Genetic Engineering: Theory & Applications
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Module 6: Recombinant DNA Technology (Part-ll)
Lec18: Protein Production in Host-(Part-II)

Hello everybody this is Doctor Vishal Trivedi from department of Biosciences and Bioengineering IIT Guwahati. And what we were discussing we were discussing about generation of genetically modified organisms. And in the last couple of modules what we have discussed so far. We have discussed about the physiology of host. As well as we have discussed about the different types of vector or the transforming agents what you can use to develop the genetically modified organisms in a particular host.

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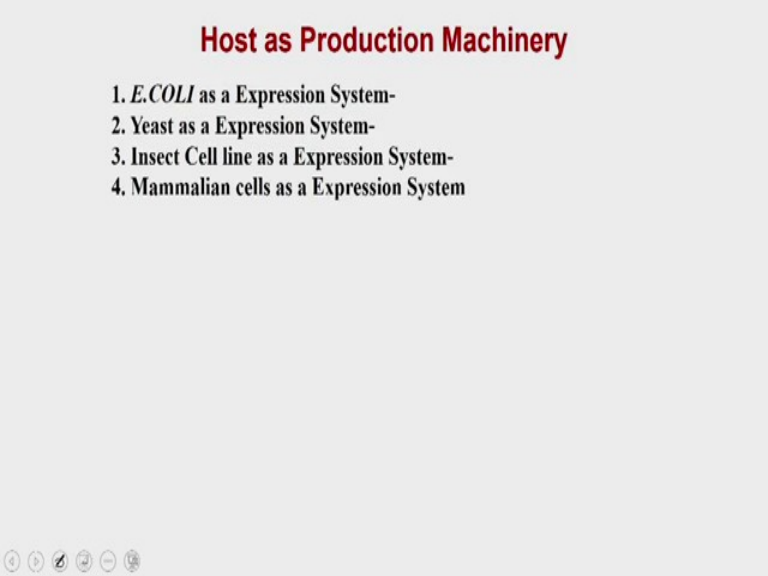

In this context we were we were discussing about how to produce the protein in different host strands. And in this discussion we have so far discussed about the E coli as an expression system. And in today's lecture we are going to discuss about the remaining expression systems such as the Yeast as an expression system, insect cell line as an expression system and lastly we are going to discuss about Mammalian cells as an expression system.

So when you talk about and the E coli as an expression system, we have discussed many aspects we have discussed about how to clone the protein into the E coli expression system, what are the

different types of host and the strategies which are available for you to produce the protein under different host systems. And following this context, now today we are going to start the lecture with the discussion about how to control the protein production in the E coli expression system.

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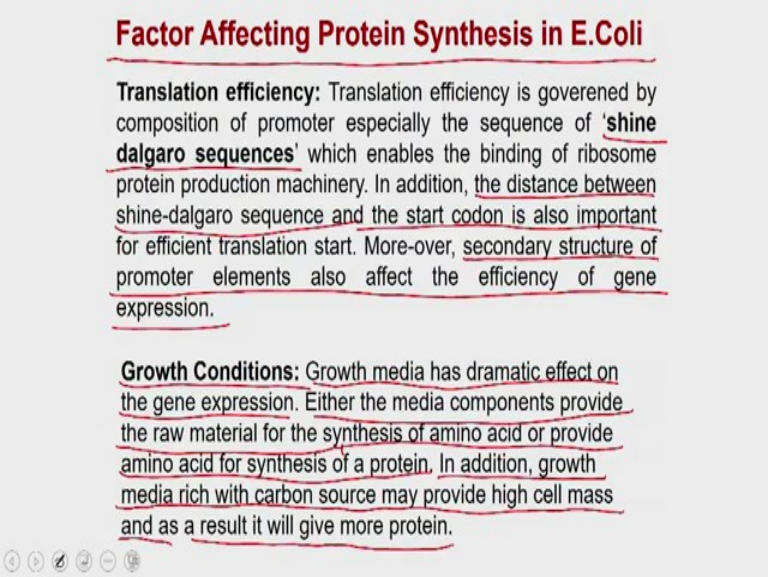

So there are many factors which are affecting the protein synthesis within the E coli. One of the major factors which we have discussed in the previous lecture as well is the strength of the promoter itself. So if you can have the two types of promoter, either the promoter which is very very strong promoter, and the strong promoter is going to facilitate the easy binding of the RNA polymerase. And that is how it is going to facilitate the more turnover number of the transcription events. And as a result you are going to have the high level of RNA in the RNA pool.

And subsequently as a result you are going to generate the more amount of protein. In addition to that if you have the if you have the weak promoter, the weak promoter is not going to support the protein production and as a result you are going to have the low level of protein expression. Both the strong promoters as well as the weak promoters has its own advantage as well as the disadvantages.

The strong promoter gives the very high amount of protein, that is why it is good for the protein production. If the protein is small in size and if it is easily getting folded, but if it is a big protein

and it requires the time for getting folded; then you need to use the weak promoter which is actually going to give the cellular machinery to fold the protein properly.

Apart from the promoter as a deciding factor to control the protein production or the efficiency of protein production, you also have to consider about the another parameter which is called as the Shine dalgarno sequences. Where Shine dalgarno sequences actually allows the binding of ribosome machinery to the recombinant DNA. And it actually controls the overall protein production from the particular clone. So the distance between the Shine dalgarno sequences and the start codon is very very important for the efficient transcription as well as efficient translation of that recombinant DNA.

Along with that the secondary structures which are been present in the promoter are also going to affect the efficiency of the protein or efficiency of the gene expression.

Apart from that the growth conditions or the growth media which you are going to use has a very very dramatic as well as significant impact onto the particular protein productions. For example you can imagine a protein which you are using and that requires a large quantity of arginine or lysine or glutamine. In those cases you have to ensure that the media components should have these amino acids or these essential amino acids. So the growth media we have already discussed about the growth media which are actually going to be used for growing the bacterial cells.

And the growth media has a dramatic effect on the gene expression. Either the media components provide the raw materials, this is like what we have discussed so far. Whether you, you are if suppose your protein requires lysine, arginine and glutamate. Then these amino acids should be present or their precursor should be present in the media. Or the provide the amino acid for synthesis of a particular protein. In addition some of the growth media are very very rich in carbon source and that actually going to generate a large number of cell mass. And once you have the large number of cell mass, subsequently or eventually you are going to have the large number of, you are going to have the more production of the particular protein from the cell.

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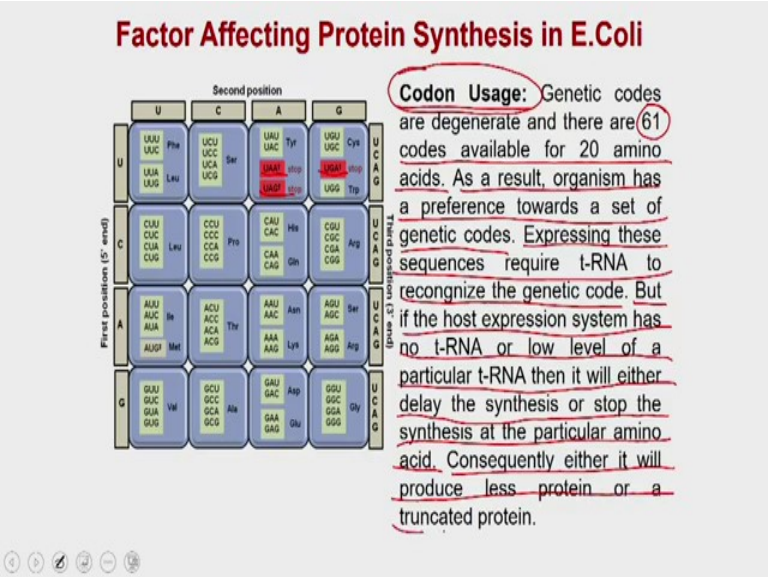

So apart from this the third factor is the codon usage. You know that the genetic codes which are 64 in number, where you have this 61 codes, 61 codes which are available for the twenty one different amino acids. Whereas the 3 codes are UAA, UGA and UAG are the stock codons. So as a result every organisms, so these 61 codons are every organism is using, but the every organism has a particular type of preference for set of genetic codes to express few to express few of the amino acids or a class of amino acids. In those cases what will happen is that if you are cloning a protein into the E coli expression system you have to ensure that the t-RNA corresponding to these codons should be available.

And this particular type of unique problem is called as the codon usage or the. So expressing these sequences requires t-RNA to recognise the genetic code. But if the host expression system does not have the t-RNA or low level of a particular t-RNA, then it will either delay the synthesis or it will stop the synthesis at a particular amino acid. You can imagine a situation that a E coli does not have the t-RNA for phenyl aniline which the gene which you have cloned into that particular particular E coli is require, then in those cases as soon as the RNA polymerase or the particular cellular bio synthesis machinery will reach to that particular phenyl aniline. It will not go into get the t-RNA, and as a result the protein synthesis will stop or it will go into generate the protein which is of the truncated in nature.

And this problem can be circumvent if you over express these additional t-RNAs into the host's strand. Either you make the chimeric expression system, where the you can clone these t-RNAs into the chromosome. Or you can supply this chromosome… supply these t-RNA genes cloned onto the separate plasmastes and you can transform your plasmate as well as these additional t-RNA plasmate. So if you do so you could be able to overcome the codon usage problem. So if that happens it will actually go into produce less protein or truncated protein.

So if you encounter the codon usage problem, you have to either over express these additional t-RNAs into the separate plasmate. Or you have to generate the chimeric host which will actually go into have these additional t-RNA genes, clone into their chromosomes. So either of these strategies will overcome the codon usage problem. And it will allow you to over express the protein. So before you decide to over express a particular gene, you have to verify as well as the you have to test that this problem it does not exist. Which means you have to critically evaluate the gene structure or the gene sequence to know the particular type of t-RNA would not be a problem. Ok. So now let us move on, so the protein which you over express in E coli could be over express as such or the native protein. Or you can have the protein which is overexpressed in the form of a fusion protein or the protein which actually going to have the additional tags.

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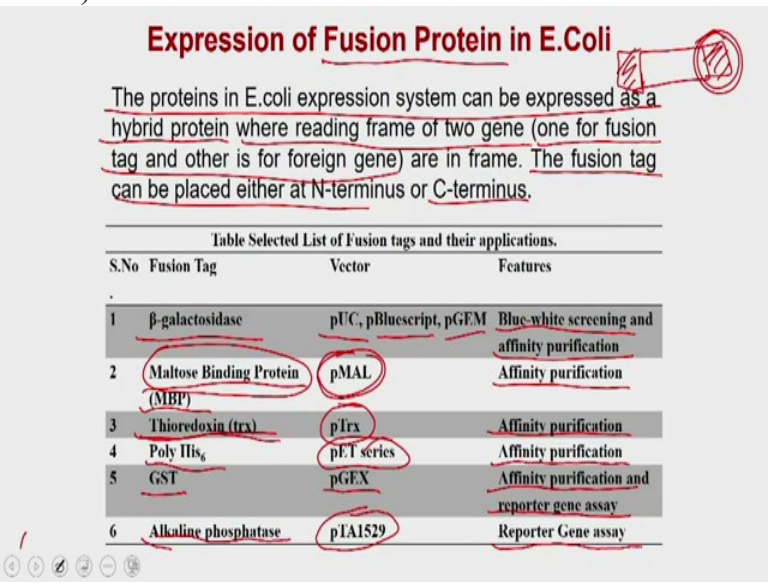

So the protein in E coli system can be expressed as a hybrid protein which is also called as the fusion protein. Where the reading frame of the two gene one gene which is foreign gene, as well

as the one of the gene which will be for the fusion tag is going to be in frame. So as a result when the protein when the cellular machinery is going to over express the foreign gene. It is also going to over express the fusion tag and as a result what you are going to have is you are going to have the chimeric fusion protein. Which means the protein is going to have the additional tag or additional protein from the other portion. And this fusion tag could be placed either on the end terminals, which means it could be placed on this side or it could be placed on the C-terminus side.

There are many different types of tags which are available for these particular applications, you can have the beta galactosidase as a as an enzyme as a tag. And the vector what you can use for this purpose is called pUC, pBluescript or pGEM. What is the advantage of this particular type of tag? You can use this tag for Blue-white screening, as well as you can use the affinity purification. Similarly you can have the Maltose Binding protein or the MBP, the vector what you can use where the pMAL is already being cloned is called as the pMAL. And it has an advantage that you can use the pMAL tag or the Maltose Binding Protein as a tag to purify using the affinity chromatography. Similarly you have the Thioredoxin or the trx as a fusion tag the vector what you can use is called pTrx. And it has an advantage that you can use the Thioredoxin tag as in the affinity chromatography.

Similarly you have the poly histidine tag and the the couple of pET series vectors can be used for and you have the advantage that it would be used in the affinity chromatography. Then you have the GST tag the glutathione s-transferase tag and the vector what you can use is the mini series of pGEX vector. And that has an advantage that you can use that in an affinity chromatography or you can use this in the reporter gene assay. Similarly you have the Alkaline phosphatase as a tag and the vector what you can use to generate the fusion protein is called pTA1529. And that has an advantage to use the Alkaline phosphatase in the reporter gene assay. So what is the advantage of producing the E coli in the fusion tag.

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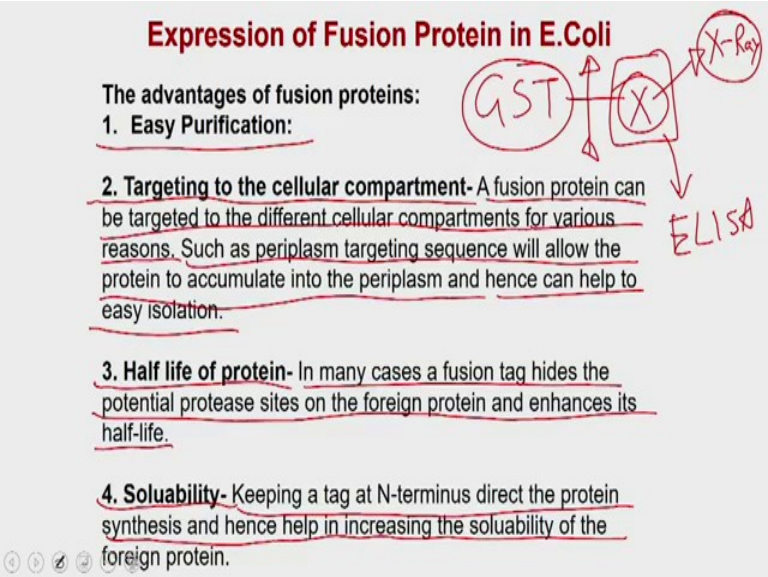

You have the multiple advantages if you are expressing the fusion protein in the form of a fusion tag. One of the easier, one of the prominent advantage is that, it actually allows you to do easy purification which means you see if you go by the conventional chromatography techniques you might have to use the different types of chromatography columns like anion exchange columns, cation exchange columns or the gel filtration columns to get the purified protein. And the disadvantage of running the multiple chromatographic column is that at every step you are you are going to lose some amount of protein and at the end your overall production of that particular purified protein is going to be lower.

Compared to this in the fusion proteins, you are going to use the affinity columns and these affinity columns are having very very exclusive affinity for that particular fusion tag, so as a result all other protein when you pass through the lysate to the column all other proteins are not going to binds to the column. Whereas the your protein will bind because it has a fusion tag and then subsequently you will wash the column and elute the protein. So in that process what will happen is first thing is you are going to get the purification which is very very high.

Number two you are going to use only single column which is that affinity column and that actually will give you very high purification compared to utilising the multiple conventional chromatography techniques. Number three it is going to be fast because you are just simply running the single column so it is going to save the time for you. Number four since you are

saving the time and the protein is getting purified at a faster rate you are not going to get the protein degradation. Which means the protein is going to saved from the proteases and other other enzymes which are present in the cell lysate.

Number five since you are doing the single step purifications, it is going to be economically viable, as well as it is going to give you the higher production. Because you are not going to lose the protein in as you are going to lose when you run the conventional chromatography columns and in every column steps you are going to lose some protein. And lastly since you are going to purify in single step you does not require a large quantity of human resource to perform the protein purification. Which means if you are going to talk about performing the purification in an industrial setup you need the lesser main power to purify the protein, compared to if you go with the conventional chromatographic techniques.

Now, since you have the tags you can actually plan the experiment in such a way that you can actually target your overexpression of your protein to a specific cellular compartment. A fusion protein can be targeted to the different cellular organelles for various reasons. So this is more more advantage for the for the academic purposes. For example, if you want to overexpress your protein to the mitochondria or endoplasmic reticulum or plasma membrane or to some specific sites, you can actually put that particular kind of fusion tag and as a result what will happen is, the once that fusion tag is present the machinery will recognize. And that is how the protein will go to that particular site within the cell.

In addition to that in some of the for example the bacterial system you can actually put the peri plasmic localisation sequences. And in that case what will happen is, once this fusion tag fusion protein is going to be overexpress, the protein will go to the peri plasmic fractions. And what is the advantage of sending the protein to the peri plasmic fractions is that the peri plasmic fractions are away from the cytosolic site. So if isolation of these protein are going to be easier compared to cytosolic protein. Number two the you are not going to face a lot of contaminations because the amount of protein which are present in the periplasmic fractions is much less compared to the amount of protein which are present in the cytosol.

So as a result if you are supposed put the periplasm targeting sequences the protein will accumulate into the periplasm and hence can help for the easy isolation. Number three, some of

the proteins are having a very very short half life. And as you know that the half life of a protein is always been decided by the number of the number of amino acids which are present on the N-terminus of the protein. So some of the proteins are N-terminus region is very very prone for proteolytic cleavage. In some cases the proteins have its own intrinsic protease activity. And as a result actually cleaves its own protein as soon as it is synthesized.

And some time what happens is these kind of things are problematic because it does not allow you to overexpress the protein in a full length. In those cases what you do is you produce a fusion protein and in that fusion protein what will happen is either depending on what is the scenario of that particular protein, either you put the fusion tag into the N-terminus side or you put the fusion tag on the C-terminus side. And either of these sides will be get blocked. So in some cases what happen is, if you put the fusion tag on to the N-terminus side, it actually enhances the overall half life of your chimeric protein, and as a result you are going to have the better survivability of this particular protein or better stability of this particular protein within the cytosol. So in many cases fusion tag hides the potential protease sites on the foreign protein and enhances its half Life.

Number four the fusion proteins are so, the tag what you are going to put are much more soluble compared to the tag which compared to the protein what you are going to you overexpress. Because most of these tags are already been optimised that they are been expressing into the E coli system. And they are expressing as a soluble factor. So once you put a fusion tag and once you generate a chimeric protein containing the fusion tag, the fusion tag component or the counterpart is actually brings the solubility into the overall system. And as a result the chimeric protein is more soluble compared to your own protein.

For example, if you put the GST as a fusion tag on to the N-terminus, GST is a cytosolic protein, it is very very soluble into the E coli system. So once you generate a chimeric protein along with this, since the GST is very soluble it actually gives that solubility parameter to your protein also. And as a result the whole chimeric protein is more, becomes more soluble. So keeping the tag at the N-terminus direct the protein synthesis, and hence help in increasing the solubility of the foreign protein. Now the question is, once you have the fusion protein and in some of the applications, the fusion proteins are not required.

For example, if you are going to use this protein for generating the ELISA. Or suppose you want to use this protein for, for solving the structure using the X-Ray, in those cases the fusion tag is going to interfere in downstream applications. For example if you would like to use this protein which is X (in) in in for a therapeutic purposes, for example if you are going to use this X as an insulin. For example are regenerating a fusion tag of insulin along with the GST, you do not want patient to receive the GST as well as the fusion tag. In those cases you have to remove the fusion tag or remove or cut this particular chimeric protein with the help of some proteases. So that the fusion tag is going to be separate.

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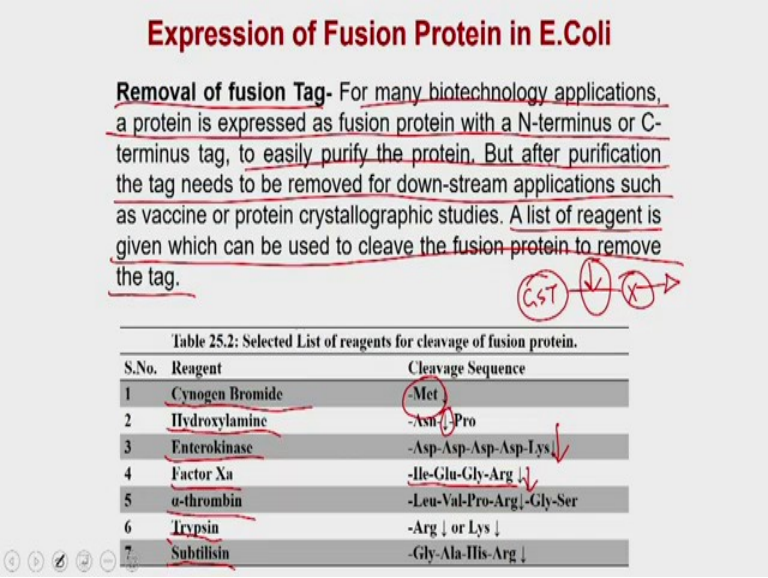

How to do that? So for removal of fusion tag, for many biotechnology applications, a protein is expressed as a fusion protein, either to the N-terminus or the C-terminus, for the easy purification. But after the purification the tag needs to be removed for downstream applications, such as if we are trying to use this protein for vaccine, for developing the ELISA or develop for… suppose we would like to use this protein for structure solutions. Or suppose we would like to use this protein simply for driving a catalytic reaction within the patient. Or into the bioreactors, or sometimes if suppose you want to use this protein for therapeutic protein, so it you want this protein to and inside the cell, inside the human body, or inside the host and drives some reactions. And in those cases the fusion tag are going to interfere or in some cases the

fusion tags are definitely going to cause the allergic reaction. In those cases the fusion tag has to be removed.

For removing the fusion tag, we have a couple of reagents, for example you can use the Cyanogen Bromide. Cyanogen Bromide is going to cleave the tag just after the metunin. Simply you have the Hydroxylamine, Hydroxylamine is going to cleave the fusion tags between the Asparagine and Proline. Then you have the Enterokinase and you have Enterokinase cleavage site. So Enterokinase cleavage site would be present between the fusion tags and once you treat this fusion tag with the Enterokinase it is going to cleave here, and as a result, GST is going to be removed and your protein x is free for the for downstream applications.

Then you have the Factor Xa, the Factor Xa cleavage site is also present and Factor Xa is always cleaving after the arginine. Similarly you have the Alpha thrombin, Alpha thrombin is cleaving the chimeric protein between the arginine and glycine. Then you have the trypsin, and trypsin is cleaving the protein just after the arginine or the lysine. And then lastly you have the subtilisin, subtilisin is cleaning after the arginine. So these are not the final list or the exhaustive list of the cleavage reagents which are available for separating the fusion tag from the foreign gene or foreign protein. Now the question is how to remove the tag from your protein.

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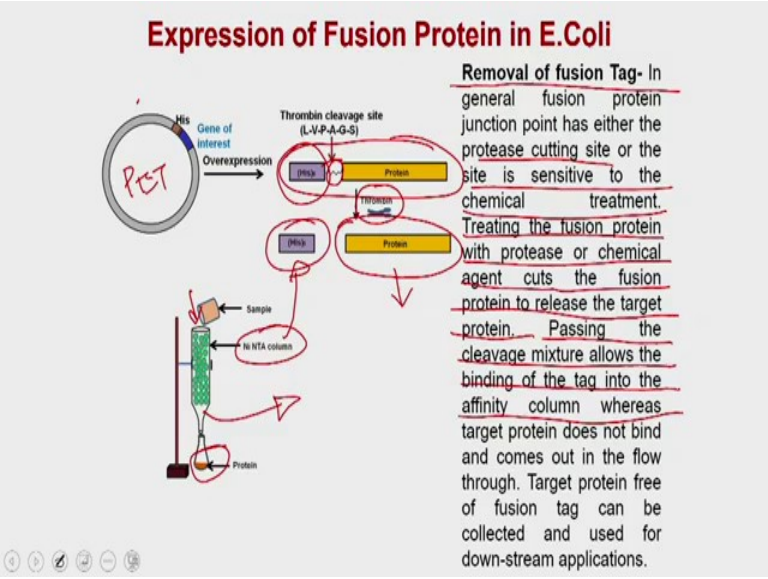

So for that you can use the same affinity column what you have been using for purifying the protein. So you can imagine that you have you have cloned a protein into a His tag, for example the pET series vector and you have over express the protein, now this is your chimeric protein you have overexpressed which it contains the histigene tags. And now what you do is you take the thrombin, Alpha thrombin and treat it. So what will happen what will happen is the Alpha thrombin is going to cleave the cleave the linker region between the histigene tag as well as protein and as the you are going to have the tag separately and as well as the protein separately.

Now what you do is take this complete complex mixture and load it onto the affinity column like Nickel NTA column. So as a result what will happen is the Nickel NTA is going to bind the histogen tag, because it has a very high affinity for histogen tag. But it does not have any affinity for your protein. So as a result what will happen is the the histogen tag is going to bind to the beads of the column. Whereas the your protein will be present in the into the flow through. And you can collect the protein from the column and you can use this protein for the downstream applications.

So how to do the removal of fusion tag, the fusion tag is… in most of the fusion tag you are going to have the protease cutting site, or the site is sensitive for the chemical treatment. So if you treat the fusion protein with a protease or chemical agent that will cut the fusion protein to release the target protein. That is what is being done. Now if you pass this cleavage mixture and allow the binding of the tag to the affinity column, which is actually in this case we have used Nickel NTA column. The column will bind your tag and it will not allow this tag to go into the flow through, whereas your protein has lost the affinity for the column because it does not have the tag now any more. And the protein will come into the flow through. So this is all about the overexpressing and production of protein into the E coli expression system. We have discussed many aspects which are very common for the different for the other expression system as well.

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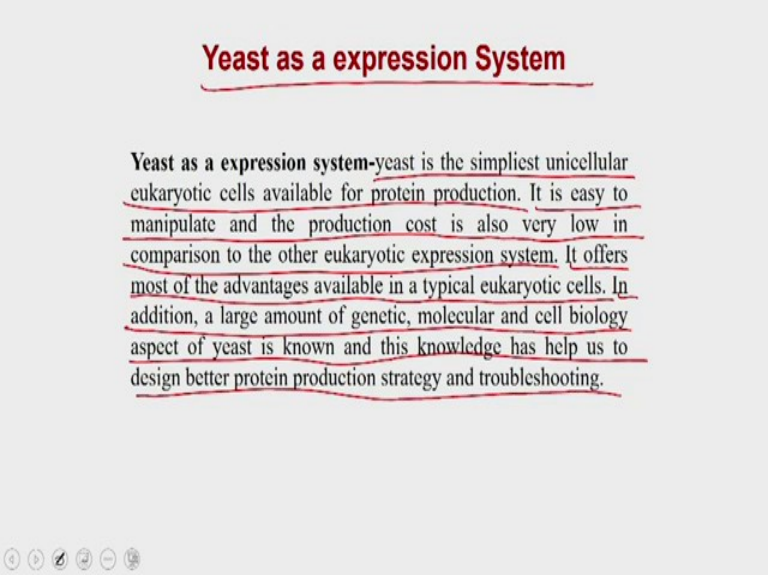

Now we will move on to the next expression system. So in the list we have the next expression system which is the Yeast as an expression system. So Yeast is the simplest Eukaryotic organisms and unicellular Eukaryotic organism which you can use for over expressing those proteins which will not going to over express nicely into the Eukaryotic system. So Yeast is the simplest unicellular Eukaryotic cell available for protein production. It is easy to manipulate and the production cost is very low in comparison to the other Eukaryotic system. So in the Eukaryotic system you have the yeast, you have the insect cell lines, you have the mammalian cells, you have the virus or bacteriophage based protein production system. Apart from… so in comparison to these systems yeast is the simplest system in terms of performing the transformation in terms of the screening the clones, as well as in terms of producing the protein, and the ultimately cost of the protein production in the yeast system is is very very low compared to the other Eukaryotic expression systems.

It offers most of the advantages available in a typical Eukaryotic cell. In addition a large amount of genetic, molecular and cellular aspects of yeast is known and this knowledge has helped us to design better protein production strategy and troubleshooting. So you know that yeast is very widely studied organism. So because of that the yeast is being genetically manipulated we have very in depth knowledge about the biochemistry of these cells. As well as we know each and every minute details of the molecular biology protocols or molecular biology strategies which

you can use to manipulate the yeast as an expression system. And as a result of this the people are using the yeast very extensively for for producing the protein which requires the Eukaryotic machinery.

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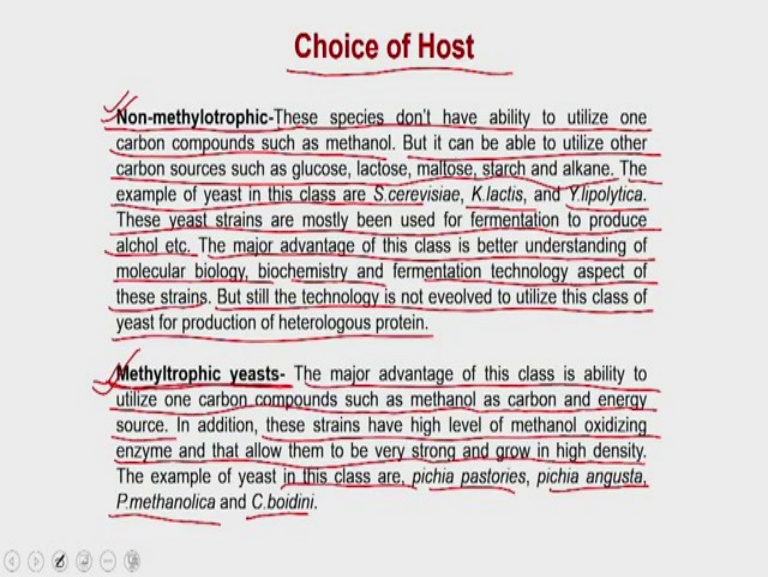

Now in the yeast you have the two choices of host one is called non-methylotrophic host and the other one is called as the methylotrophic host. Non methylotrophic host means these yeast molecules or yeast organisms does not depends on does not accept the methanol as a one carbon source or carbon source. And instead of methanol they use the other carbon sources. So these species do not have the ability to utilise one carbon compound such as methanol. And that is why these are called as non- methylotrophic yeast strains. But it can be able to utilise other carbon sources such as glucose, lactose, maltose and starch and alkane. The examples in this category are saccharomyces cerevisiae, K.lactis and Y.lipolytica. These yeast strains are mostly been used for fermentation to produce the alcohol. The major advantage of this class is better understanding of molecular biology, biochemistry and fermentation technological aspects of these strains.

So the non-methylotrophic host strains or non-methylotrophic yeast strains are not using the methanol as a one carbon source, instead of that it is using the other carbon sources such as glucose lactose and all other kinds of sugars as well as the alkane. And mostly these strains are been used for fermentation process these strains are been used for producing the alcohol and their fermentation related products, and these strains are less popular in terms of utilisation, in

terms of their utilisation for the protein production. So these strains are not been utilized in terms of production of heterologous proteins.

Now methylotrophic yeast, methylotrophic yeast as the name suggests these yeast strains are using methanol as a one carbon source. So these strains have the advantage that they have the ability to utilize one carbon compound such as methanol as carbon and energy source. In addition, these strains have high level of methanol oxidizing enzyme and that allowed them to be very strong and grow in a high density. The example of yeast it in this class are pichia pastoris, pichia angusta and pichia methanolica and C. boidini. So these are the strains, the methylotrophic strains which people very often use for protein productions.

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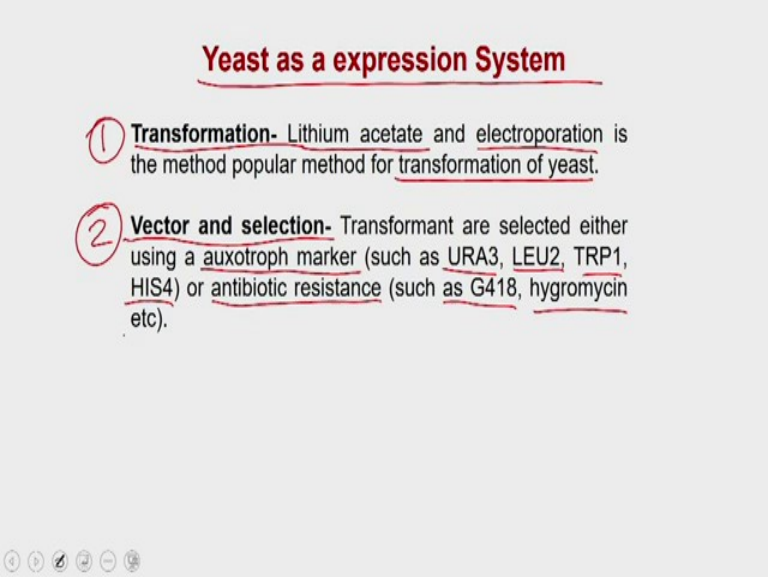

Now if you want to use the yeast as an expression system, the first step is that you are going to put the you are going to transform the recombinant DNA what you have generated into the yeast vectors. So we have discussed a couple of yeast vector in the past and we have, we have also discussed about the transformation methods where you are going to use either the stereoplast based transformation methods or the Lithium acetate based transformation methods. So in for protein production mostly people use the Lithium acetate or the electroporation as a popular method for transformation of Yeast. The transformations are then selected based on the kind of vector what you are going to use. So in the second step you are going to select the vectors as well as the transforms host strains, either you will, so mostly people use the auxotroph marker such as

URA3, LEU2 or TRP1 or HIS4, or sometimes they use the antibiotic resistance gene such as the G418, hygromycin etc.

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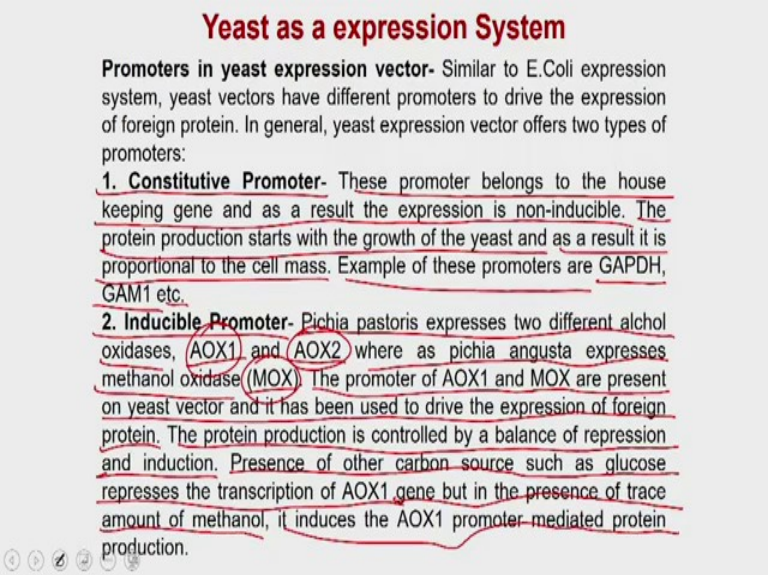

Once these two steps are over then you have to use the promoters, so just like as we discussed in the in the E coli expression system. There are different types of promoter also present in the yeast as an expression system. So you have the two different types of promoter present in the Yeast expression system. Either you have the constitutive promoter or the inducible promoters. So in the constitutive promoters, these constitutive promoters are the promoter which are present in the house keeping genes. So these promoters actually belongs to the house keeping gene and as a result the expression is non-inducible. Which means they are going to give you the protein but that protein production would be in proportion to the number of cells what you are going to have. You are not going to be able to use any inducible agent, for example if you remember in the case of E coli we were using the IPTG or lactose or some kind of inducible system where we were using the that kind of environmental changes like for example when we were using the CI3 promoters, we were changing the temperature and as a result changing the temperature it is actually also driving the protein production. But in these cases you can not do that because most of constitutive promoters are the promoter which are belonging to the housekeeping genes.

And you know the purpose of housekeeping gene is to produce the protein at a consistent rate and throughout the life of the particular organism. One of the classical example is the LDH, so

LDH protein is a housekeeping gene or actin or myosin, most of the structural proteins which we which we are present or which are actually be a part of the formation of plasma membrane are actually belonging to the housekeeping genes. So housekeeping genes are never been inducible because they are been consistently producing the protein. The protein production starts with the growth of the yeast and as a result it is proportional to the cell mass. Examples of these promoters are GAPDH, GM1 etc.

Now the second class is the inducible promoters, inducible promoters are the promoter where you can actually some class of inducers to produce the protein in a large quantities. Examples are pichia pastoris expresses two alcohol oxidases like AOX1 and AOX, whereas the pichia angusta expresses methanol oxidase MOX and all these three genes, if you take the promoter out of these genes, these promoters are going to be inducible promoter because as soon as you add the alcohol these genes are been, the promoter which is present in front of these genes are going to generate the large quantity of these proteins. The promoter of AOX1 and MOX are present on yeast vector and it has been used to drive the expression of a foreign protein in a inducible matter.

The protein production is controlled by a balance of repression and induction. Presence of other carbon sources such as glucose represses the transcription of AOX1 gene, but in the presence of trace amount of methanol, it induces the AOX1 promoter mediated protein production. So inducible promoter just like it we remember. If you have the very large small quantities of lactose that actually is going to give the Ligy expression in the E coli system. Similarly here if you have the alternate carbon source for example if you have the glucose the East is not going to use the methanol as a carbon source, instead it will use the carbon, glucose as a carbon source. In those cases the glucose is going to repress the activity of AOX1 promoters. But if you use methanol or tiny amount of methanol and that actually is going to induce the AOX1 promoters. And as a result you are going to see the protein production if you have cloned the protein in front of the AOX1 promoters.

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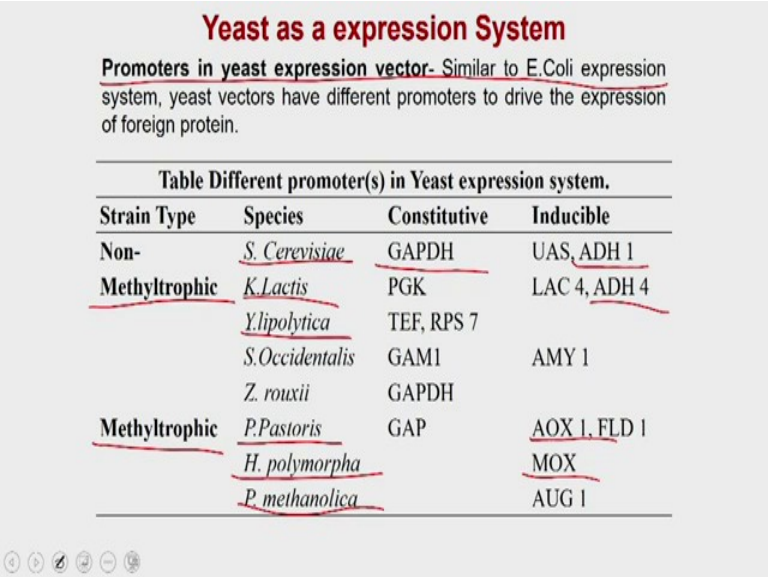

So this is just a summary of different promoter as well as the expression systems present in the E coli expression system you have non methylotrophic strains such as S. cerevisiae, K.lactis or Y.lipolytica. As whereas the you have methylotrophic strains such as pichia pastoris or pichia methanolica. As you have the constitutive promoter such as GAPDH and all other kind of promoters where as you have been visible promoters such as ADH1, ADH4 and AOX1, MOX can all these promoters can be used to drive the protein production into the yeast as an expression system.

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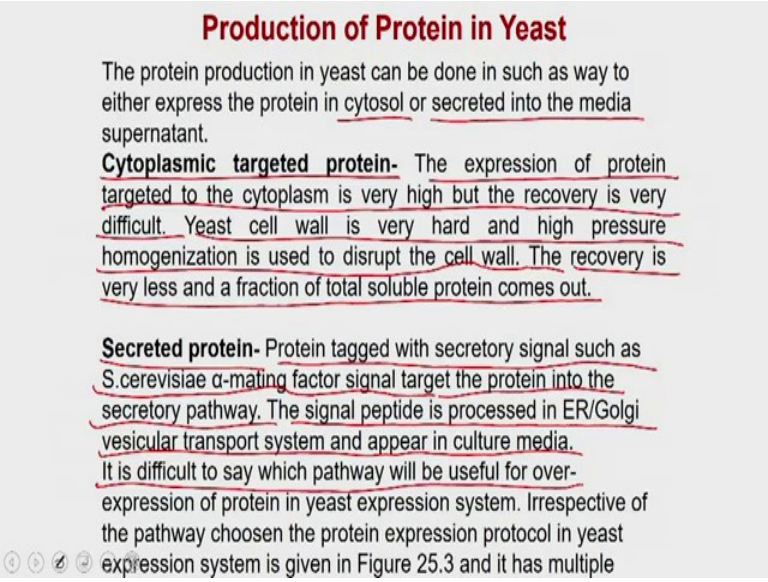

You have the choice that you can produce the protein either into the cytosol or you can have the choice that you secrete the protein into the external supernatant. And that it depends whether you have the any kind of tag which is present on the protein. So if you have a tag which is present on the protein, that protein will go into secret out. So cytoplasmic targeted protein, the expression of the protein targeted to the cytoplasm is very high but the recovery is very difficult. Yeast cell is very hard and high pressure homogenization is required, a homogenizer is required to disrupt the cell wall. The recovery is very less and the fraction of total soluble protein comes out. So if you over express the protein as cytosolic protein, the protein production is going to be very very high but the disadvantage is that since the E coli as the yeast has a very strong cell wall, cell wall is not going to be broken very easily and as a result even if you use the high pressure homogenizers like French press and all other kind of homogenizers, even then the recovery of the protein from the yeast cell is going to be very less.

Now if you are putting a secretory components like protein. If you protein tagged with a secretory signal such as S.cerevisiae Alpha mating factor that actually will promote the secretion of the protein into the secretory pathway. And that actually signal peptide is processed in E coli vesicular transport system and appear in culture media. So in that case the protein will be secreted out, it will present in the culture media. But it depends about the protein which is going to over express, it is very difficult to you decide whether you would like to the cytoplasmic

pathway or the secretory pathway. Because both are these pathways have their own advantages as well as the disadvantages. And that actually you have to decide depending on the type of protein which you would like to over express in yeast expression systems.

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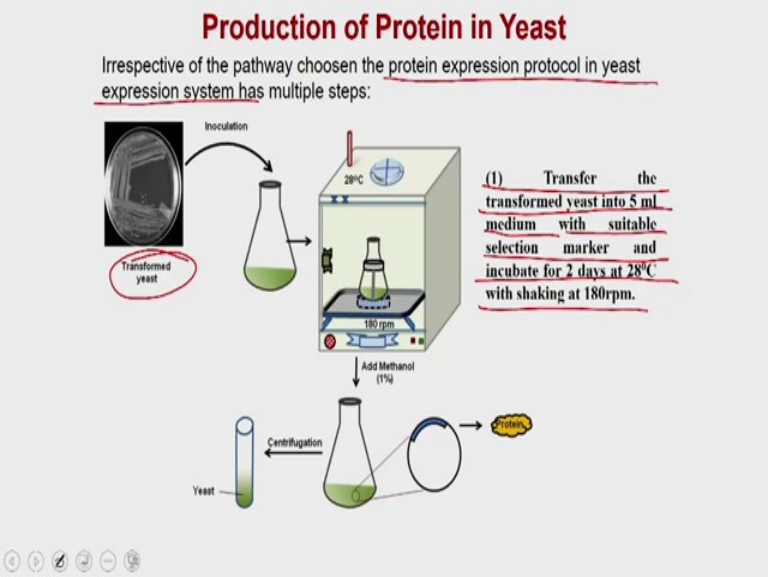

Now we have the multiple steps which you have to follow for the protein production in the yeast expression system. The first step is that you do the transformation of the yeast with the recombinant DNA. So once you generated the transformed yeast the first step is the transfer the transform the yeast into a 5 ml media with suitable selection markers and incubate that for two days at 20 degree 28 degree Celsius with shaking at 180rpm. So the first step is that you first transform the bacteria first transform the yeast with the foreign DNA for Recombinant DNA. Then inoculate that into a 5 ml media selection marker and then incubate that on 28 degrees Celsius for 180rpm. That actually will allow yeast to grow at a very high density.

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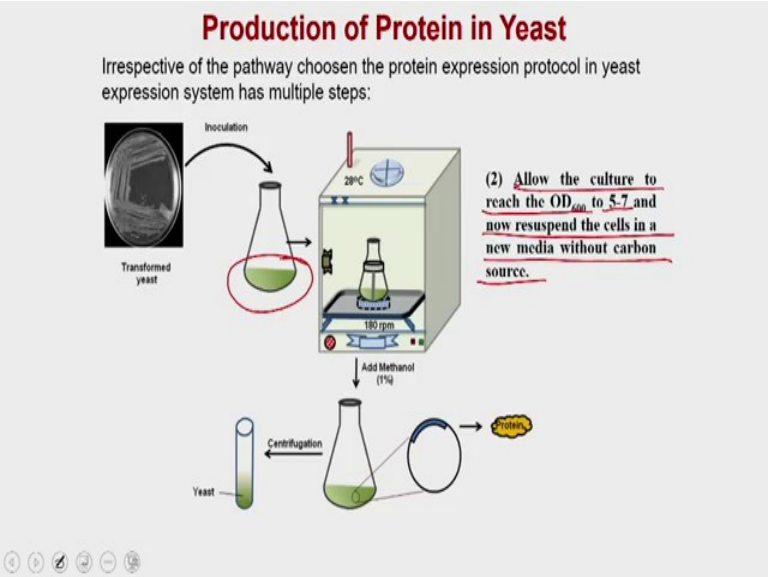

Once it reach to a specific density then you allow the culture to reach the OD of 5 to 7 and now resuspend the cells in a new media without carbon source. So in this particular type of media you are not going to use the methanol as a carbon source here you are going to use the regular carbon source such as glucose or other kind of carbon sources. And then you allow the yeast to grow to a OD of 5 to 6, once it reaches to a OD of 5 to 6, then what you do is pellet down this yeast cells, put it into a new media. And in this new media you are not going to provide the glucose or other one carbon sources for the carbon source.

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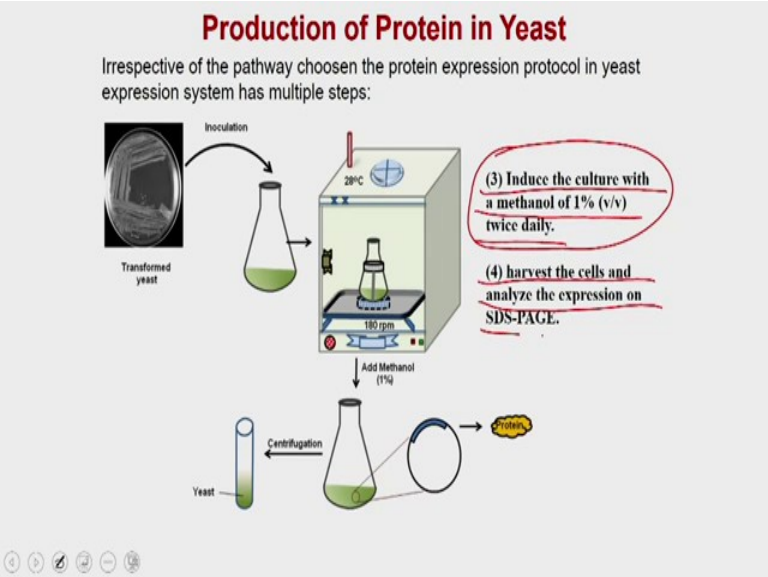

Instead you were going to induce the culture with methanol the one percent methanol twice daily. As a result what will happen is the… since you know the methanol is very toxic. But for these particular type of strains, because they have the ability to metabolize the methanol. You have to use the very small quantity of methanol only the one percent methanol you have to use that to twice daily. And that will actually induce the promoters of AOX1 or MOX and that actually will allow the production of the protein. Once the protein is being produced for in couple of days, because that actually will take time. Then you harvest these cells by centrifugation and analyse the expression on the SDS-PAGE. Just like as we discussed in the E coli system as well.

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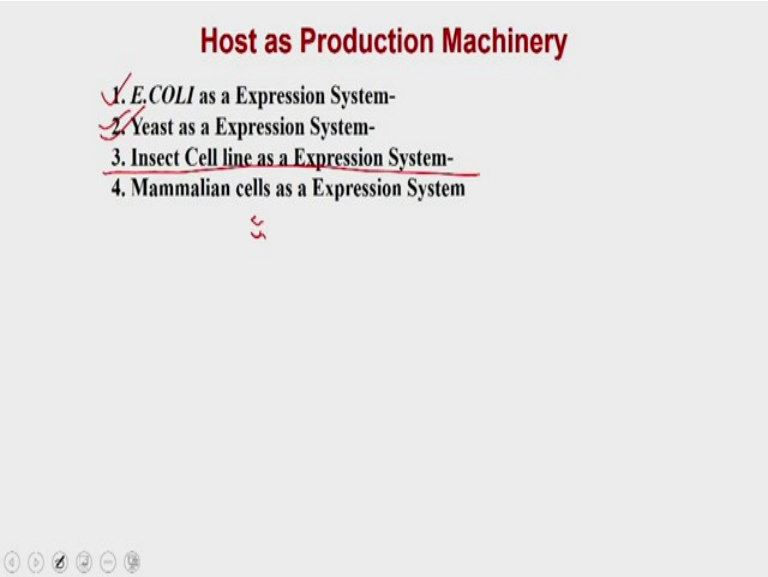

Now so far we have discussed about the E coli as an expression system, we have discussed about the yeast as an expression system. And now we will move on to discuss about the insect cells as an expression system. If you would like to use the insects as an as an expression system you have to use the what we what you have to do is you have to perform the multiple steps to achieve the protein production in insect cell lines.

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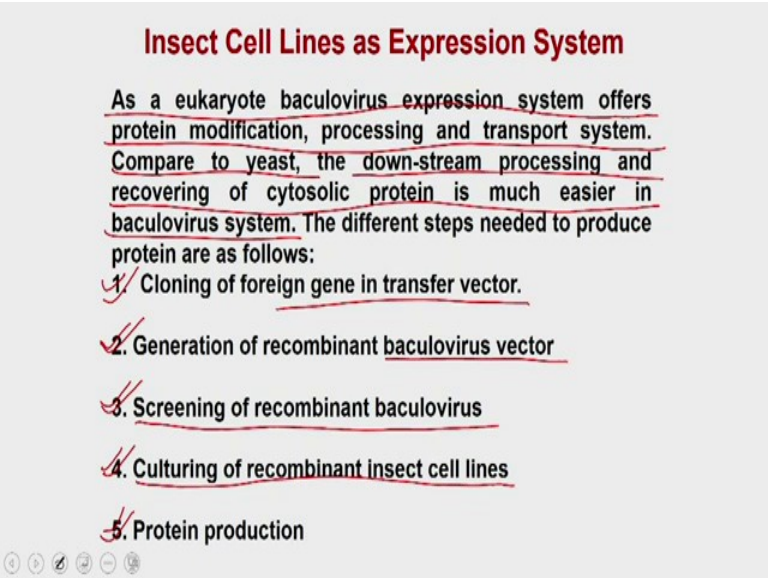

So as a eukaryote baculovirus expression system offers protein modifications, processing and transport system. Compared to yeast the downstream processing and recovery of cytosolic protein is much easier in baculo expression system. You know if you remember we have discussed that the yeast has a very very strong cell wall. And because of that the recovery as well as the processing of these cells are much more difficult. Which is actually going to be simplified in the baculo expression system or the insect cell line as an expression system. Because insect cell lines are easy to break and the recovery is much better.

So if you would like to produce the protein you have to you follow these steps. First you have to clone the foreign gene into the transfer vector. Then you have to generate recombinant baculoviruses or baculo vectors. Then you have to screen the recombinant baculovirus. Then you have the culture the recombinant insect cell lines. And ultimately you have to do the protein production.So let us start with the first step and the first step is the cloning of foreign gene in transfer vector.

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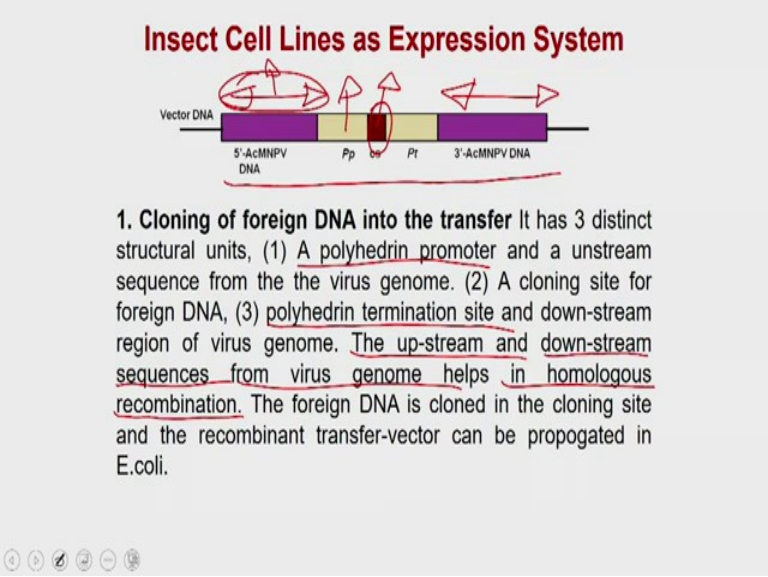

We have already discussed about the design of the vectors which are available in the baculo expression system. In the baculo expression system you have a vector which is actually e containing the the flanking sequences which are present from the viruses. So you have the N-terminus flanking sequences you have the C-terminus flanking sequences. In between you are going to have the promoter which is for the polyhedrin promoter, a polyhedrin promoter then you

have in front of the promoter you have the cloning site and then and next to the cloning site you have the the polyhedrin termination side. So that this polyhedrin promoter is going to drive the production of the gene which you are going to clone into the cloning site. And the upstream as well as the downstream sequences which means these sequences are required are from the virus genome and that actually helps in homologous recombinations. So what you have to do is for the cloning of the foreign DNA into the transfer vector, you clone your particular protein into the cloning site.

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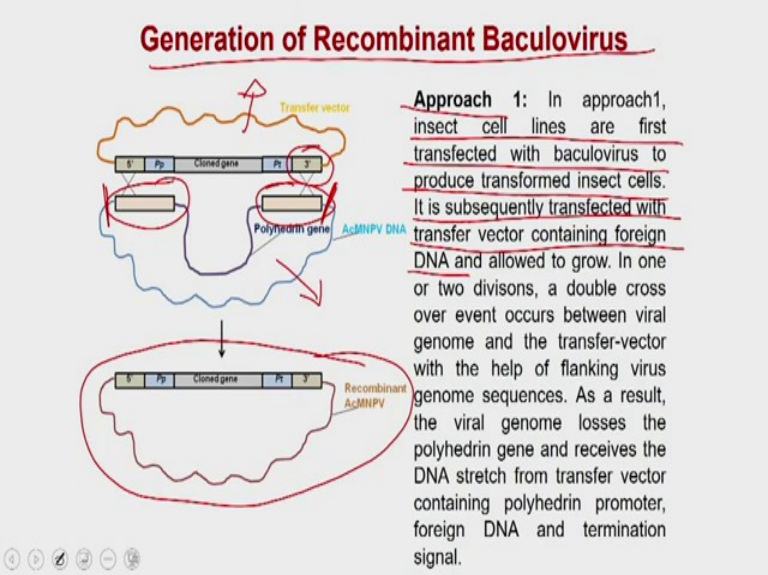

Now the next step is the generation of recombinant baculovirus. You have the two approaches to (prod) to generate the recombinant baculoviruses. In approach one what you are going to do is you can imagine that you have the insect cell lines. So first in the first step what you do is take the insect cell line and first transfected with the plane baculovirus and that actually will go into produce the transformed insect cell line.

Now in the second step what you do is you transfect that these cells with their transfer vector containing the foreign DNA. Now what will happen is once these two vectors, the (transformed insect cell lines which were you gave already put the viruses, and these transformed cells they will be transfected with the transferred DNA, containing the foreign gene once they will replicate for 2 to 3 cycles, what will happen is there will be a homologous recombination between these two factors these two regions. Which means the upstream as well as the downstream region are

going to do the homologous recombination. So this is transfer vector which you have transformed in the second step. And this is your plane virus. So as a result what will happen is this whole cassette from this from this place is going to be replaced with a your foreign gene and as a result you are going to generate the recombinant DNA or recombinant virus which actually will go into contain your gene or your cassette instead of the virus intrinsic polyhedrin gene. And as a result now what you do is you take this virus and you can use that for protein production.

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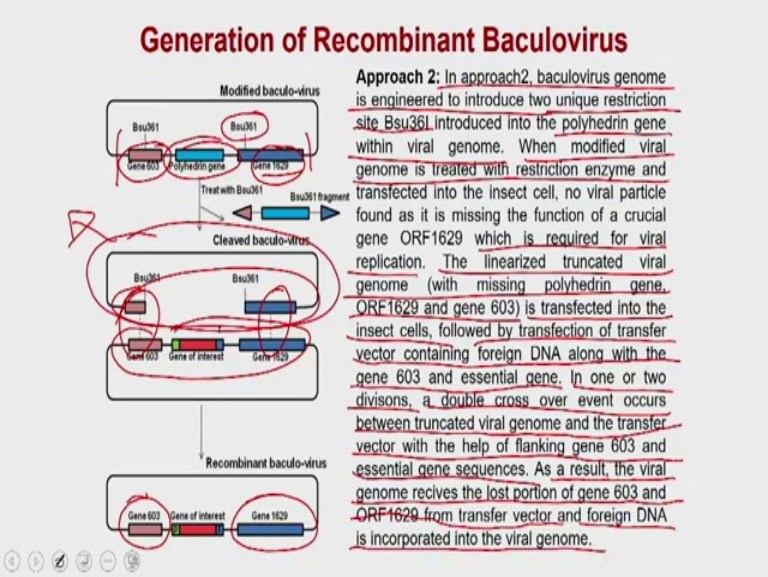

In the approach to what you have to do is in approach to the baculovirus genome is engineered to introduce two unique restriction site Bsu361 on to the gene 603 as well as on to the gene 1629. And both of these gene are present in the polyhedrin gene within the viral genome. When modified viral genome is treated with the restriction enzyme and if you take this modified baculovirus treated with the Bsu361, what will happen is it is going to be cleaved off from here. And as a result you are going to remove the complete casatte of polyhedrin gene. So you are going to lose this particular fragment. Now once you if you if you transform this particular fragment the cleaved fragment, you are not going to get any viruses, because you are actually missing the crucial function of the gene 1629 which is required for the viral replication.

Now this linearized viral genome or linearized truncated viral genome with missing polyhedrin gene ORF1629 and the gene 603 is transfected into the insect cell followed by a transfection of transfer vector containing the foreign DNA along with the gene 603 and essential gene. In one or

two and division, so once you take this particular gene and transfect into the insect cell lines. Once they will go with the one or two cycles. This particular region is going to go with the homologous recombination. And as a result the gene this particular cassette it is going to be replaced with this cassette and you are going to produce the recombinant baculovirus where you are going to have the gene 603 in the front and the gene 1629 on the backside. And in between you are going to have the gene of interest cloned within the within the promoter of polyhedrin gene. And now this Recombinant virus baculovirus is is ready for or protein productions.

So a double Cross event will occur between the truncated viral genome and the transfer vector with the help of flanking gene 603 and essential gene sequences. As a result the viral genome receives the last portion of gene 603 and ORF 1629 from the transfer vector and the foreign DNA is incorporated into the viral genome. So as a result you are going to generate the recombinant baculovirus genome, Recombinant baculovirus, so now whether you use the approach one or whether you use the approach two you have generated the recombinant baculovirus. Now these baculoviruses can be used to for protein production.

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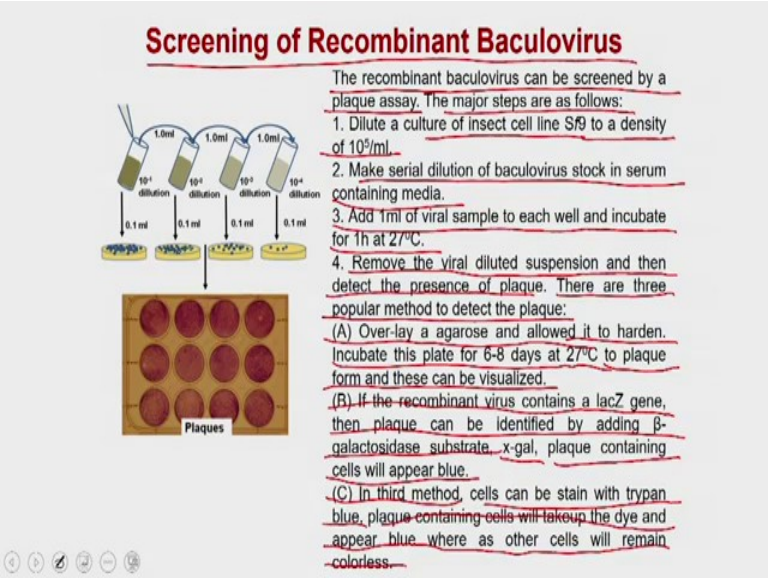

Now the subsequent step is screening of these baculoviruses. Recombinant baculoviruses can be screened by a plaque assay. The major steps in the plaque assay is, that first you dilute the the culture of insect cell line sf9 to a density of 10 to power 5. Make serial dilution of baculoviruses

stock in serum containing media. Add 1 ml of virus sample to each and incubate for 1 hour at 27 degree Celsius. Now remove the viral diluted suspension and then detect the presence of plaque.

There are three popular methods to detect the plaque. What are these methods? You overlay the agarose and allow it to harden. Incubate this plate for 6 to 8 days at 27 degree Celsius to platform and these can be visualized. Number two if the Recombinant virus contains a lacZ gene, for example then the plaque can be identified by simply adding the beta galactosidase substrates such as x-gal. And x-gal is going to give you the blue colour. So the plaque containing cells will appear the blue. In third method cells can be stained with trypan blue and plaque containing cells will take up the dye and appear blue where are the other cells will remain colourless. So plaque is nothing but the holes within the cell. So once you add the trypan blue the trypan blue you will directly go into the cell and it will give you the blue colour.

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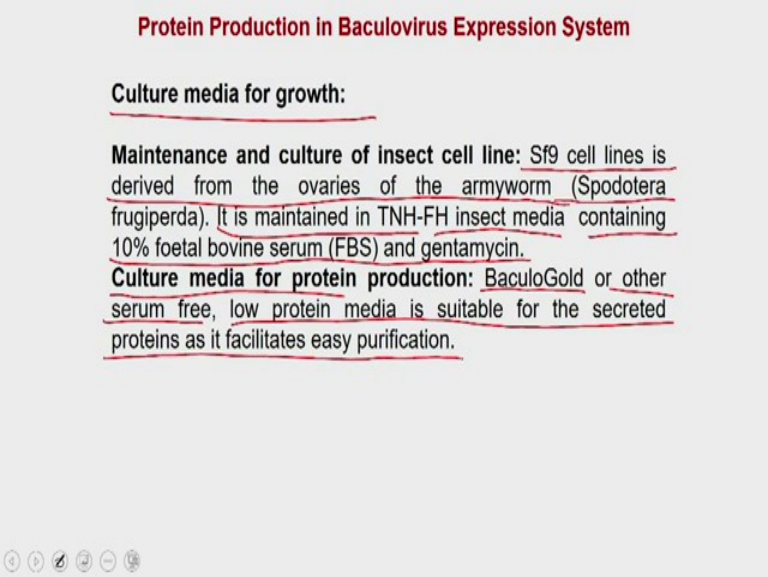

Now once you are done with the screening you can use this Recombinant baculoviruses for protein productions. For before you get into the details of protein production you have to do the culture media for the growth. We have discussed different culture media in a in a previous lecture which you can use to grow the insect cell lines. So insects cell line sf9 is been derived from the ovaries of the armyworm. And it is maintained in TNH-FH insect media containing 10 percent FBS and gentamicin.

For protein you can use either the baculogold or other serum free media, because if you keep the serum in the protein production media and if the protein who is secretory in nature. You are going to have the high level high level of cross contamination or high level of contamination from the serum protein itself. Low protein media is suitable for or for the secretory protein as it facilitates the easy purification.

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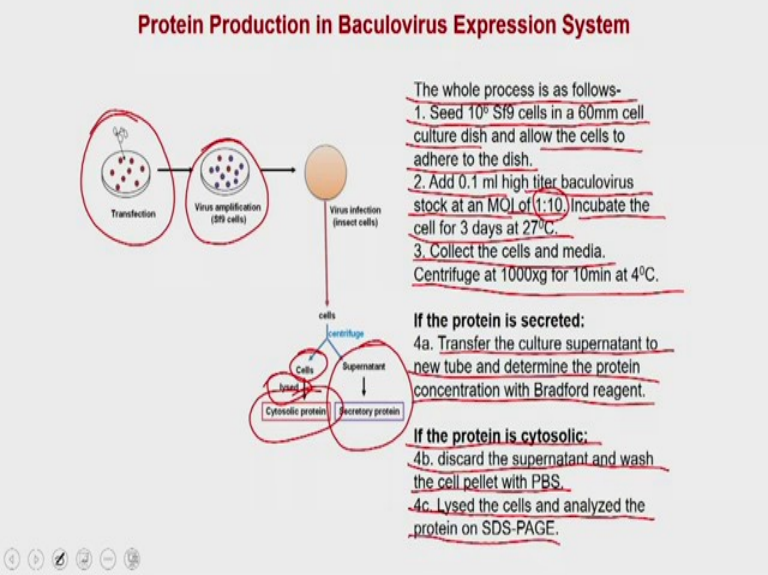

Now let us see what are the different steps we have, so the whole process of protein production is as such. You first what you see is 10 to the power 6 sf9 cells in a 60 mm cell culture dish and allowed the cells to other to the dishes. Now add point one ml high titre baculovirus stock at a MOI of 1 to 10. Now incubate the cells for 3 days at 27 degree Celsius, so that the virus can infect the insect cells and it can propagate within the insect cell. Now collect the cells and media centrifuge at 1000 for ten minutes at 4 degree. So the first step you do plating in a 60 mm dishes and allowed the cells to adhere and then you make the single layer. Then you do the transfection with the help of baculovirus at the MOI of 1 is to 10. Once this cells are been transfected then what you do is you culture them and collect the the supernatant as well as the cells.

Now you have the options if the protein is secretory in nature which means the protein is going to be present in the cell culture, so you transfer the culture supernet into a new tube and determine the protein concentration with a Bradford reagent. Which means if your protein is secretory in nature you can just collect the culture supernatant and you come estimate the amount

of protein present. If the protein is cytosolic in nature then you can recover the insect cells, you lyse these cells by using the different lysate method. And that actually is going to give you the cytosolic protein. So if the protein is cytosolic in nature discard the supernatant and wash the cells pellet with the PBS. Then the lysed the cells and analyse the protein on the SDS-PAGE. So with this… so these are the different steps which you have to follow to over express the protein into the baculo expression systems.

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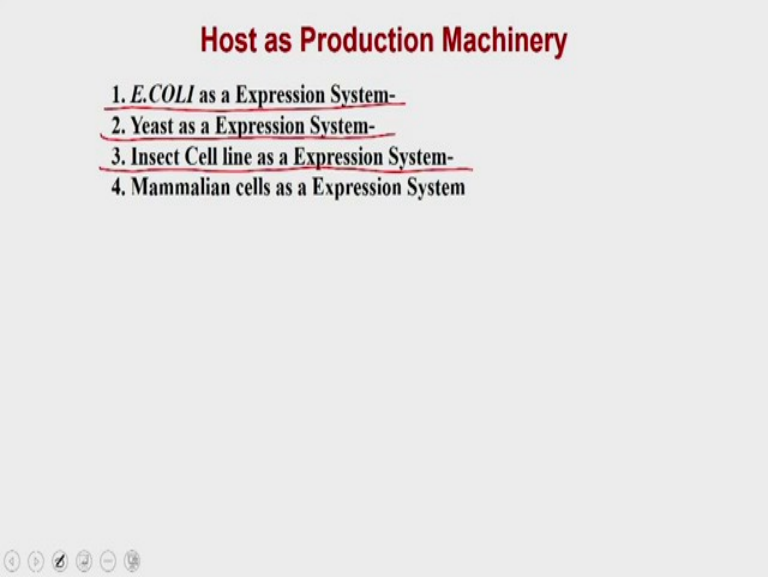

So, so far what we have discussed, we have discussed about the E coli as an expression system, we have also discussed Yeast as an expression system. And lastly we have discussed about the insect cell line as an expression system. We have discussed about the different approaches and different options that are available in all these different expression system. And now in a subsequent we are going to discuss about the mammalian cells as an expression system.

And with this I would like to conclude our lecture here. And in our subsequent lecture we are going to discuss about the mammalian cells as an expression system. Thank you.