Enzyme Science and Technology
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Module - IV
Enzyme Production (Part 2: Over-expression)
Lecture - 21
Over-expression of Enzyme in host (Part-II)

Hello everyone, this is Dr. Vishal Trivedi from Department of Bioscience and Bioengineering IIT, Guwahati. And what we were discussing, we were discussing about the different properties of the enzyme in the course enzyme science and technology. And in this context, so far what we have discussed, we have discussed about the history of the development of the field of the enzymology.

And then subsequent to that, we have also discussed about the nomenclature, classifications. And in this particular couple of modules, we are discussing about how you can be able to produce the enzyme in the bulk quantities. And in this context, we have discussed about the cloning of the enzyme into the, we have discussed about the cloning of the enzyme into a suitable vector.

And subsequent to that, we have also discussed about how you can be able to deliver this d this particular clone into the suitable host. And in the current module, we are discussing about how you can be able to produce the enzyme by the over expression. And for the over expression, we have said that we are, you know, we have couple of choices of the host what we can use for the particular vector.

And we can have the bacterial expression system, we can have the mammalian expression system, we can have yeast expression system, and we can also have the yeast expression system. So, in the current module, we are discussing about these host strains and how you can be able to produce the enzyme in the bulk quantities.

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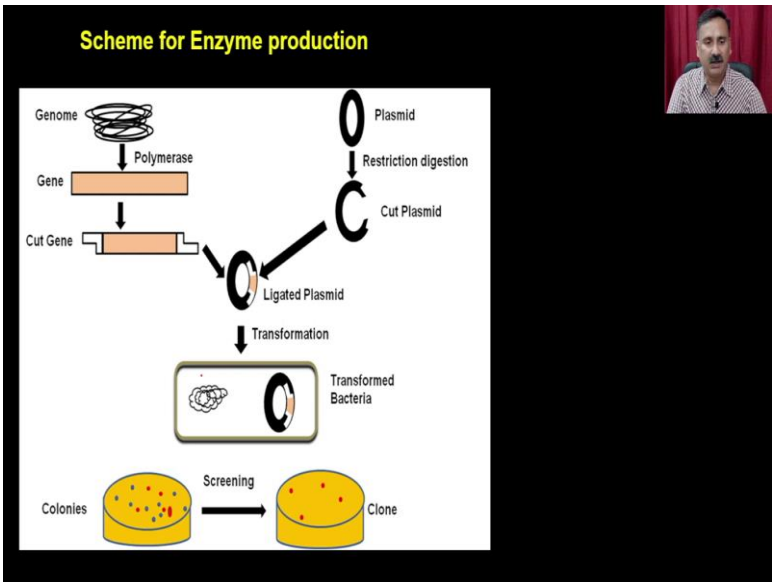

So, this is what we have discussed so far. We have discussed about how you can be able to isolate the enzyme or the gene, which is coding for the enzyme, either by the screening, the genomic or the decision library or with the help of the BCR. And once you have the gene, you can actually be able to digest the gene with the restriction enzyme and that is how you are going to have the sticky ends.

Same you have to do for the vector and you have to have the sticky vectors. And then when you put them together, you are going to have the, you know, the ligated plasmids and that ligated plasmid you are going to deliver to the host of your choice. And depending on the different types of requirement, you can actually be able to choose the host; either it could be a bacterial expression host or the eukaryotic expression host.

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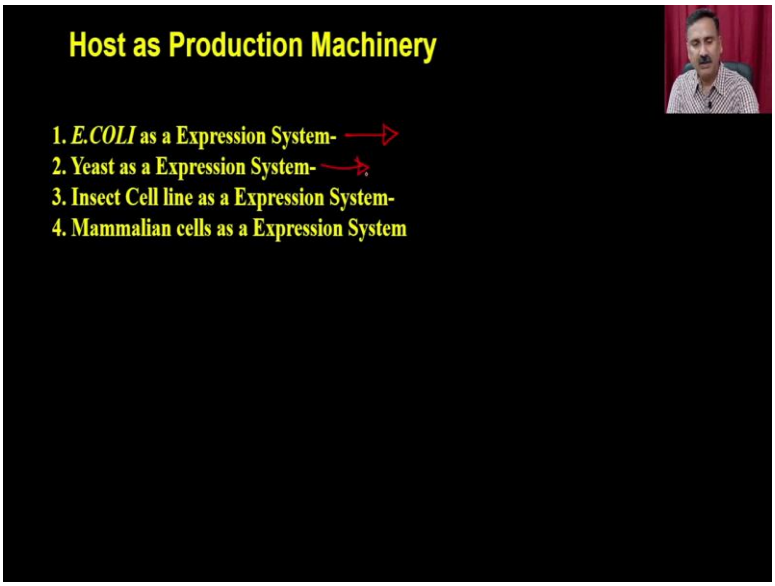

So, in the previous lecture, we have discussed about the E.Coli as a expression system. We have discussed about how you can be able to E.Coli for transformations, screening and how you can be able to observe the or monitor the over expression with the help of the SDS page. In today's lecture, we are going to discuss more about the yeast as an expression system.

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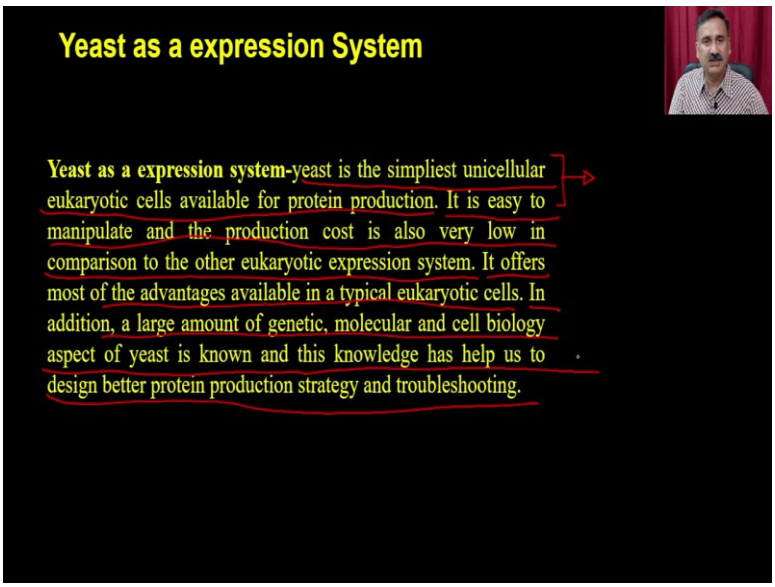

So, when we talk about the yeast as an expression system, the yeast is the simplest unicellular eukaryotic cell available for the protein production. So, the manipulations in

case of the yeast are very simple because they are behaving just like a bacteria because they are unicellular. But they have the eukaryotic cell like features and it is easy it is easy to manipulate and the production cost is very low in comparison to the other eukaryotic system right.

Remember that when we were discussing about the gene delivery, we said that it is going to, you can be able to use the multiple chemical methods and as well as the other kinds of methods to deliver the gene of your interest into a yeast cells. It offers most of the advantage available in the typical eukaryotic cell, right? So, it has a eukaryotic machinery, it has the nucleus, it has all other organelles, what are present in the eukaryotic system.

And in addition, and it also can be able to provide you the glycosylation of the protein. So, it also be a added feature. So, in addition, a large number of genetic molecular biology and cell biology aspect of the yeast is known and this knowledge has helped us to design the better protein production strategy and troubleshooting.

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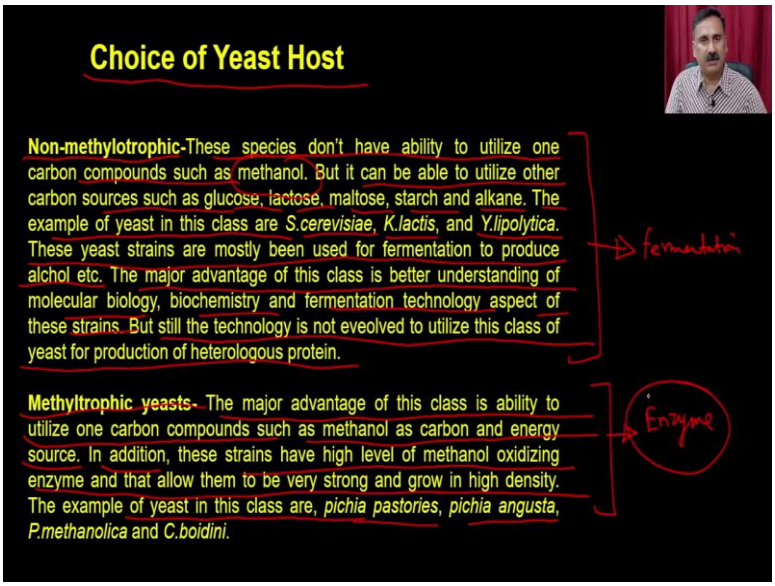

In as well as the choice of the host yeast host, you can have the non-metallotropic host or the metallotropic host. So, non-metallotropic host, the yeast species do not have the ability to utilize one carbon compound such as the methanol, but in it can be able to utilize the other carbon sources such as glucose, galactose, maltose, starch and alkenes.

The examples of the yeast in these classes are S cerevisae, K lactis and the Y lipolytica. These yeast strains are mostly being used for the fermentation to produce the alcohol. The major advantage of this class is better understanding of the molecular biology, biochemistry, and the fermentation technology aspects of these strains.

But still the technology is not evolved to utilize this class of yeast for the production of heterogeneous proteins. So, these are the non-metallotropic yeast which are mainly being used for the fermentation. And the classical example is S cerevisiae which is being used for the fermentation to produce the alcohol.

Then you have the methyltrophic yeast. So, the major advantage of this class is that its ability to utilize one carbon compound such as methanol and energy sources. In addition, these strains have the high level of methanol oxidizing enzyme and that allow them to be very strong and grow in very high density.

The examples of yeast in this class are pichia pastories, pichia angusta, pichia methanolica and C boidini. So, these are some of the strains which can be used for the enzyme production because they can be utilized the methanol and they can use the methanol when they utilize the methanol as a carbon source. They also have the downstream promoters and other kinds of machinery and that is how they can be able to get induced.

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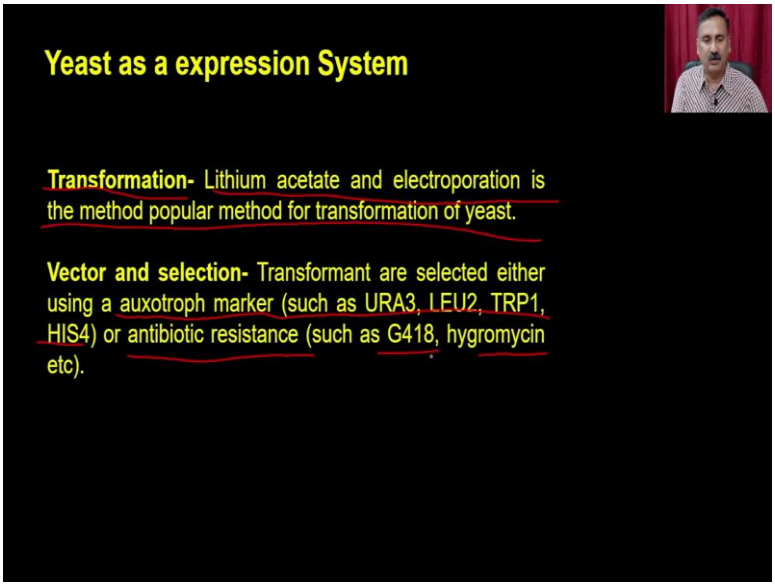

As far as the transformation is concerned, you can be able to use the lithium acetate and electroporation are the methods which are popular for the transformation of the yeast and the vectors. You can actually be able to use the different types of vectors what you are going to use and they can be selected based on the some of the auxotroph markers. So, you can use the you know and the antibiotic resistance such as G418 or hygromycins.

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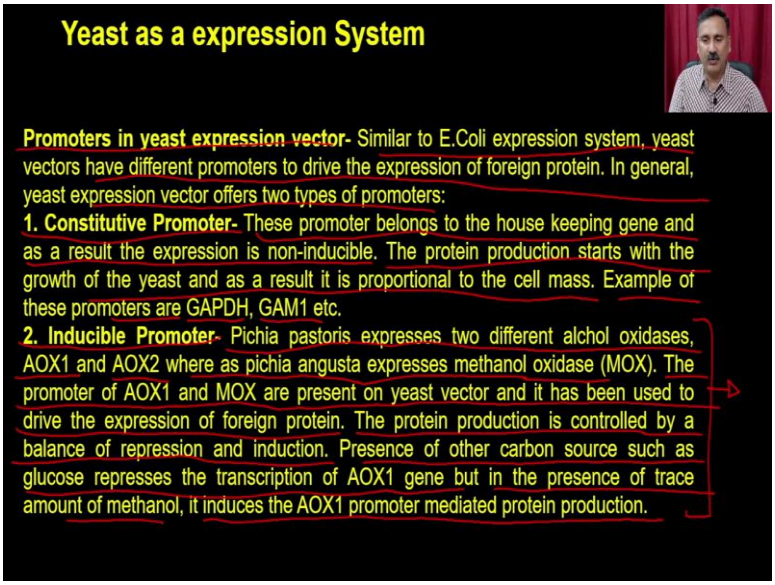

Then the promoters: So, promoters in the yeast expression vector. So, similar to E. Coli expression system, yeast expression vector also have a different promoter to derive the expression of the foreign proteins. In general, the yeast expression vector offer two types of promoters.

You can have the constitutive promoters or the inducible promoters. So, constitutive promoters are the promoter which are, you know, constitutively going to express the protein of your interest and that is going to be linked to the growth of the yeast. So, these promoters belong to the housekeeping gene and as a result, the expression is non-inducible.

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 and GAM1. And then you have the inducible promoters. So, pichia pastoris expresses two different types of alcohol oxidase. AOX1 and AOX2 where as, the pichia angusta expresses the methanol oxidase or the MOX.

The promoters of the AOX1 and the MOX are presented on the yeast vector and it has been used to drive the expression of the foreign protein. The protein production is controlled by a balance of repression and induction. The presence of the other carbon source such as the glucose, represses the transcription of AOX gene. But in the presence of trace amount of methanol, it induces the AOX1 promoter mediated protein production.

So, in the inducible promoter, you can have the multiple choices. Either you can use the AOX1 and AOX2 promoters or you can use the MOX promoters.

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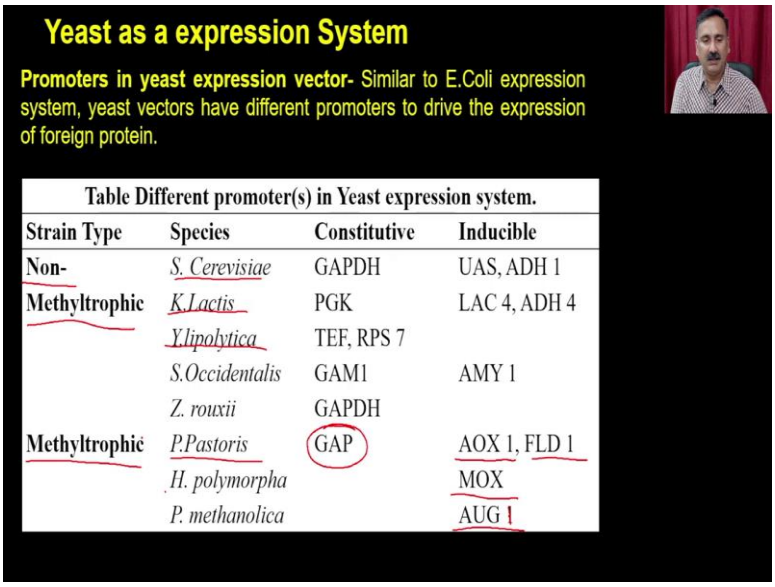

So, these are the tables where I have given you the species and whether they are using the constitutive promoters or the inducible promoters. And in the non- methyltrophic strains, you can have the S cerevisiae, K lactis, Y lipolytica and all that. And that is going to use an array of the constitutive promoters as well as the inducible promoter.

As well as the methyltrophic strains, you can have the pichia pastoris and the pichia methanolica and it is going to use the gap or the induce gap promoters, right? GAPDH promoter or the inducible promoter, you can use the AOX1, FLD1, MOX and AUG2, AUG1. So, these are the some of the promoters, what you can easily use and the different strains. So, depending on the combinations, you can be able to define the device and strategies to promote to produce a protein in the yeast expression system.

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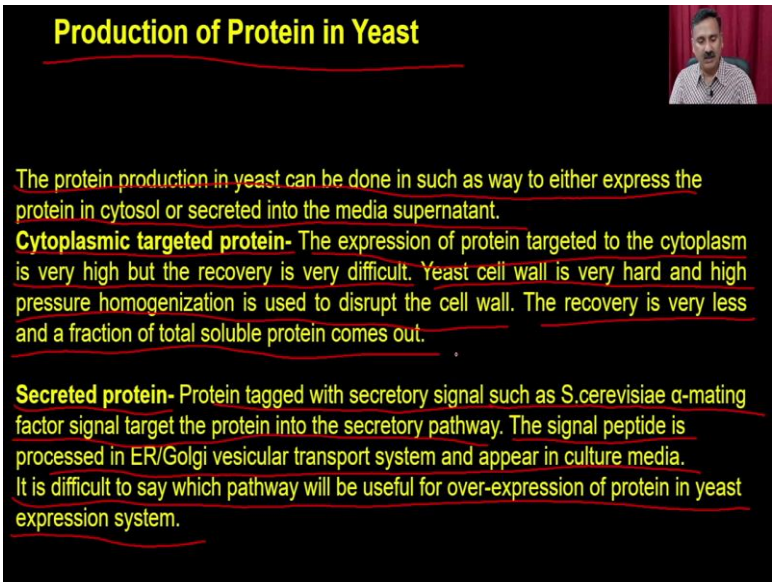

The production of the protein in the yeast, the protein production in the yeast can be done in such a way that either the protein is present in the cytosol or secreted into the media in the supernatant. Cytoplasmic targeted protein- this means these proteins are going to be expressed inside the yeast cells. The expression of the protein targeted to the cytoplasm is very high, but the recovery is very difficult. The yeast cell's wall is very hard and high pressure homogenization is the use to disrupt the cell wall.

The recovery is very less and a fraction of total protein comes out. So, if you are going to put the protein into the cytosolic targeted protein, it is going to be very high, but recovery is very low because the yeast has the cellulosic cell wall and it is very difficult to break. And you can have the secreted protein.

So, protein tagged with the secretory signal such as S cerevisiae, alpha-mating factor signal, target the particular protein into the secretory pathway. The signal peptide is processed in the ER-Golgi vesicular transport system and appears in the culture media. It is difficult to say which pathway will be useful for our expression of the protein in yeast expression system because both of these pathways have their own negative and positive because in the cellular targeting system, the recovery is going to be low.

Whereas in the secreted proteins, because the protein is going to be present in the media, it will not have any kind of protection so, it will actually going to be get degraded by

some of the proteins and other kinds of cells. And it is also going to you know experience the environmental changes.

For example, if there will be change in the pH of the media, that change in the pH is going to affect the quality of the protein, what you are going to put into the secretory pathway. Irrespective of the pathway, or you are going to use, you can actually be able to follow a couple of steps to over-express the protein in the yeast.

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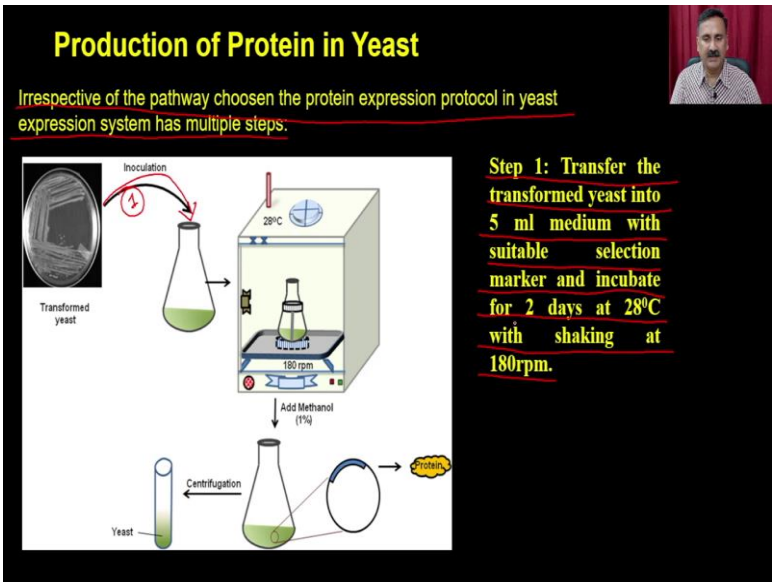

So, irrespective of the pathway, you can actually be able to choose, you can be able to express the protein in the multiple step method. For example, in the step 1, you are going to transformed you are going to the transformed yeast into a 5 ml medium with a suitable selection marker and incubate for 2 days at 28 degrees Celsius for checking a.

So, in the step 1, you are going to transfer you know the transformed yeast into a suitable media. Then you put it for the checking at 28 degrees Celsius for 2 days, and that is actually going to give you the good cell mass.

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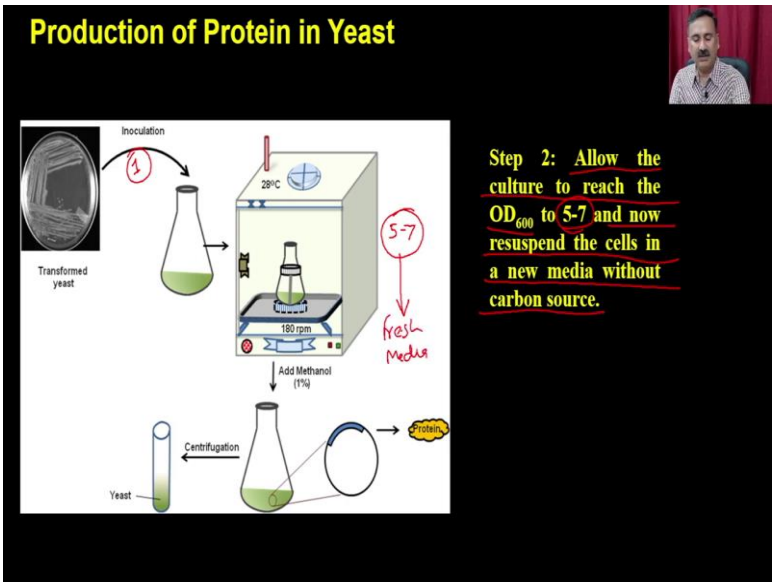

So, you in the step 2, you allow the culture to reach at the OD 600 at 5 to 7, and now they resuspend the cell in a new media without carbon source ok. So, in this case, first you are going to grow, first we are going to inoculate the cells into the into a media, let them grow for 2 days into at 28 degrees Celsius and 180 RPM. And once the OD is going to be at 5 to 7, then you can actually be able to transfer this into a fresh media, and that is going to be the step 2.

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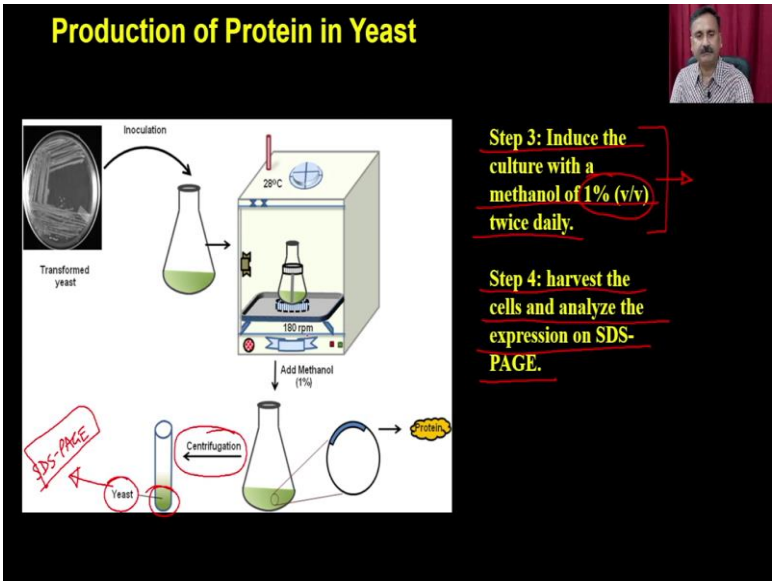

And then the step 3, you are going to induce the culture with a methanol of 1 percent twice daily. So, you have to be very careful at this stage because when you add the methanol, methanol is very toxic. So, you might have to do a calibration curve before you actually going to induce the cells with the 1 percent methanol. So, you can actually be able to induce the cells with methanol, 1 percent methanol with volume by volume twice daily ok.

So, then in the step 4, you are going to harvest the cells and analyze the expression on the SDS page. So, you are going to centrifuge the cells that will give you the cell pellet, and that cell pellet, you can actually be able to grab open. And that is how you are going to express the expression of these proteins into the SDS page. So, this is all about the protein production in the yeast expression system.

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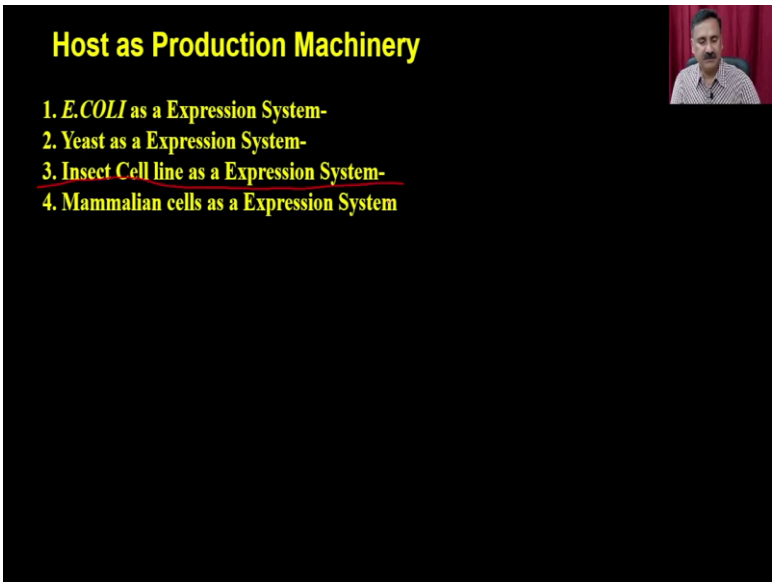

And now let us move on to the next system. And the next system is the insect cell line as an expression system.

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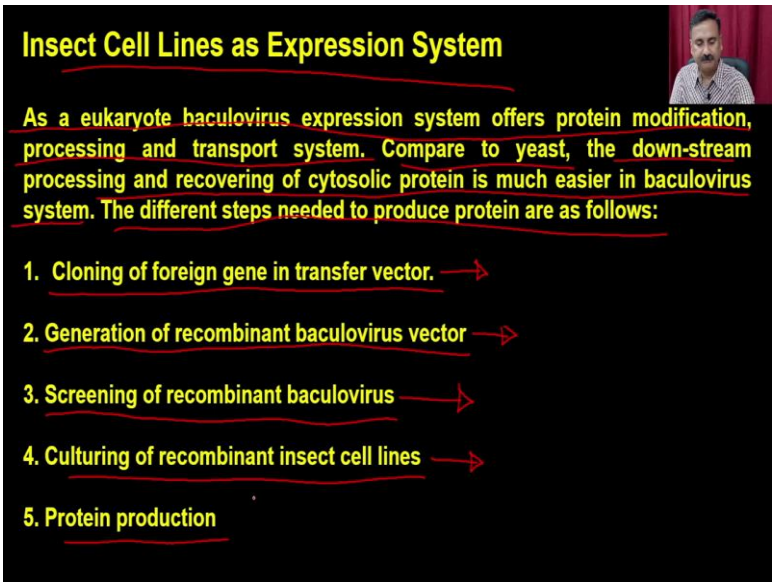

So, in the insect cell line as an expression system, as a eukaryotic baculovirus expression system offer protein modifications right, processing and transport system, compared to the yeast, the downstream processing and recovery of the cytosolic protein is much easier in the case of baculovirus expression system. The different steps need to produce the protein are S volumes.

In the step 1, you are going to clone the foreign gene into the transfer vector, right? And then you are going to have the recombinant baculovirus expression system. So, once you clone the protein into a transfer vector, your that gene is going to be transferred onto the, and it is going to be used for production of the recombinant baculovirus vectors.

And then you are going to screen the recombinant baculovirus vector expression in the step number 3, then you are going to culture the recombinant insect cell lines. And then in the step 5, you are going to do the protein production.

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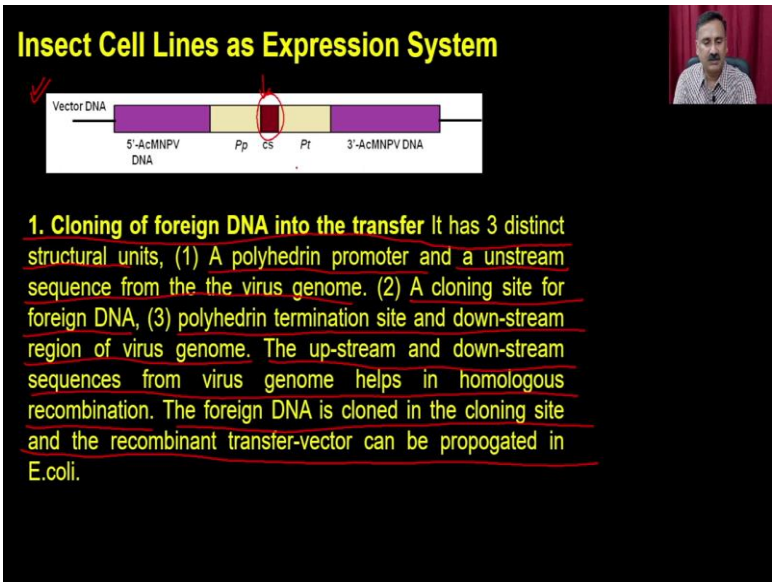

So, this is the, you know, the vector what we are going to use. And this is a region where you are going to, you know put your protein of interest. So, in the step one, you are going to clone the DNA into a transfer vector. It has distinct structural units. So, you can have the polyhedrin promoter and upstream sequence from the viral genome. Then you can have a cloning site for a foreign DNA. And then you can have the polyhedrin termination site and downstream region of the viral genome.

The upstream and the downstream sequences from the viral genome helps in homologous recombinations. The foreign DNA is cloned into the cloning site and the recombinant transfer DNA can be propagated. And once you have cloned it into this particular vector.

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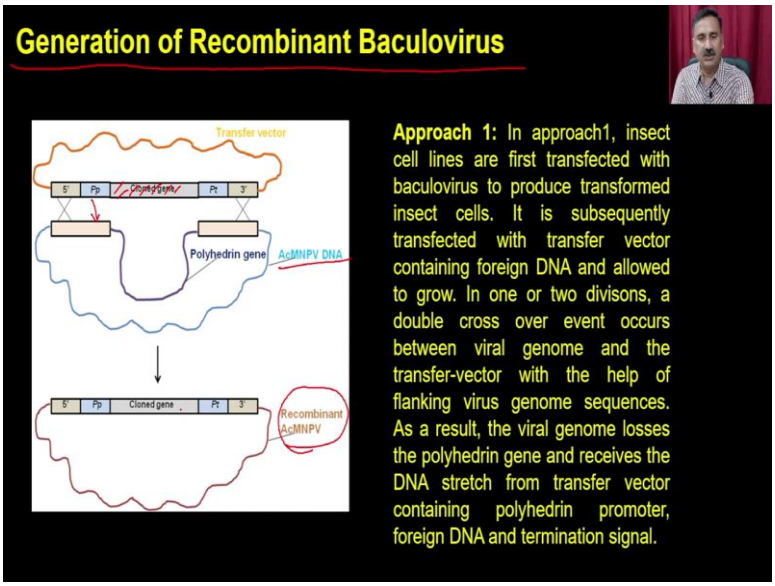

Then you can actually go to the next step and where you are going to have the generation of the recombinant DNA. So, in that case, what you are going to do is you are going to have this transfer DNA. So, you are going to put your protein into the gene into a transfer vector and utilizing the 5 prime and as well as the 3 prime, you know homologous recombinations.

This particular cassette is actually going to be put it into the ACPMP DNA. And that is how the it is actually going to produce a recombinant vector virus. So, this is the portion what is being, you know, going to replace with the help of from the sequence what is present onto the transfer vector. And that is how you are going to have the recombinant vector and this recombinant vector is going to be used for the infection into the insect cells.

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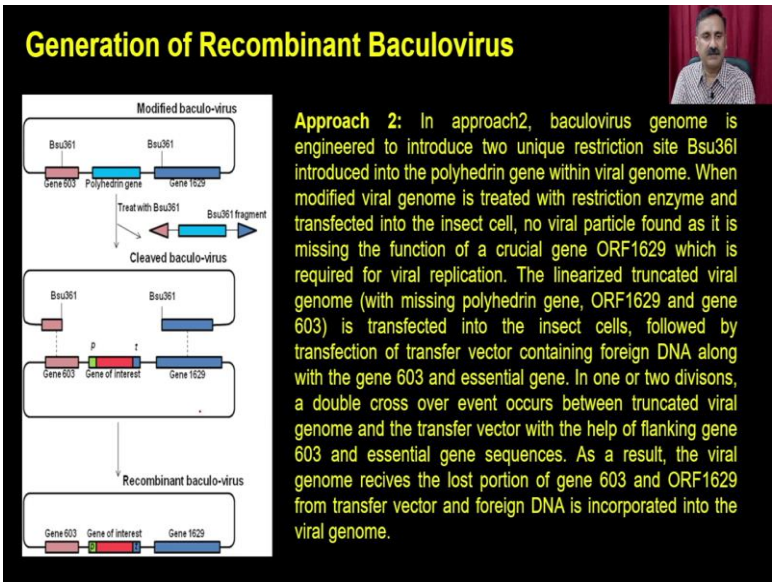

This is approach number 2, ok. So, how you can be able to use the different cassettes and you can be able to generate the recombinant baculovirus. Then you can actually be able to do the screen of the recombinant baculovirus.

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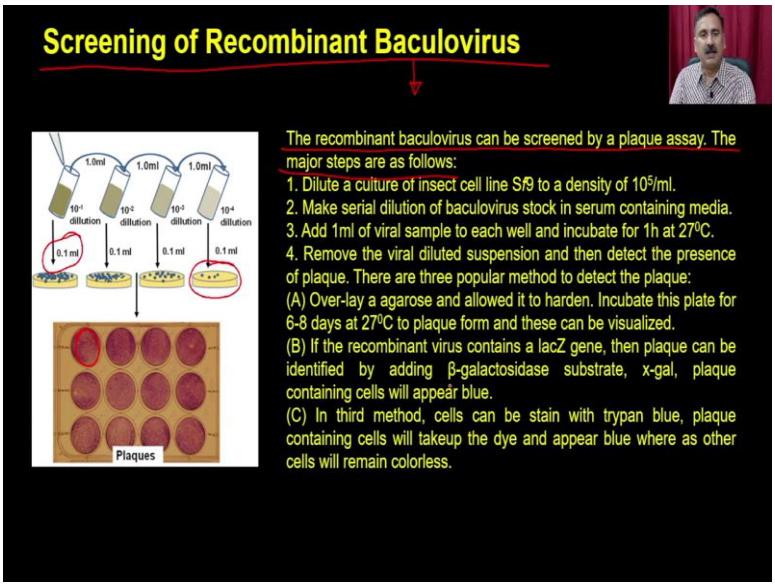

So, the recombinant baculovirus can be screened by a plaque assay. And what you are going to do is first you are going to do a serial dilution of the virus and serial dilution you can do by multiple method and then once the serial dilution is over, then you can

actually be able to take the small elicort and then you can check whether it is forming the plaque or not.

And by doing so, you can be able to screen the recombinant baculovirus which actually has the gene of your interest and that is how you can be able to use that for the further protein production.

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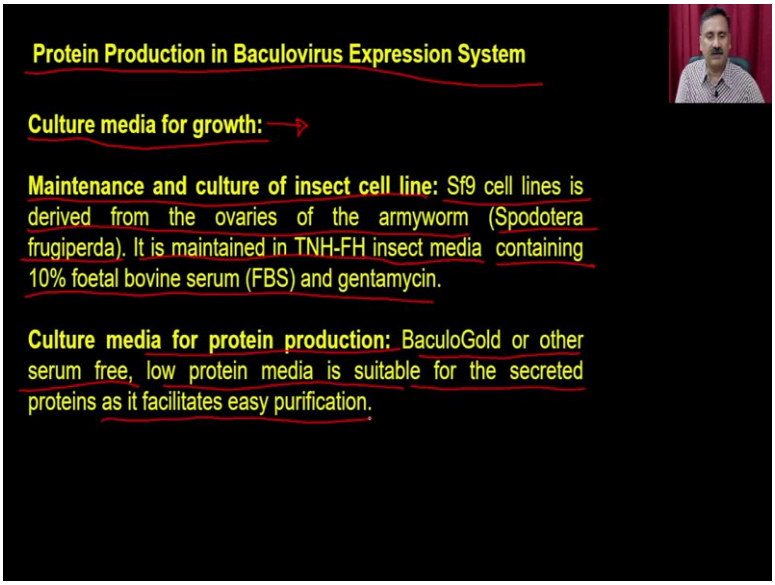

Then the how you are going to do the protein production in baculovirus expression system. So, you are going to use the suitable culture media for growth of the insect cells. So, maintenance and the culture of insect cell lines so, Sf9 cell line is derived from the ovaries of the armyworm spodotera frugiperda. It is maintained in the TNH-FH insect media containing the 10 percent FBS and the gentamycin.

And the culture media for the protein production so, you can use the baculogold or other serum free media which actually contains a low protein media is suitable because it is actually going to reduce the amount of contaminating protein and that is how it is actually going to facilitate the easy purification.

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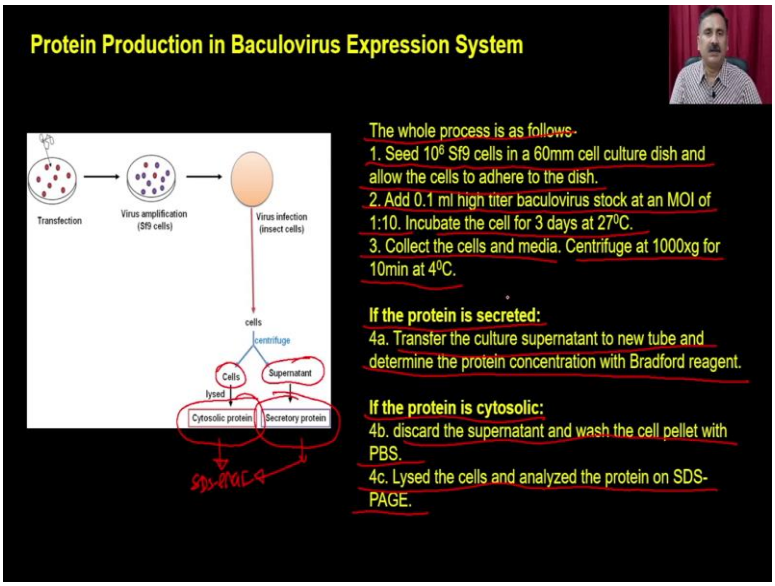

So, the whole process would be like this ok. In the step 1 you are going to see 10 to the power 6 Sf9 cell in a 60mm cell culture dish and allow the cells to adhere to the dish ok. Then in the step 2 you are going to add 0.1 ml higher titer baculovirus stock at the MOI of 1:10. Incubate the cells for 3 days at 27 degrees Celsius.

In the step 3 you collect the cells in media. Centrifuge at 1000g for 10 minutes at 4 degrees Celsius. Now, at this stage you can have the 2 choices. Either you want to put the protein into the cytophilic protein or you can actually be able to put the protein into the secretary pathway.

So, if the protein is secretory you can actually transfer the culture supernatant to new tube and determine the protein concentration with the help of the brat ford. If the protein is cytosolic then you can actually discard the supernatant and wash the cell pellets with the PPS and lysed the cells and analyze the protein on the SDS page. Which means if the protein is cytosolic you can actually be able to lysed the cells.

You collect the first cells with the certification and then you can actually lysed the cells and check the expression of the proteins into the SDS page. If the protein is secretory then you can actually be able to collect the supernatant and that supernatant will have the secretory protein and that also you can be able to check all to the SDS page. So, this is all about the expression system what we have discussed. We have discussed about the E.Coli as an expression system in the previous lecture.

And in today's lecture we have discussed about the yeast and as well as the insect cell line as an expression system. In our subsequent lecture we are going to discuss about the mammalian expression system and then we also going to discuss about how you can be able to detect the expression of the protein with the help of the different analytical technique. So, with this I would like to conclude my lecture here.

Thank you.