Principles of downstream techniques in Bioprocess – a short course
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Lecture – 04
Cell Breakage

This class we are going to talk about cell breakage, issues involved in cell breakages, various techniques that are practised for breaking cells and a couple of problems as well.

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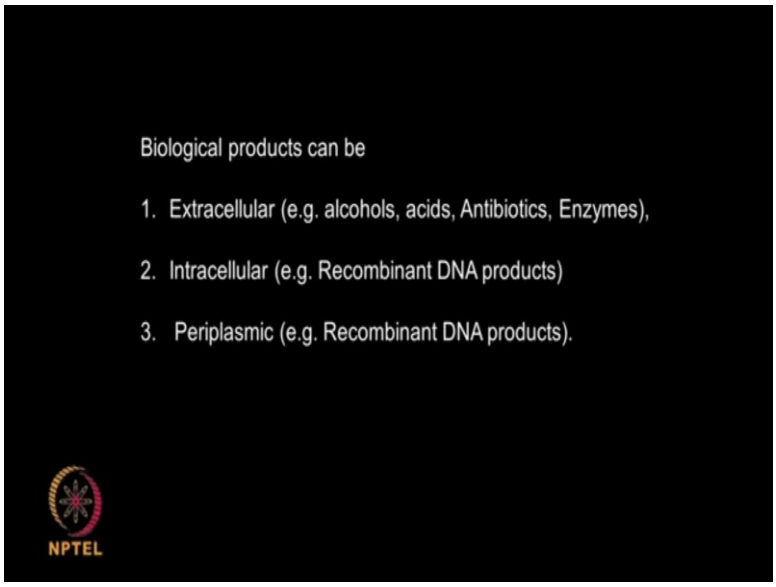

So why do we need to do the cell breakage? If the metabolites or the product of our interest is inside the cells and unlike when the product comes out into the medium then it is called intracellular material. Okay. Sometimes the material could be, not exactly inside but it could be between the two layers of the cell or that is called periplasmic so intracellular material products or things like recombinant DNA products the periplasmic could be recombinant DNA again.

The extracellular could be like alcohols, acids, antibiotics, enzymes; they all come out into the extracellular medium. So there is no problem if it is an extracellular product, all you have to do is after the fermentation, remove the cells by centrifugation or filtration and then start processing the liquid. Whereas if it is an intracellular or periplasmic material then what we have to do is, we have to harvest the cells that is; we need to collect the cells not the liquid medium. We collect the cells and then we have to break the cells to recover the product.

So you have to be very careful actually especially when you are breaking the cells you may be generating heat, you may be generating some unwanted metabolites or you may be affecting the pH. So the product if it is a bio molecule like an enzyme or a protein you should not get denatured. So

there are many techniques available for breaking cells mechanical technique thermal technique enzymatic technique. And I will show you some of those techniques and they are very very important nowadays because many products may be inside the cells rather than being outside in the extracellular medium.

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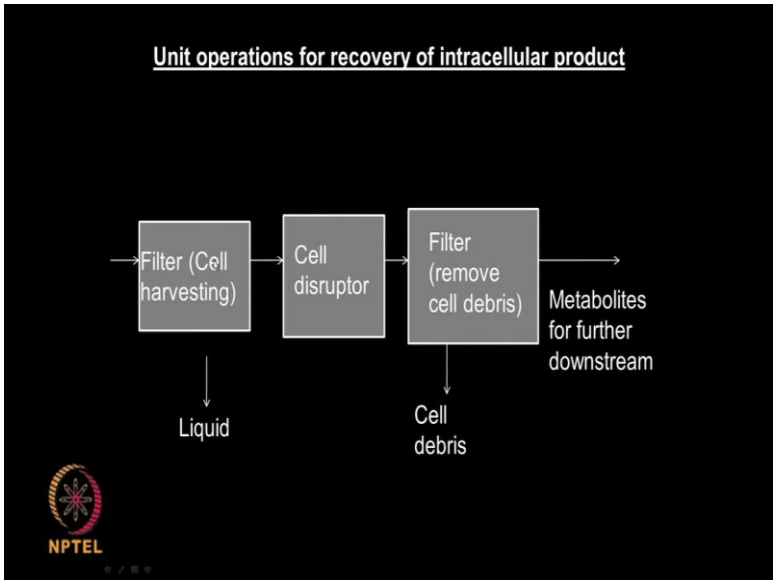

So what do we have to do after the fermentation we have to harvest the cells that is generally done through centrifugation or a filtration throw the liquid out collect the cells break the cells or disrupt the cells or homogenize the cells, then you again use a filter, remove the cell debris, biomass and so on and then get your metabolites in the liquid form. In the downstream and then you take it down do whatever you want to do you may use a membrane process or you may use a liquid-liquid extraction for concentrating.

And then you may go into chromatography for purifying. So these are the extra steps that are followed if the product is a intracellular material okay unlike a extracellular material.

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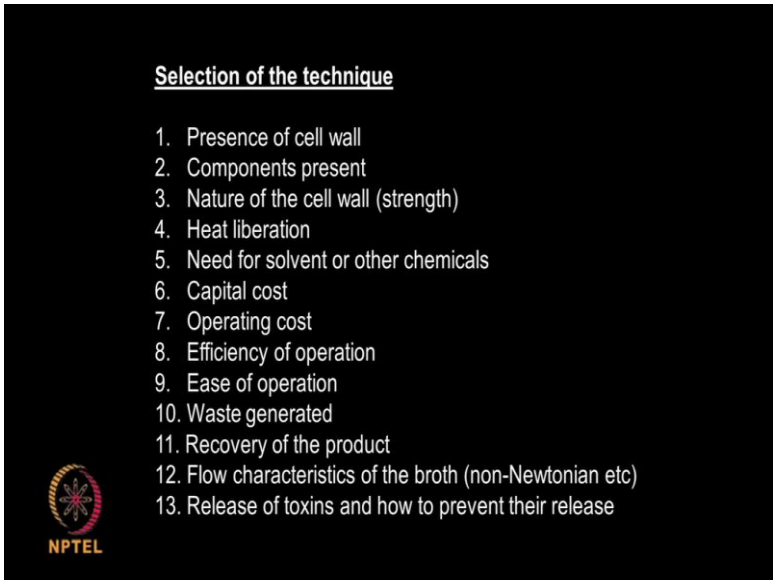

So how do you select the technique? There are like as I said it could be a mechanical it could be a chemical it could be a thermal it could be enzymatic. How do you decide? So does the bacteria have cell wall? What are the components present in the cell wall? How strong is the cell? For example plant cells may be very very fragile whereas certain yeast cells could be very strong in the cell wall okay. How much heat is liberated? Because if it is a thermally labile then when you generate heat because of the disruption process the product could get denatured.

Do we need any solvent or any other chemicals because there are chemical methods by which you can break the cell? What is the cost involved? Capital cost, operating cost that means how much chemicals or consumables or heat or cooling I need to have that the operating cost. How efficient is the process? How easy is the process? How much waste I generate because I will be generating lot of cell debris and then unwanted intracellular material, they are all waste.

How I recover the product after removing the product from inside the cells? How am I going to re recover once I have done a cell deception because you are liberating lot of intracellular material, the entire broth would be non-Newtonian, that means it could attain certain viscosity which may be difficult to process so I need to understand that. Am I releasing toxins when I am doing a cell deception I need to understand that if I am releasing toxins then the intracellular product may get denature because of that toxin so you decide based on all these various criteria okay.

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Now if I take bacteria all you know there are two types, right one is a gram positive, other one is gram negative okay. They are separated based on the gram staining is used so gram positive bacteria remain staining by crystal violet after washing this gram negative not remain stained after washing. So obviously they have two different types of cell wall okay.

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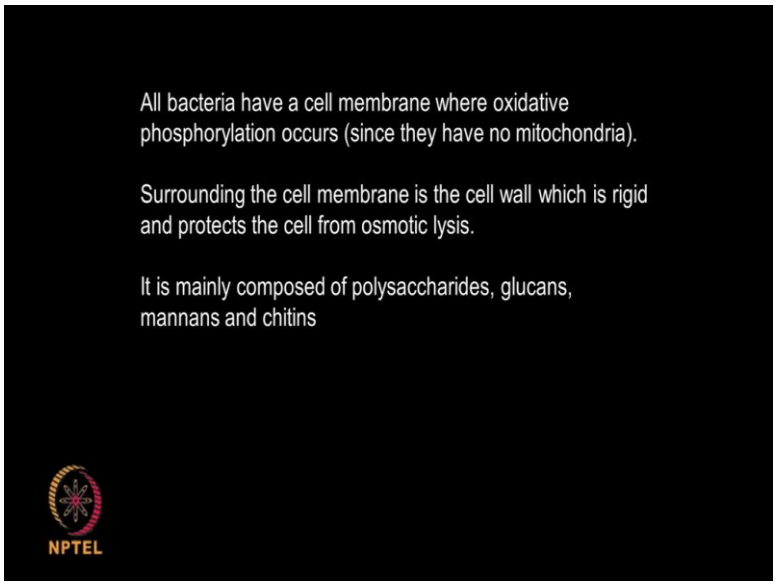

So all the bacteria have cell membrane where the oxidative phosphorylation occurs because they do not have mitochondria. Now surrounding the cell membrane is the cell wall which is really rigid and it protects the cell from osmotic lysis and other physical processes actually okay. So they could be made up of polysaccharides, glucans, mannans and chitins and so on. So there is a cell membrane and then after that you have the cell wall the difference between the gram positive and the gram negative is the presence or absence of the cell wall.

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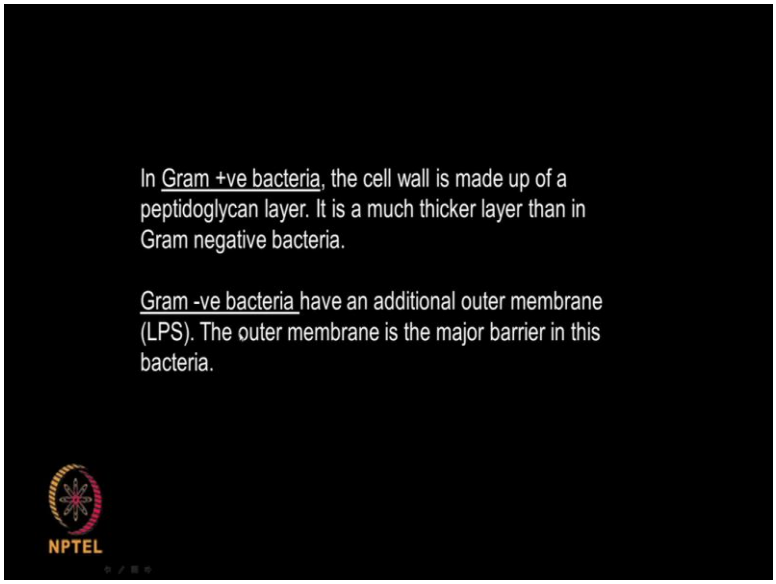

Okay so in gram positive the cell wall is made up of a peptidoglycan layer now this layer is very very thick whereas in gram negative bacteria they have something on top of that the layer may be very thin but they have something on top of that that is called the outer membrane. Generally it is made up of lypho polysaccharides and this is major barrier which prevents drugs or other toxins entering the gram negative. That is why gram negative bacteria are much more resistant to antibiotics

Because of the presence of this outer membrane okay whereas gram positive bacteria has the peptidoglycan layer and they do not have this lypho polysaccharide outer membrane.

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So the region between the inner and outer membrane this is called the periplasmic space actually. So in gram negative bacteria, they store lot of degradative enzymes in the periplasmic space, gram positive bacteria lack a periplasmic state space because they do not have this lps layer outside. But

they secrete lot of enzymes and they carry out the extracellular digestion that is the difference you need to understand.

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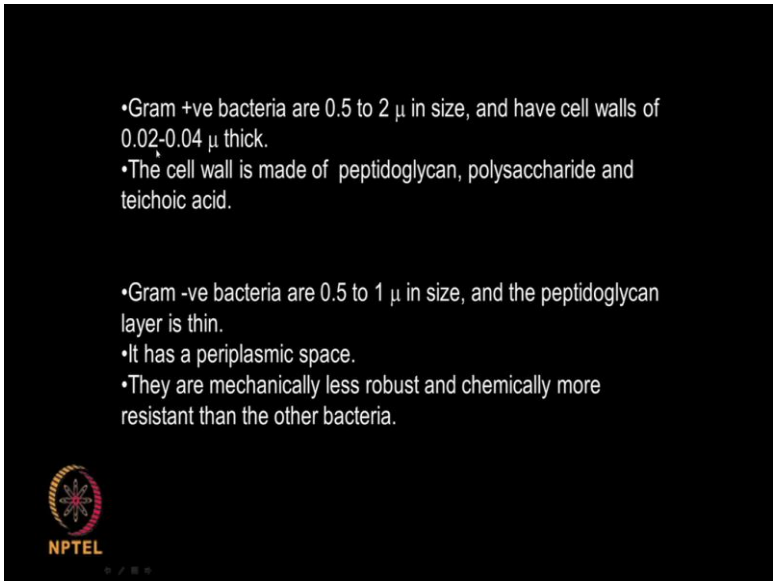

So gram positive bacteria are generally 0.5 to 2 micron in size and they have cell walls of quite small 0.02 to 0.04 micron thick and the cell wall is made up of peptidoglycan which is polysaccharide. If you take gram negative they are much smaller 0.5 to 1 micron in size and the peptidoglycan layer is very thin and it has got a periplasmic space and on top it there is a impious layer also membrane also.

They are mechanically less robust but they are chemically more resistant than the bacteria so if I use mechanical techniques to break the cell walls gram negative will break faster because the peptidoglycan layer is thinner whereas chemically they are much much stronger than this. So mechanically gram positive is much stronger chemically gram negative is much stronger so depending the type of bacteria which you try to process you can go into a mechanical method or a chemical method.

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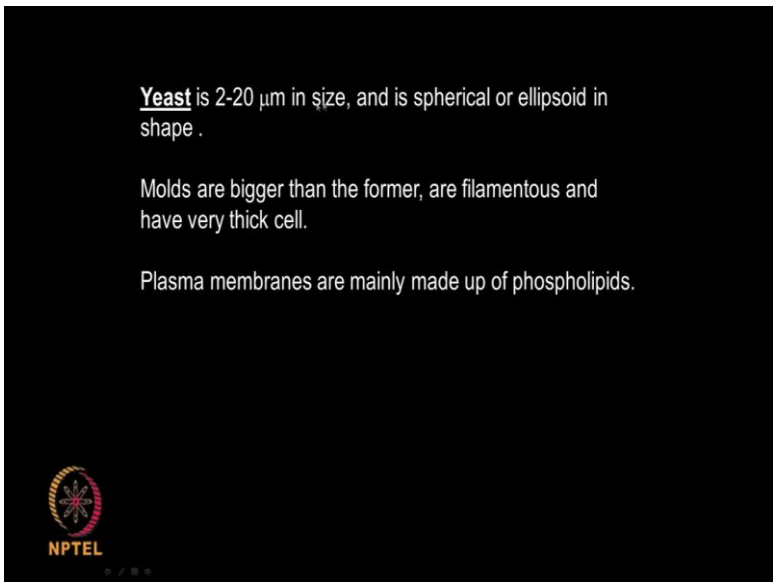

Now yeast if you take they are much larger in size they are about 2 to 20 micron in size and they are very spherical or ellipsoidal unlike the other one bacteria and moulds are very big okay. And they are sometimes filamentous also and they have it takes cell wall so filamentous they will create lot of problem especially in homogenize or French press where you have a very small losses because these filamentous organism may go and block those nostrils. Take plasma membranes they are mainly made up of phospholipids okay.

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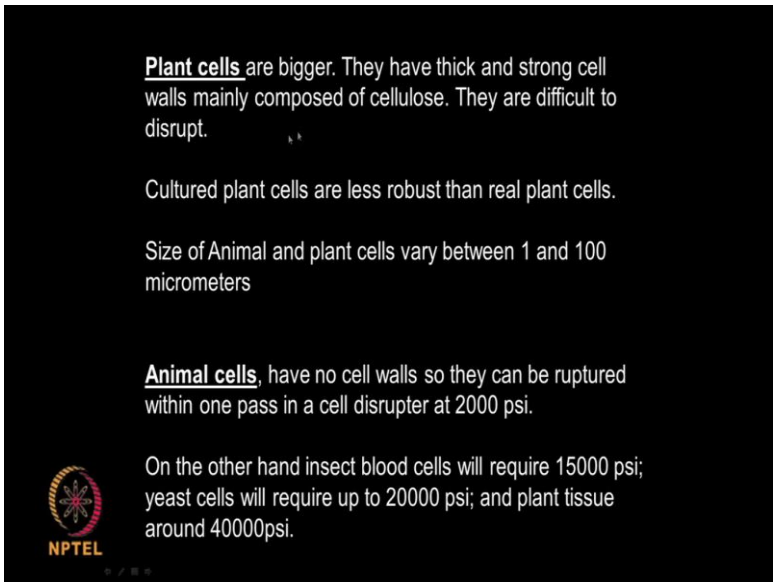

Now plant cells if you take they are very big they have thick and strong cell walls mainly composed of cell walls they are difficult to disrupt. Whereas cultured plant cells are less robust than real plant cells. If you take animal and plant cells the size varies between 100 to 1 micron. So you see they are very big, so animals have no cell walls, animal cells have no cell walls. So they can be ruptured very easily in one pass itself actually at about 2000 psi.

Whereas insect blood cells will require 15000 psi each cells will require 20000 psi and plant cells

will require 40000 psi. You see depending upon the thickness or the toughness of the cell wall you will require different types of pressures to rupture or break the cell walls so animal cells will require lowest then comes yeast cells then comes finally the plan cells.

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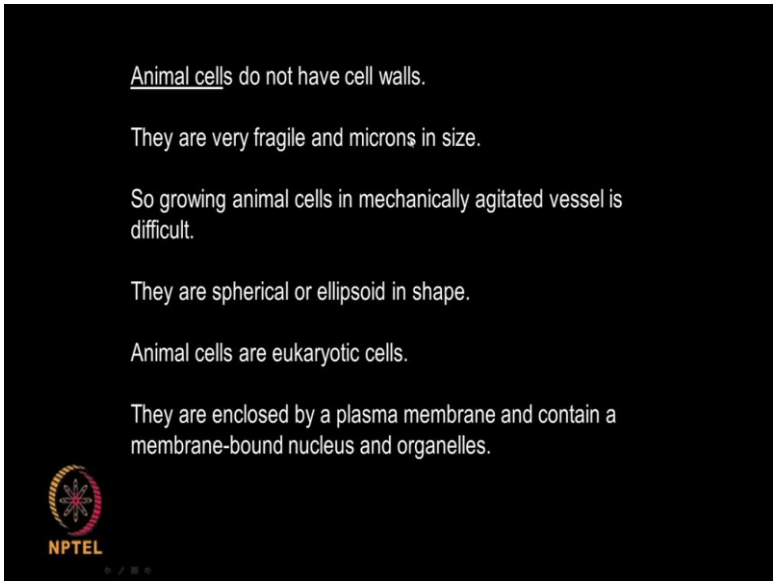

So animal cells they do not have cell walls they are very fragile and they are quite big in micron size so growing animal cells in mechanically agitated vessel is difficult that is why generally animal cells are grown in non agitated vessel. They are very spherical or ellipsoid in shape they are eukaryotic cells actually. They are enclosed by a plasma membrane and they contain membrane bound nucleus and organelles.

So if I am going to recover products and if they are membrane bound then I need to collect the membrane and I need to recover the product from the membrane to which it bound to

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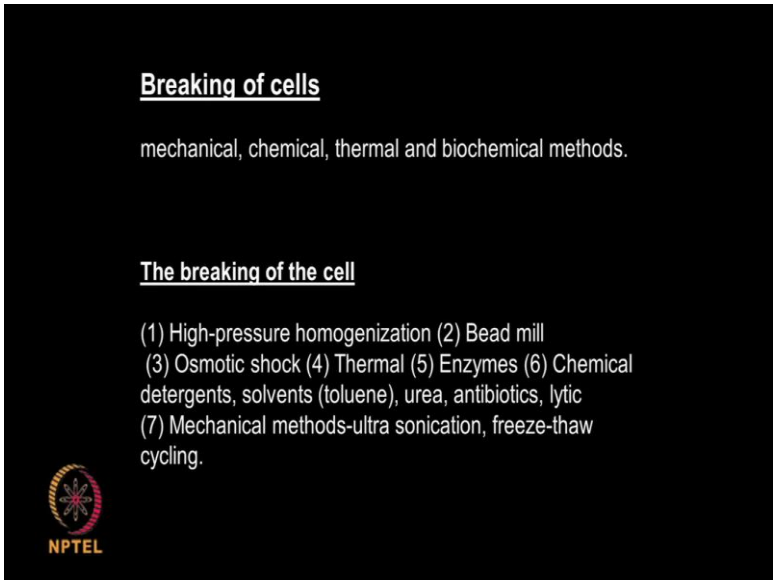

Now breaking of cells as I said there are different techniques available you have mechanical techniques chemical techniques biochemical technique okay. So you can use any one of these techniques depending upon your requirement and type of cells you are trying to make whether it is plant or animal or bacteria and so on. So there are techniques like high pressure homogenization, bead mill, osmotic shock, thermal method, enzymatic method, chemical method.

Like you may use chemical detergents, you may use solvents like toluene urea antibiotic lytic or you can also use mechanical methods like ultrasonication, freeze, thaw cycling, so all these are large number of methods available and some of these methods are for laboratory use and some of the methods are for large scale plank use.

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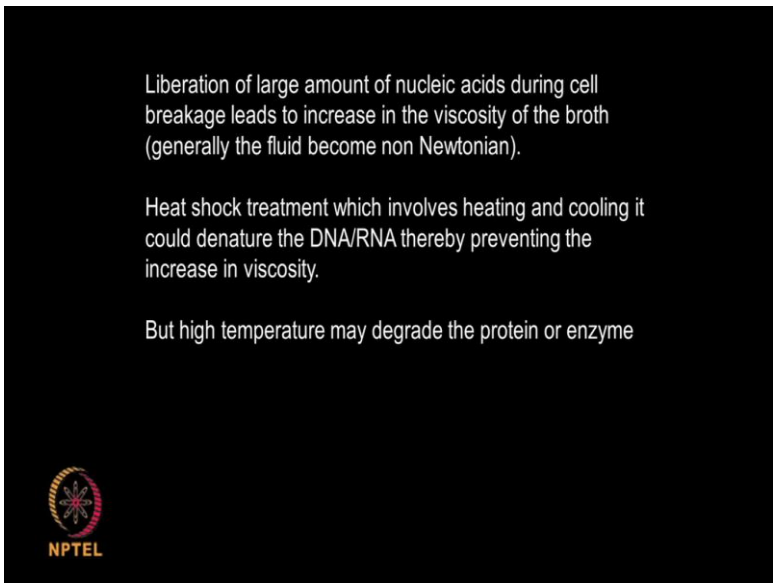

So we will briefly touch about each one of the method okay. Now lot of nucleic acids may be liberated when you break the cells so obviously they will increase the viscosity of the broth and then your broth becomes a non-Newtonian liquid. If we take heat shock type of method where you

are heating it and cooling it and heating it and cooling it there by the cell walls are damaged and broken it may denature the DNA and RNA present inside and that will also increase the viscosity.

If you use high temperature or if the temperature rise happens because of the breaking of cells then obviously it is going to degrade the protein or the enzyme present inside the cells so all these are issues which we need to think about.

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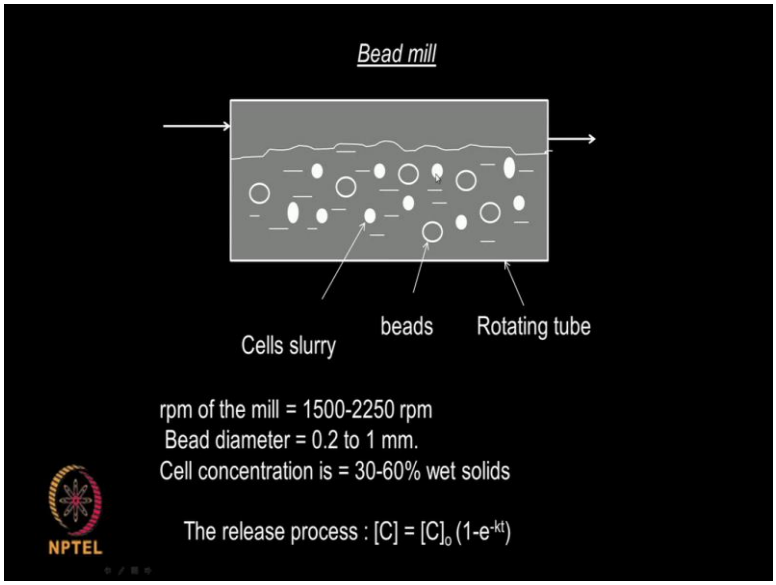

So what is a bead mill? bead mill is a long cube hollow cube made of lot of beads present inside okay these beads are made up of ceramic or metal so there are it takes slowly around 1500 to 2250 rpm so the slurry comes inside so when it rotates the beads metallic or ceramic beads heat each other they also heat the cells thereby disrupting the cells thereby disrupting the cells actually okay.

So this quite method a good method for industrial application we can have cells of about 30 to 60% by weight wet solid percentage

So the release of the intracellular material is considered to be a first-order process okay generally we take it as first order process. C = c0 *1 - e power kt. That is how it is assumed actually. So the concentration will slowly increase exponentially and reach the maximum value k is nothing but a constant which determines the first-order rate actually. K is sometimes defined as 1 by tow where tow is the characteristic time okay so this is the first-order process and generally this type of equation is able to describe the release of the intracellular material okay.

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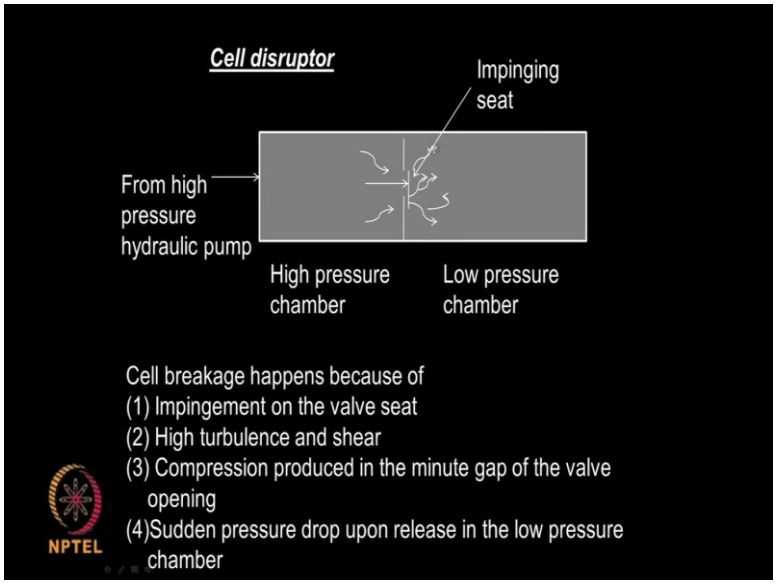

If you take cell disruptor what happens is, there is a chamber high pressure chamber, there is a low pressure chamber. So the cells are compressed to very high pressure and they are introduced through this nostril and released on the side. Okay when they are released on this side they come out from high pressure to low pressure there is a seat also present valve seat it is called the cells hit that valve seat so many things happen.

The cells hit the valve seat the cells moved from a very high pressure to low pressure there is a lot of turbulence and shear produced because of the small nostril so there is a compression that is taking place so in the small nostril so because of all these reasons the cells rupture and so the industrial material is liberated actually. This is a very good method when compared to the bead mill so you are not really destroying too much of intracellular material unlike the beadmill method.

Actually the heat produced is slightly less that the beadmill but still the heat is produced by a lot during this operation.

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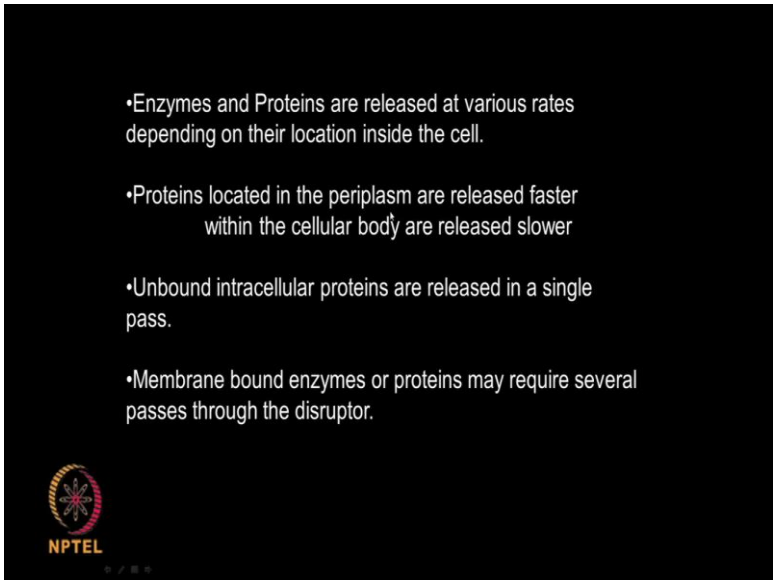

So enzymes and proteins are released at various rates depending upon where they are located so if the proteins are located at the periplasmic they are released first. If the proteins are unbound first they are released in one pass. If the proteins are bound to the cell wall you need many passes through the same cell disruptor to reduce the protein so at various time periods the products keep coming out actually okay.

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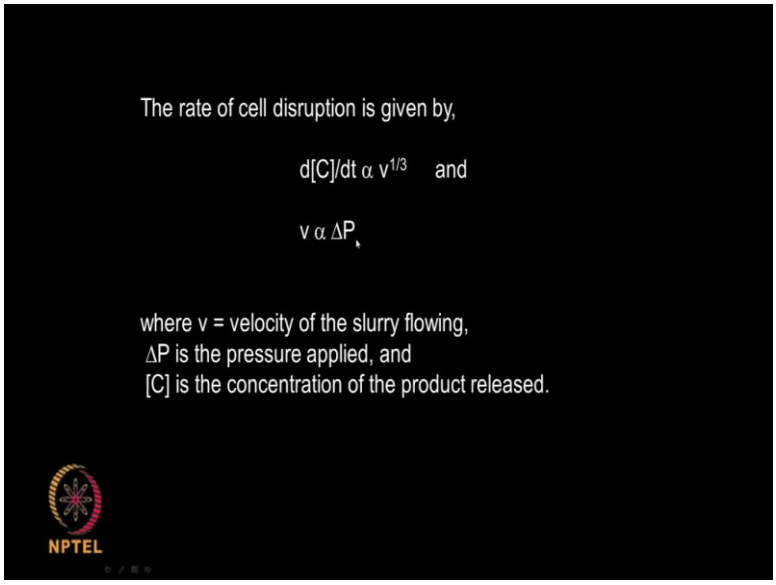

So again we consider this the cell disruption process as a function of pressure that means the amount of pressure, we put in the or the delta p that is the pressure difference at high pressure as well as in the low pressure region okay.

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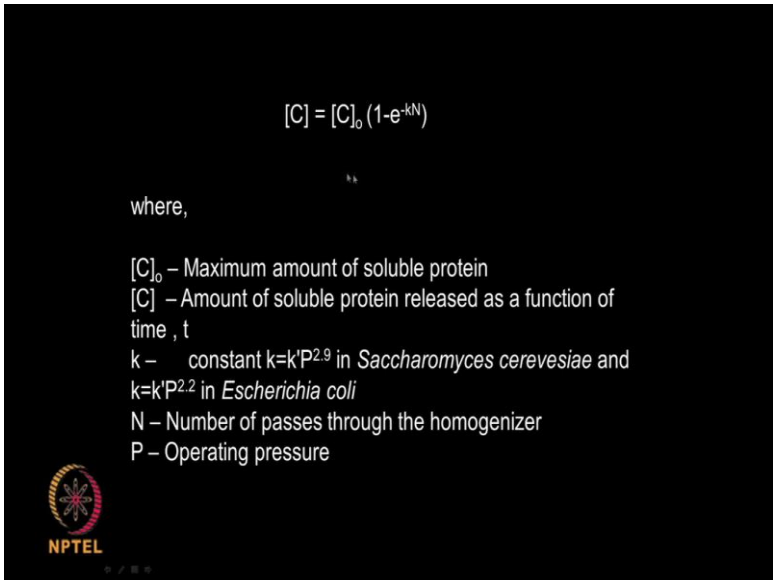

And the concentration of the product released is again considered as proportional to velocity raised to the power 1 / 3 and the velocity or the throughput is proportional to the pressure and again the concentration release is assumed to be a first-order process C = C0 1 e power – kn. N is the number of passes okay, so in the previous beadmill we had e power - kt here, we are having e power – kn. Again it is considered as a first-order process and the k depends upon the type of organism whether you are using a bacteria.

Or whether you are using a yeast the k values will differ and the k value will depend upon the pressure. For example if you look here we see that k is proportional to the pressure raised to the power 2.9 for the yeast like saccharomyces and whereas it is raised to the power p raised to the power 2.2 if it is a escherichia coli bacteria right. So the k depends upon the type of organism which is being broken and n is the number of passes and p is the pressure.

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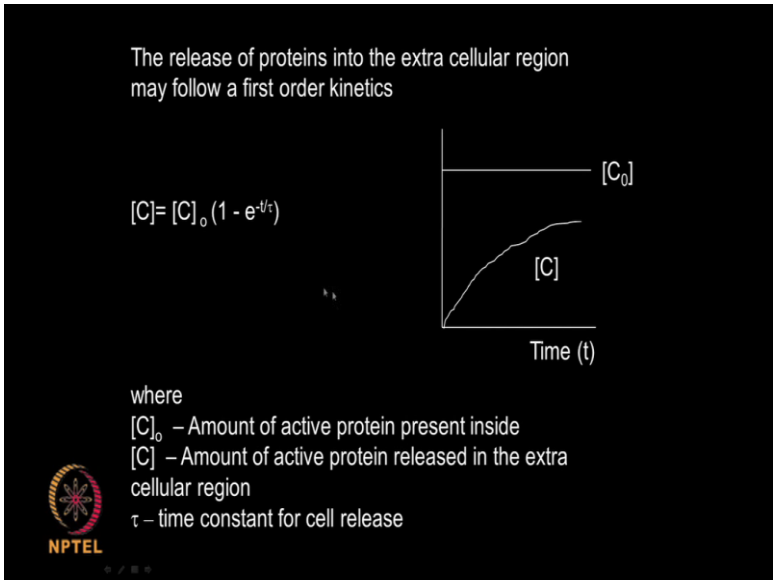

Okay so the release of the protein in the extracellular region you always consider as first-order process like this so c = c0 1 - e t by tow. Tow is called the time constant for cell release okay or e power kt again k is inverse of tow. c0 is the maximum concentration or the amount of active protein present inside the cells and c is the amount of active proteins released in the extracellular region.

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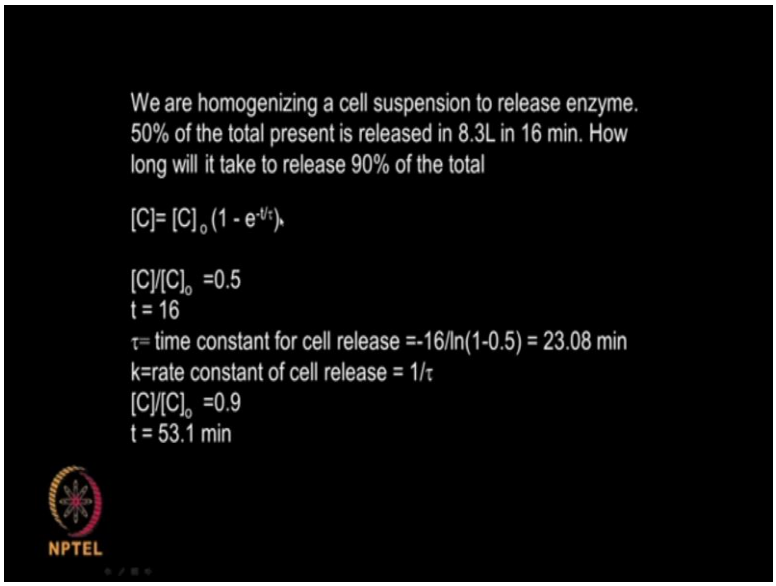

Okay let us look at a small problem so what I am saying is we are homogenizing a cell suspension to release enzymes 50 percent of the total present is release in 16 minutes now if I want to release 90 percent how much it will take okay. 50 percent of it is released in 16 minutes for 90 percent how much time is required so the intracellular release follows a first-order model so we all know what is first-order model. C = c0 1 - e t by tow. c0 is you total concentration as possible.

T is the time and tow you call it as the time constant or characteristic time and so this is how your first-order model looks like. Now the problem says 50 percent so c by c0 is 0.5 and the problem say

in 16 minutes 50 percent is released so t is 16, so what do we do? By substituting to this equation and we estimate tow that is the time constant. So very simple, so we substitute all these and we end up with the time constant of 23.08 minutes.

Now the second part of the problem is how long will it take to release 90 percent of the total, that means C by c0 will be 0.9 and now we know tow which is 23.08, so we substitute that into this equation and we are supposed to estimate this time t so we do that we get the answer of 53.1 minute. So for 50 percent release it takes 16 minutes for 90 percent release it takes 53.1 minute so you can see from this problem but as we want more and more percentage release the time will be longer and longer and longer.

Because any first-order process as you know it will go up exponentially and it will slowly keep on increasing so if you want to have much higher percentage release time required will keep going up and up and up and so on actually.

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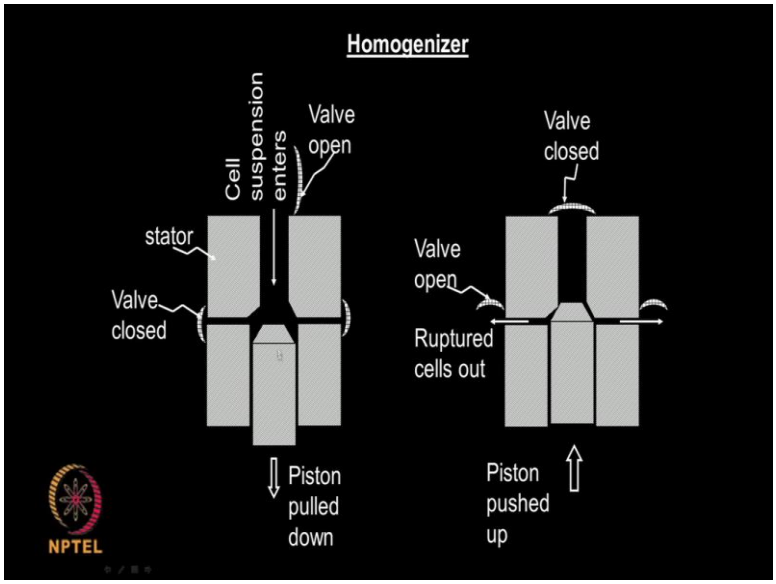

Now there is another down these cell disrupting technique that is called homogenizer. So what it does is, it sucks in a cell suspension okay through this piston and then it compresses your cell suspension so the cells have to move out through the small nostrils so during that process the cells get ruptured okay. So it moves up and down this piston moves up and down the cells are drawn in and then cells are compressed and then sent to these nostrils and during that process cells get disrupted

So this process is much better than beadmill like process its very violent breaking cells here. It is very systematic breaking of cells so the increase in viscosity is not match but still heat is liberated,

so what they do is after one pass the contents are cooled again, it is sent back for second pass then again cooled and so on actually that is how you handle this type of equipments after each pass it cooled.

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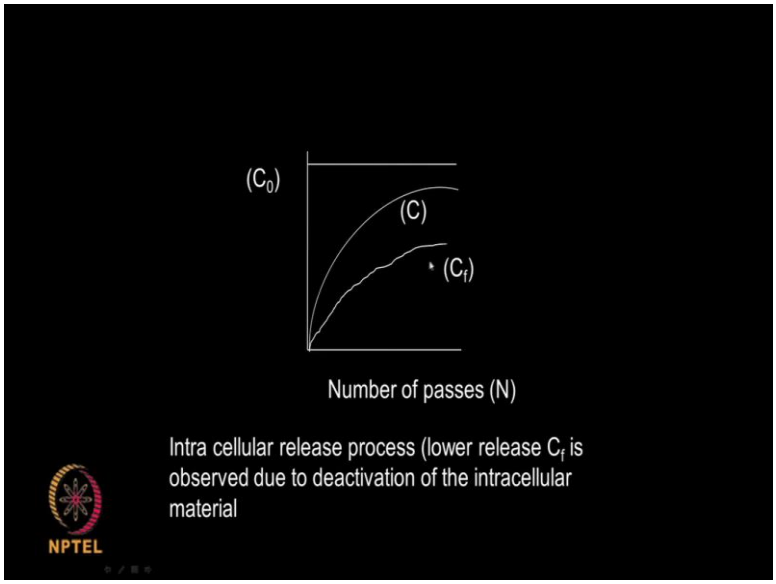

So again it is a first-order process so but then if the heat is liberated and its getting deactivated then the real curve will look like this because of the deactivation whereas there is no deactivation it will look like this so the deactivation is generally assumed considering arrhenius type of behaviour you know you all know how the arrhenius equation looks like right

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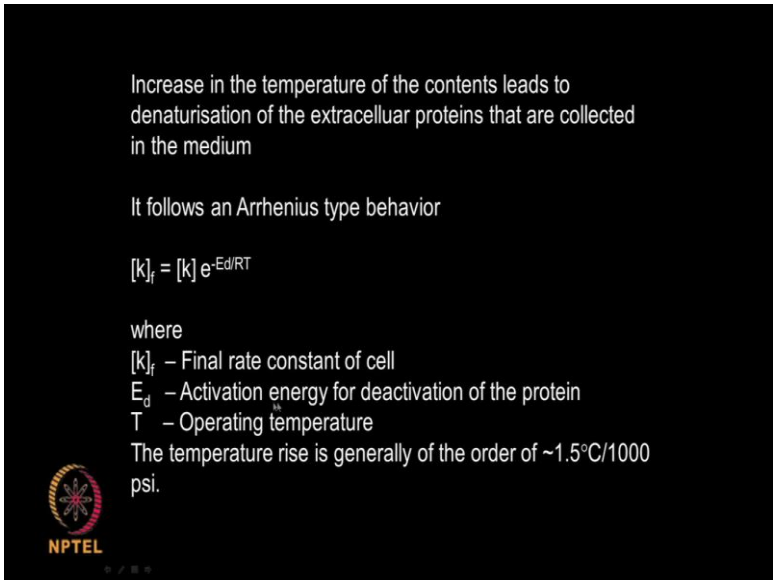

This is a typical arrhenius equation k f = k * e power - Ed by rt. Ed is the activation energy for deactivation that is deactivation energy so if the temperature is high then t will come there your rate constant will go down so obviously the cell release will be much lower than what is expected because of the decrease in k. Okay because of the heat that temperature rise and Ed. So generally they say about 1000 psi can give you a temperature rise of the order of 1.5 and especially for

enzymes and proteins a temperature rise is leading to deactivation of the product okay.

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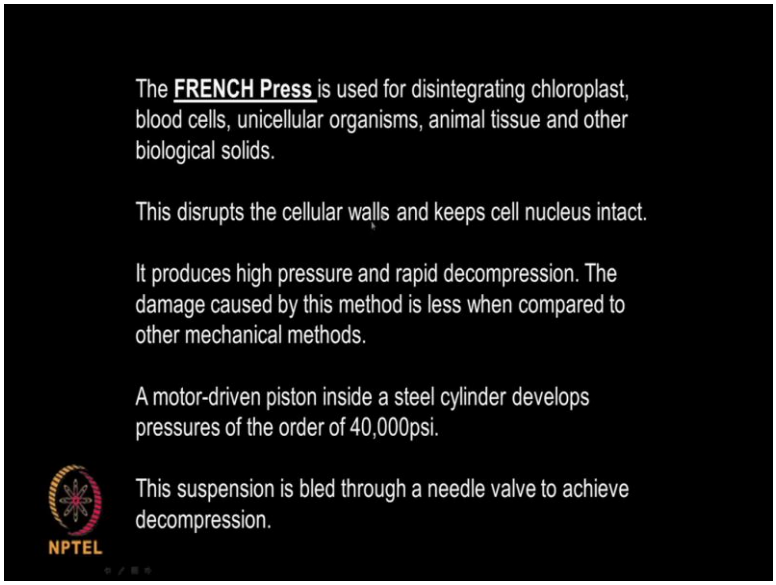

So that needs to be considered. French press this is again at the techniques used for disintegrating chloroplast, blood cells, unicellular organisms, okay so it disrupts cellular walls but it keeps the cell nucleus is intact, it produces high pressure and rapid decompression. So very high and quickly de-compressive. By doing that it is not really damaging the whole cell actually okay. So the cell suspension is bled through a needle valve to achieve a decompression.

So the viscosity does not change too much unlike the mechanical methods so this is a good method to resort to.

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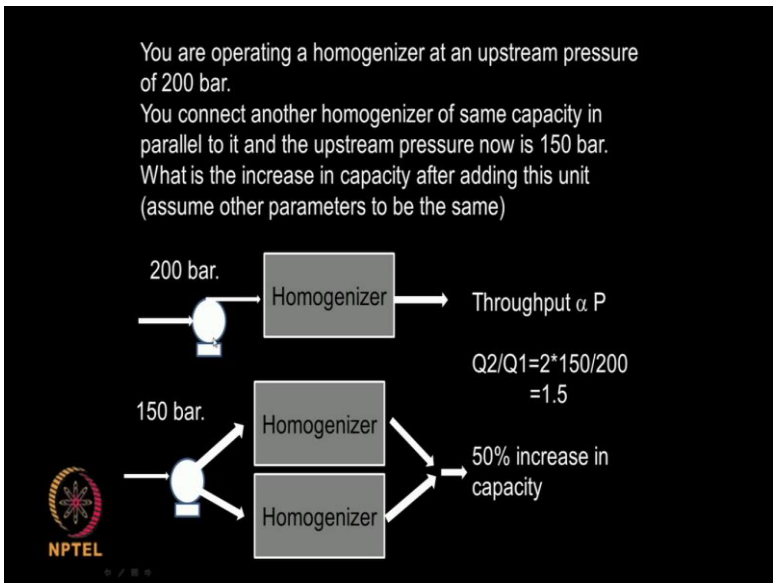

Now another problem you like to see imagine that I have a homogenizer okay and it is operated under 200 bar. Now I connect another homogeneous same homogeneous another homogeneous of same capacity in parallel so what happens is the pressure come down to 150 so when it does that I

am asking what is the increase in capacity after adding another. So is it going to be 2 times it won't be 2 times because your pressure has come down and we all know that the capacity is proportional to pressure right.

So the pressure has come down from 200 to 150 correct so that means how much 25 percent less but you have added 2 homogenizer so it will be 2 times for 50 percent. So 2 into 150 by 200 that is 1.5. Why is 150? That is the new pressure, 200 is the old pressure, 2 come because we have put 2 homogenizer. So the capacity has gone up to 1.5, so it has gone up from 1 to 1.5 that means there is an increase in capacity of 50 percent. So it will not be double the capacity increase but it will be only 50% increase because the pressure has come down from 200 to 150 bar.

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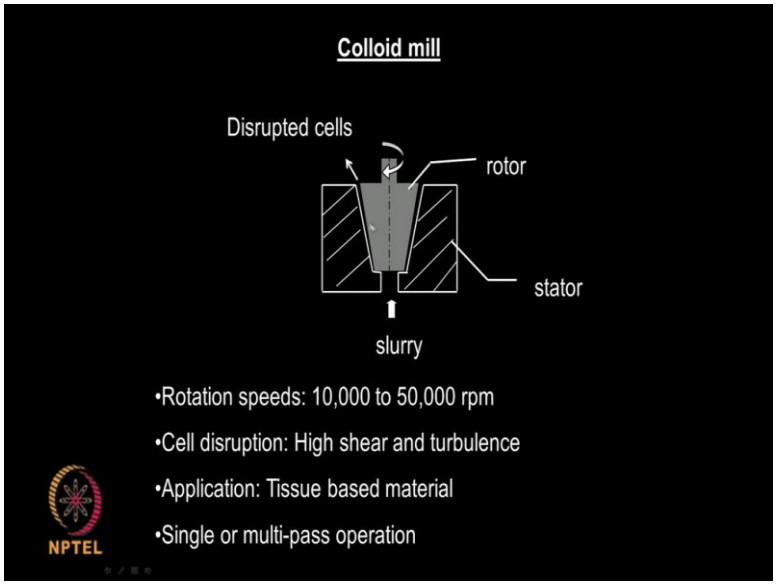

You have colloid mills that is another mm method where you have this type of a rotating cylinders and there is a small gap, so the cells are rubbed in that small gap the cells get broken down. This is very good for tissue based material, you can have single pass or multi pass. It is happening because of the high sheer and turbulence that is produced inside between the rotor and the stator here that is called the colloid mill.

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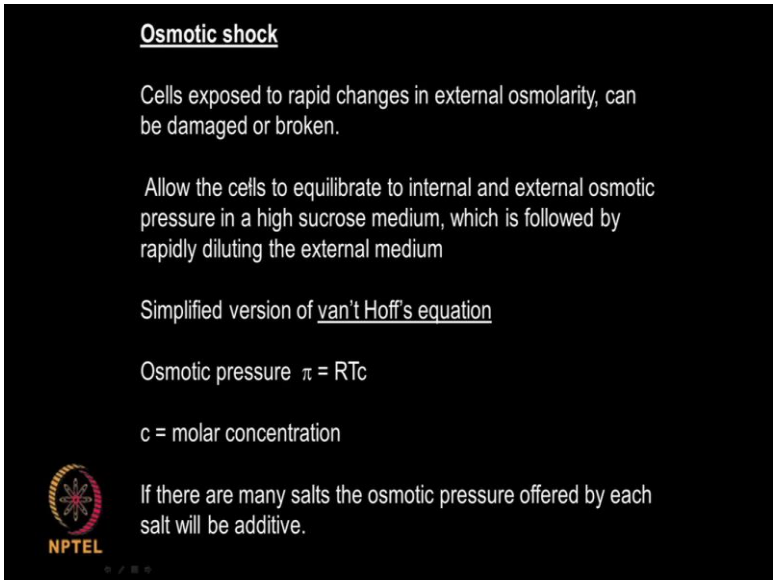

You can have osmotic shock, so what you do in osmotic shock is, you move the cells from certain osmotic pressure to another osmotic pressure. So during that process water may enter into it and it may break the cells so osmotic pressure is based on the Vant Hoff's equation, if you remember pi equal to rtc. T is the temperature r is the gas constant c is the concentration of the salt in molar. So we can calculate the osmotic pressure for any given concentration of the salt actually.

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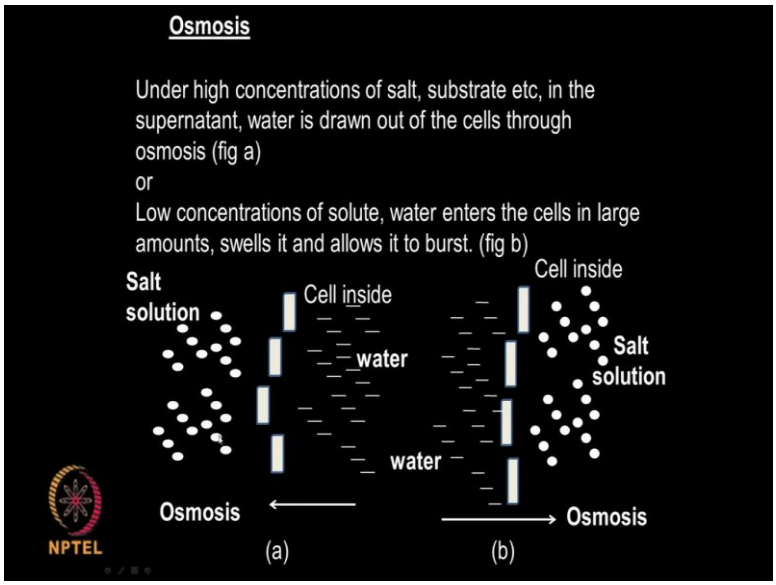

So what happens here you have a salt solution okay and you have cells inside water here okay now this salt solution is in this side so obviously, there will be osmosis happening here and so water will enter the cells and cells get water get entered the cells in large amounts swells it and allows it to break. This is a very good method because it does not involve heat liberation or it does not involve uncontrolled leakage actually.

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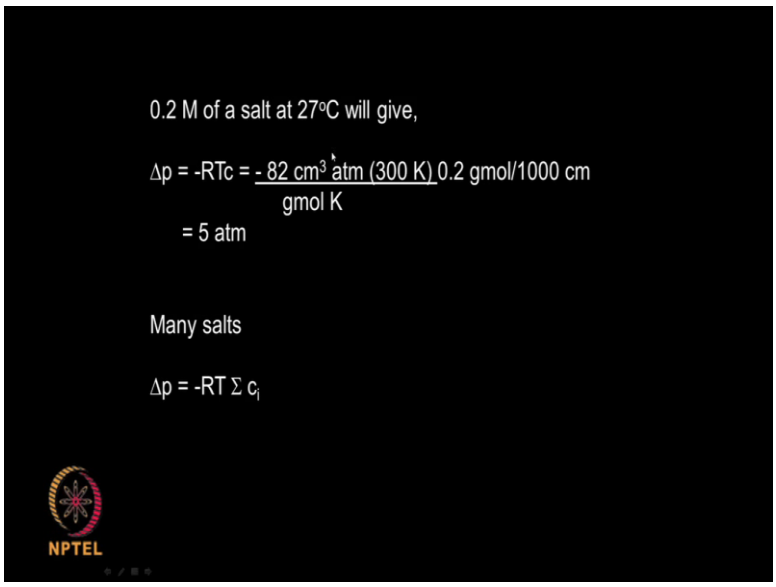

So we can calculate the osmotic pressure using the Vant Hoff's relation rtc so if I 0.2 molar salt means I can put c I here in 0.2 okay r is 82 temperature is 27 that is 300k. So for a 0.2 molar solution I will get an osmotic pressure of 5 atmosphere. So if you have any salts we keep calculating osmotic pressure on each salt and we need to add up, so you can see quite high pressure that is generated with salt and this method makes use of the cons concept of osmotic pressure.

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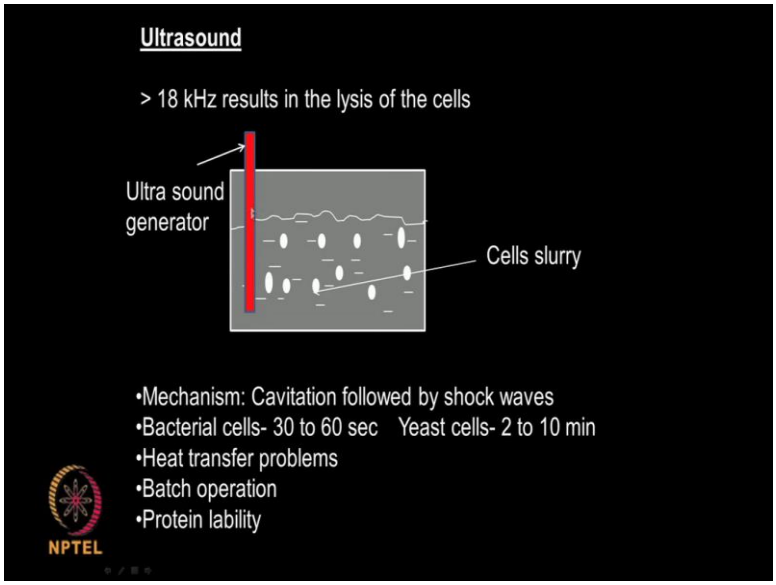

Ultrasound this is generally used in laboratory scale you have an ultrasound generator so when it produces ultrasound cavitation are produced shock waves are produced so sales get disrupted. So heat transfer is the problem that means heat liberation is very high sometimes proteins may get denatured it also produces radicals because ultrasound can produce radicals. So these radicals could be harmful for the cells so generally this is done in batch and generally it is done in small scale, laboratory scale it is not done in industrial scale application okay. So that is a big problem here but it is done regularly in a lab scale.

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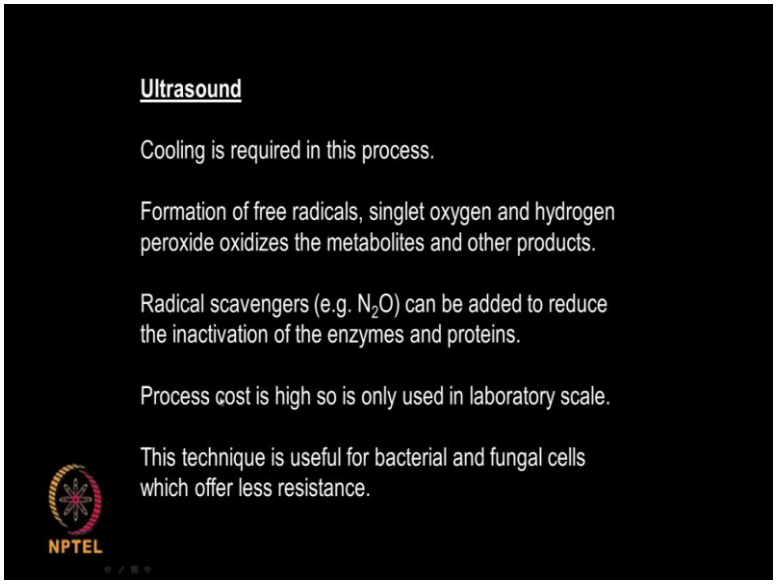

So you need to use radical scavengers because it produces radicals which may be harmful for certain enzymes and proteins.

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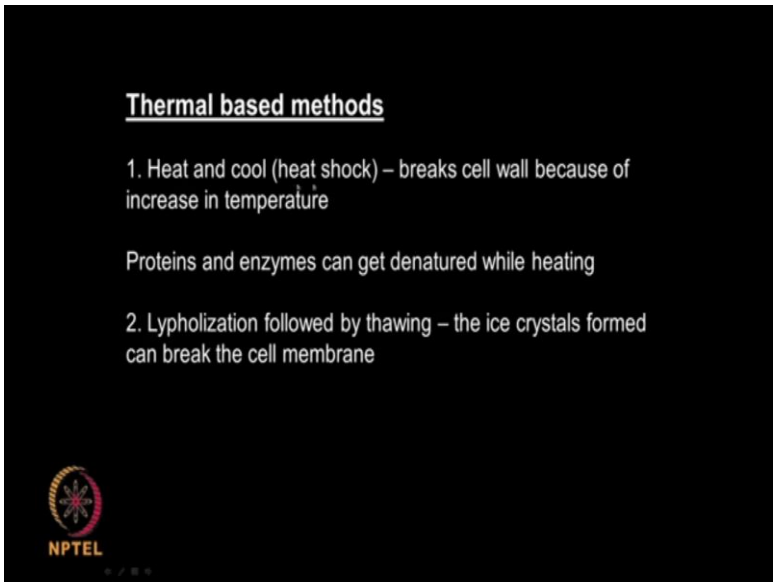

Then thermal method where you are heating, that is called heat shock method. You heat and cool and heat and cool, so during that process cell wall is break and the problem is proteins and enzymes can get denatured. Another is lyophilisation you will cool it and then you thaw it cool it and thaw it and the ice crystals that are formed can break your cells okay. This is easier method because this is a better method because the protein is not undergoing a thermal degradation but this is more expensive than the heat shock method actually.

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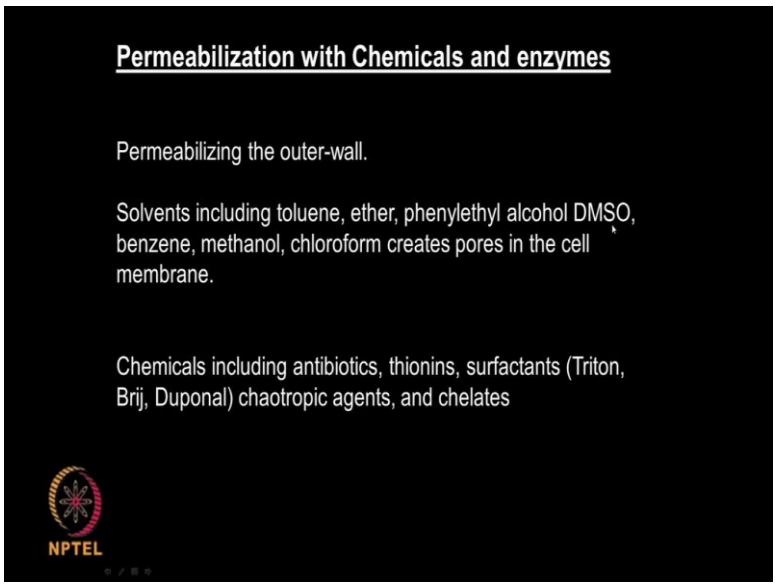

Now permeablization with chemicals you can use chemicals solvents like ether toluene, phenyl, ethyl alcohol or chemicals like thionins, antibiotics, chaotropic agents, chelating agents, surfactants, like triton so all these can be used to sort of dissolved cell wall or completely make the cell wall for us. This is the very good method because it is very very method which does not produce heat it does not produce any harmful products but then the chemicals should not harm the protein or the enzymes which you are interested in which is present in the soil.

So otherwise chemical methods are quite useful method because they are very simple method to use and they are very mild methods to use okay.

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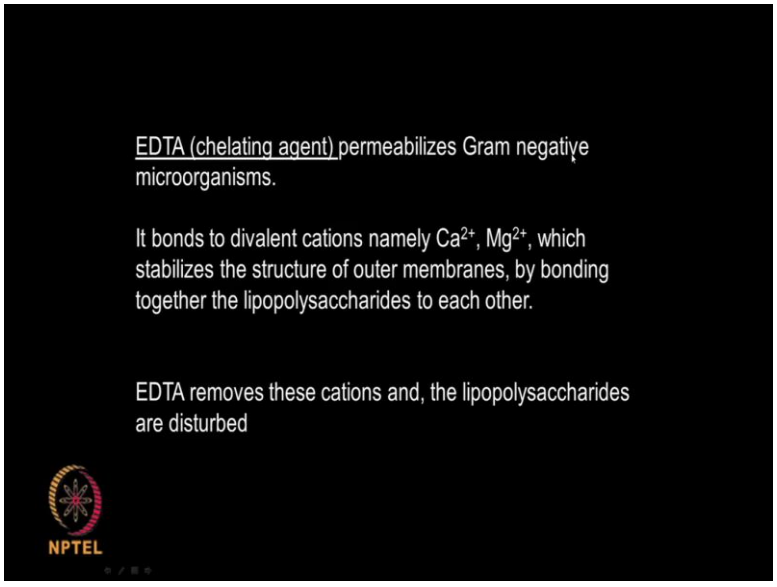

EDTA is a chelating agent what it does is it permeablizes the gram negative bacteria it binds to the cations like calcium magnesium which are present in the membrane thereby it is disrupting the membrane stability that way it is releasing the internal contents actually. So it removes the cations

lipopolysaccharides are disrupted so that is a very good yield.

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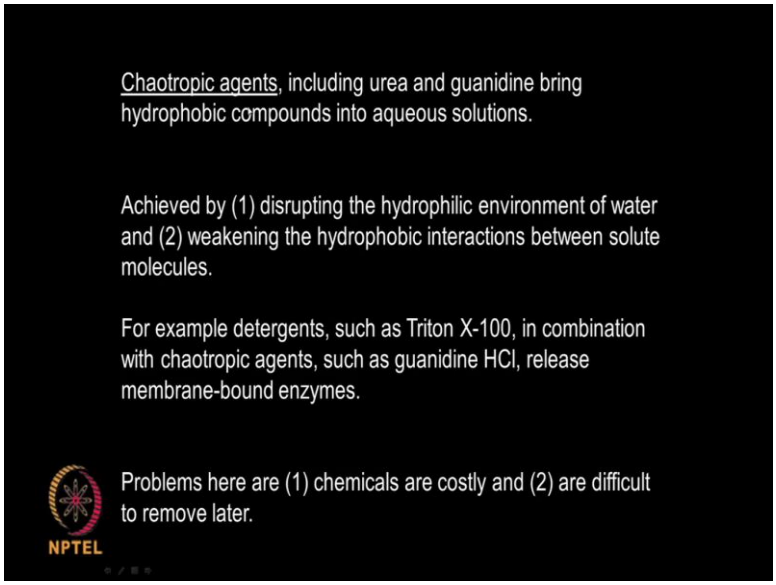

You also use chatotrophic agents like urea, guanidine, debric hydrophobic compounds into aqueous solutions, okay so the disrupt the hydrophilic environment of water, the weaken the hydrophobic interactions between solute and molecules okay. So like detergents triton x, you can use chatotrophic agents like guanidine, hydrochloride and so on actually but then the chemicals could be costly and then later on you may have to remove the chemicals from your product. So that may require certain intelligent thinking actually.

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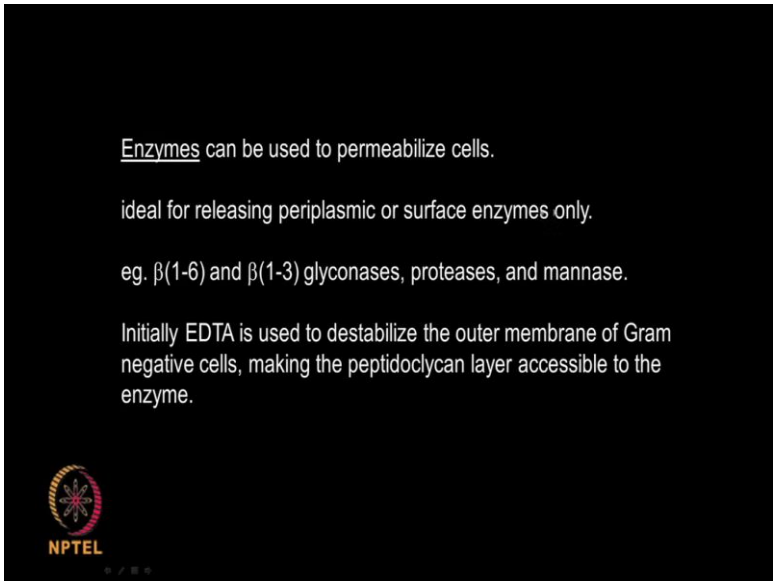

You can use enzymes as well to permeabilize cells enzymes of course can be expensive, so you have to be careful to use cheap enzymes for example, we can use enzymes like beta 1-6, beta 1-3 glyconase, you can use protease, mannase to destabilize the cell wall or make the cell wall of the bacteria so that the internal material are released. But then the problem is you need to later on

remove that enzyme that could be another issue you need to consider because if your intracellular material is an enzyme.

And you are using an enzyme method to break the cell wall then how do you remove these tow enzymes we need to keep that point in mind actually.

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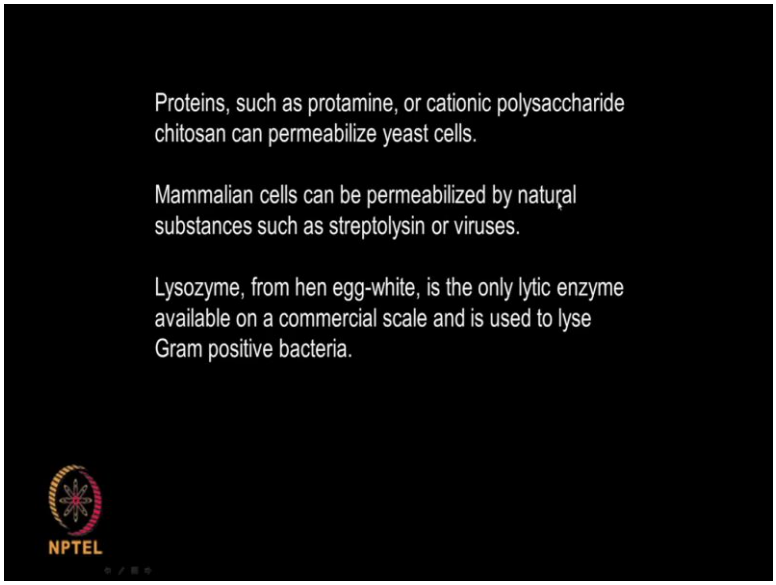

So protein such as protamine cationic polysaccharide chitosan can permeabilize yeast cells. So mammalian cells can be permeabilized by natural substances like viruses or streptolysin. Lysozyme, this is an industrial process lysozyme from hen egg-white is lytic enzyme and it is used to lyse gram positive bacteria it is used in commercial industrial scale actually so lysozyme is quite cheap and it can nicely make the cell wall of gram positive bacteria (poros) and thereby allow the release of the intracellular material okay.

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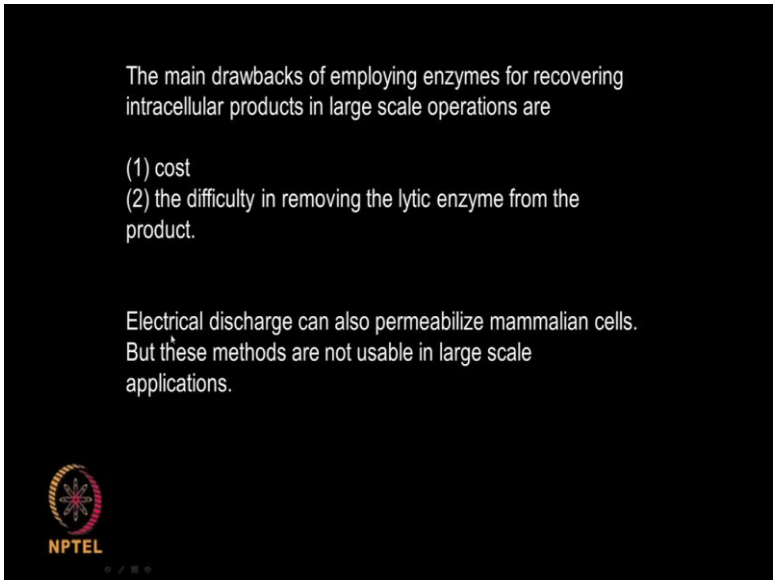

So main drawback cost could be a main drawback of this and then removing these enzymes from your final product could be a drawback. Of course there are other exotic methods like using electrical discharge methods permeablizes cells but then they are in the current we use in large scale may be in may be very small scale theoretical scale one can think about using those methods actually. Okay so with that we conclude the cell breakage it is a very important downstream technique especially if your product is inside the cells.

So you collect your cells that means harvest then you use one of these methods either mechanical, chemical, thermal or high pressure homogeneous type of method okay and then you release the intracellular material so that it comes into the (())(30:12) and then you follow the usual downstream purification and recovery and so on actually.

Thank you very much.