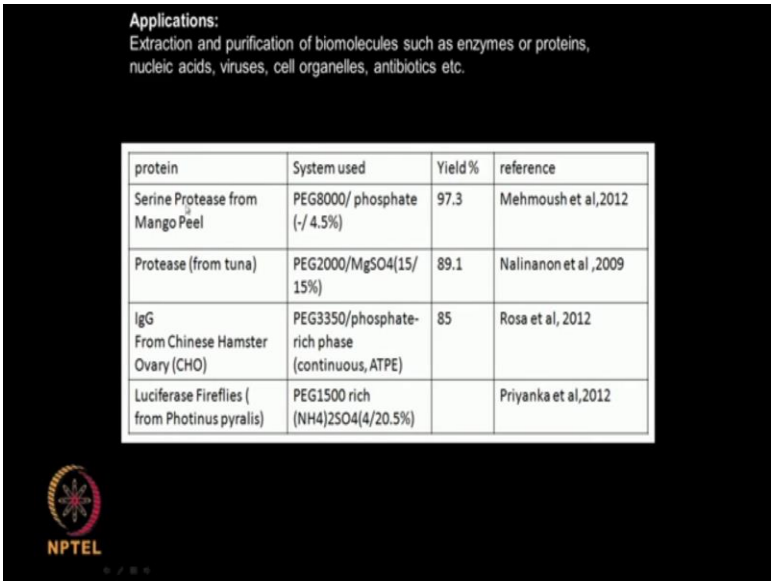

So we are there are many examples of this actually applications examples certain proteas protease, igg luciferase, fireflies using PEG and phosphate PEG, magnesium sulphate PEG, phosphate PEG, rich with sodium sulf, ammonium sulphate and so on actually.

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So extractive fermentation we can combine fermentation extraction in situ systems PEG sulphate PEG phosphate PEG-phosphate as you can see for a large number of antibiotics here cephalaxin, xylanase, asparaginase, 6-phenyl, alpha purone actually.

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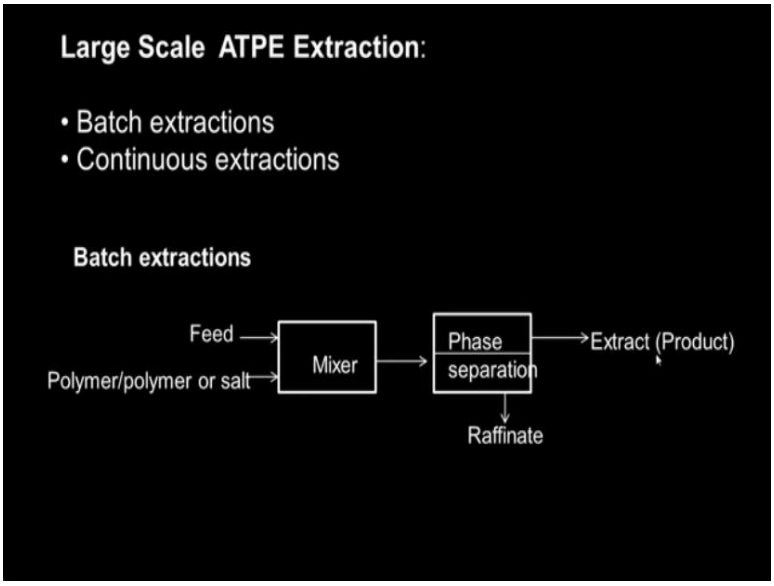

So how do we do it? We can mix them together that is a batch process, we mix them mixer and then phase separation extract raffinate.

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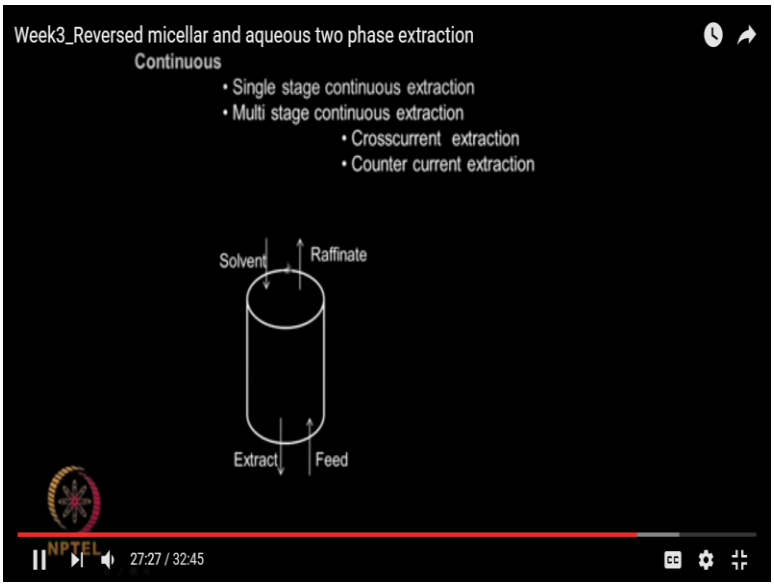

Or we can have continuous system continuous tubular, so we have solvent extract feed raffinate cross current counter-current you know all about it actually.

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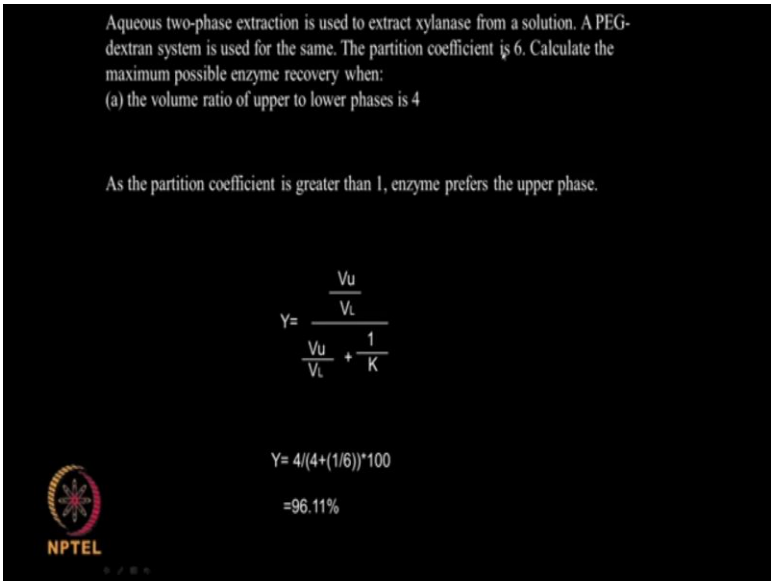

So let us look at problems very important aqueous two phase extraction is used to extract xylanase from a solution a PEG dextran system is used the partition coefficient is six calculate maximum possible enzyme recovery when the volume ratio of upper to lower is now you need to use this equation yield is equal to Vu / Vl / Vu /Vl + 1 / K. Here yield is given as n the first part is K is given as K Vu / Vl is given as 4.

So I want to calculate the yield. So I put 4 I put 4 + 1 / 6 and I calculate Y okay and of course fi the partition coefficient is greater than 1 so it will partition to the upper phase that you need to keep in mind. So what do you get? 96 %. So with the with the ratio of upper to lower of 4 am able to get 96 % of the protein extracted the partition coefficient is 6.

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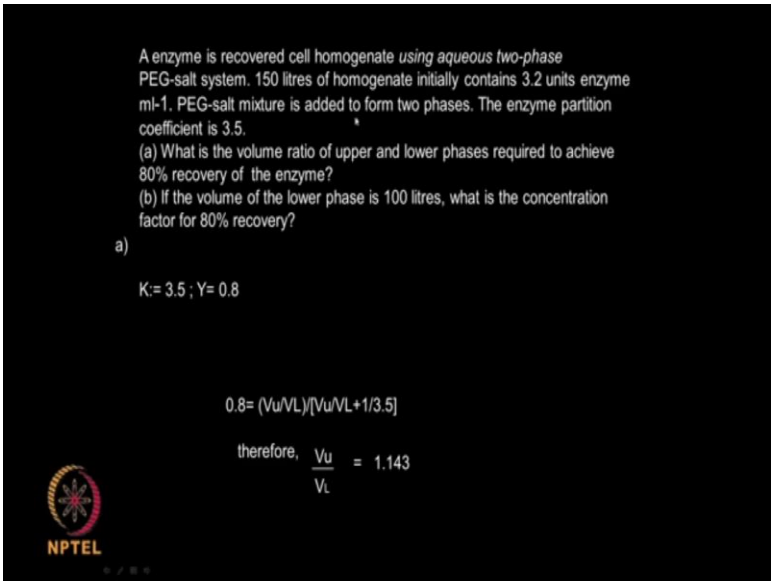

Now let us look at another problem. Enzyme is recovered using aqueous two phase am using 150 litres of homogenate initially which contains 3.2 units of enzyme PEG salt mixture is added to form 2 phases then the enzyme partition coefficient is 3.5 so K is 3.5 yield is 80 %. What is the volume ratio of upper to lower to achieve 80 % yield, so yield is 80% K is 3.5so I take that equation on the left hand side I know 0.8. So I need to calculate Vu / Vl it comes to 1.14 so 1.143 if my ratio of Vu / Vl I will get 80 % of recovery.

Now if the volume of the lower phase is 100 litre, okay volume of the lower phase is 100 that means Vl is 100 liter. What is the concentration factor 80 % recovery okay so Vl is 100litre Vu I can calculate okay then what is the concentration factor for 80% okay.

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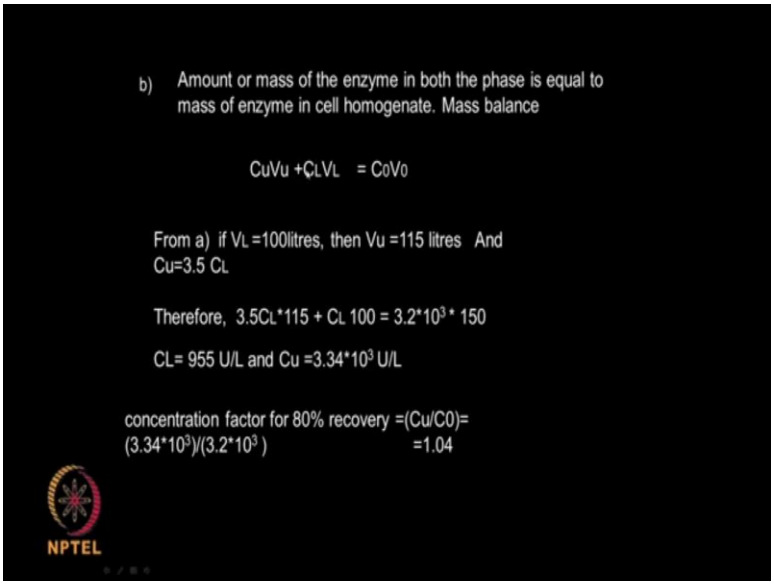

What is concentration you know CuVu + ClVl is equal to Cl not v0 whereas Cl not is the original concentration, total amount of one total volume initially. So Cl not v0 gives you the total amount of solute. Now the total amount of solute in the upper, total amount of solute in the lower, okay, so we know Vl is 100 Vu is115 because we calculated Vu / Vl as 1.15. Now Cu is equal to 3.5cl okay therefore I substitute here 3.5cl into 115 + Cl into 100 is equal to 3.2 into 10 power 3 into 150.

So Cl is equal to so much and Cu is equal to so much okay. That is concentration of the pro the enzyme in the upper concentration of enzyme in the lower okay concentration factor for 80 % recovery I just do Cu / Cl not that become 1.04 that is Cu is this divided / Cl not. Cl not is given here okay 3.2 so I get 1.04 value so you understand, so we know we calculate Vl from the first part we know Vu / Vl is 1.15 so we calculate Vu.

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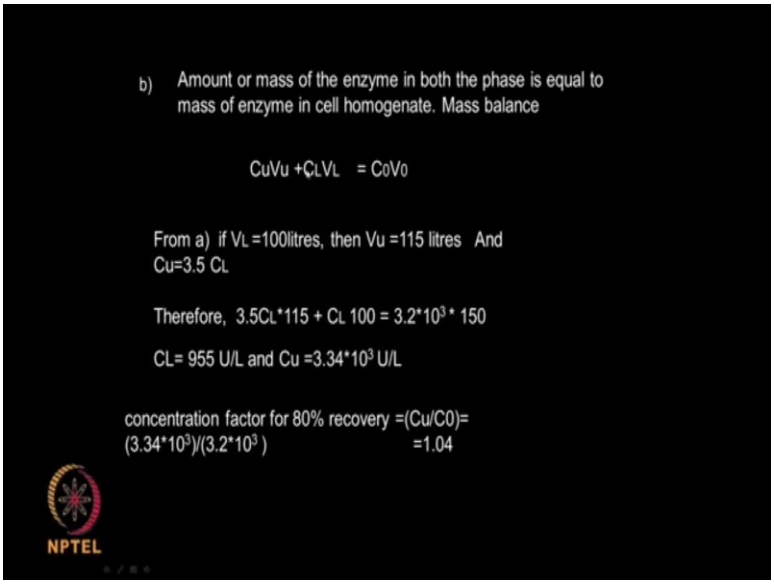

Now Cu / Cu and Cl are related okay by this 3.5 into Cl okay because this is enzyme partition coefficient is 3.5 okay therefore I substitute in this one so from I can get Cl and Cu okay. Now the concentration factor is Cu upper Cl not okay Cl not is given as here 3.2 units. So I substitute here so get concentration factor of 1.04 that means that is the increase in the concentration of the enzyme when I extract into the upper layer.

So it is a very useful so problem to look at so it gives you an idea of what should be the ratio of the PEG for upper and the lower and also it gives you the enhancement of the concentration after the extraction. So we looked at 2 different techniques which uses aqueous one is reverse micellar where we use a surfactant and the second one is called two phase aqueous where we use a PEG like a biomolecular polymer so extraction in both the cases involve major majorly water unlike the previous study where we used solvents.

So the advantage is we can extract proteins and enzymes without worry worrying about losing their activity. So they have lot of applications in bioprocess industry.

Thank You Very Much