Eukaryotic Gene Expression: Basics and Benefits

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Lecture No. # 30
Eukaryotic Protein expression system - 1

Welcome to this lecture series on Eukaryotic gene expression basics and benefits. Today, we are going to discuss about Eukaryotic protein expression systems part one. This is the thirtieth lecture in this series. In the previous lecture, we had actually discussed about prokaryotic expression system. Although this lecture series about Eukaryotic gene expression basics and benefits, I discussed about prokaryotic expression systems (( )) in the last class, because a number of Eukaryotic proteins have been expressed in prokaryotic expression vectors, and they are available as drugs and (( )) drugs in the market.

And as we will see in this class the first choice of recombinant production is always to use a prokaryotic expression system, because of all the expression system they are currently available today, it is the prokaryotic expression system, which are least expensive and most economical way of producing recombinant protein. So, it is only after you are certain that the prokaryotic expression system is not the right choice for you, that you go and choose a Eukaryotic gene expression system for making your recombinant protein. So, it is very very important to understand the prokaryotic expression systems very well. So, that is why in the last class, we had discussed at length the various, the various plasmid vectors, page vectors cosmic vector, that are used for not only for basics to understand, to gene expression and gene regulation, but also they making a variety of recombinant proteins of interest to human and animal health. So today, let us now start looking at Eukaryotic expression systems, and this is as I said, this is the number of first in this series of lectures.

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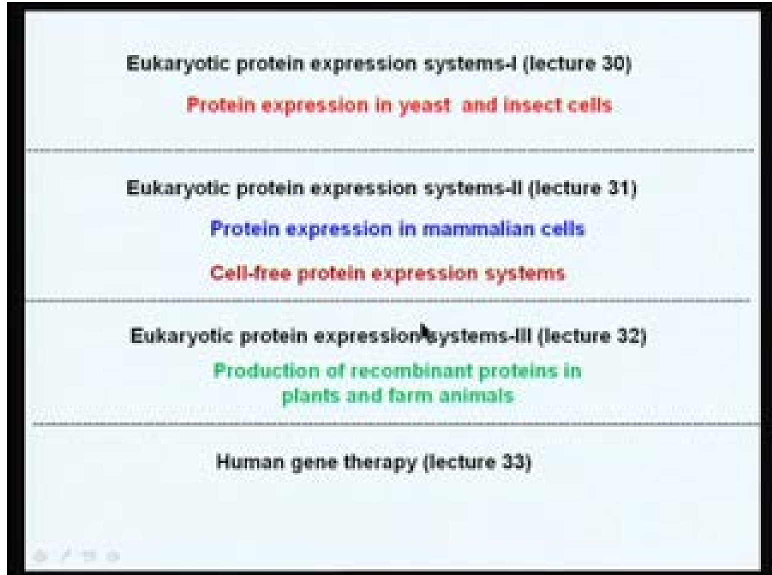

So, I have this entire talk on Eukaryotic protein expression systems into at this 3 or 4 different paths. Today, we are going to talk about protein expression in yeast and insect cells, and in the next class we are going to talk about protein expression in mammalian cells are. Following that we are going to talk how expression vectors have designed to make recombinant proteins in plants, and farm animals, and then ultimately in all these lectures , we are going to discuss about cloning a gene of your interest into either a e coli vector, or a yeast vector, insect vector, or a mammalian expression vector. Make protein in large amounts purify the protein, and then use it as a therapeutic. But in the final of the lecture series, we are going to talk about what is called as a human gene therapy, where instead of making your therapeutic protein of interest in another organism, or in cell lines purifying it and giving it as a therapeutic drug, why cannot we express the gene of your interest directly in the human body, so that the protein of our interest is made right inside our body. So, there is no need for you to make a recombinant protein elsewhere.

And then, (( )) as therapeutic into the humans, but let us introduce the gene right away. Express the gene right away in the human body, and see whether you can or get the therapeutic vector therapeutic benefit. This has tremendous benefit in the areas of cancer therapy, in the area of treatment of genetic disorders, and on and so forth. So, after we understand how protein expression is made in various Eukaryotic systems, we will now try to understand how genes are directly being introduced into humans, various tissues of

humans in viral, as well as non-viral vectors, and what are the implications of this new areas of research called Human gene therapy for the benefit of mankind.

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Now as I told in the number of classes in this lecture series that one of the very clear benefits that came out of understanding Eukaryotic gene expression is our ability to introduce genes into any cells of our choice, any organism of our choice, and then express the corresponding protein in large amounts, purify it, and use a therapeutic.

This is one of the major benefits that came out of recombinant DNA technology as well as our understanding of gene expression both in prokaryotes as well as Eukaryotes. As I have listed here, for example, a number of protein or biotech drugs or bioformastic or pharmaceuticals which have been produced using this kind of a recombinant DNA technology. A number of blood factors like clotting factors, like factor 8, factor 9, which are tremendous implications for treatment of patient suffering from hemophilia have been produced using this recombinant DNA methods. Thrombolytic agents like () kinases tissue , plasminogen activator have been produced using this kind of a protein expression systems. A number of hormones like insulin, glucagon, growth hormone, various Gonadophins have been produced. Several Hematopoitic growth factors, Erytyopoietin variety of colony stimulating factors, g C f s g s g m C f s C f s, and so on, and so forth have been expressed purified, and being used as therapeutic agents. A

number of Interferons again have tremendous applications in the antiviral therapy, Interleukin based products which as interleukin 2.

A number of vaccine protein, the surface proteins of a number of viruses have been expressed in e-coli as well as yeast systems, and is being used as vaccines. Examples are Hepatitus B Vaccine and a (( )) Vaccine for cervical cancer, and with Human papilloma virus antigens have expressed in each systems. A number of Monocional antibodies have been expressed and many of them are actively being used for treatment in various Various cancers. And in addition, there are number of Mischellaneous products which have been expressed, and basically, strategy has been to take the gene of your interest, put it in a expression vector, introduce this expression in vector into the (( )) choice, make the protein large amounts, purify, and then use it as a therapeutic product. So, this our ability to engineer genes, and then engineer their expression into organism of our choice, has set a huge biotechnology industry for making bio therapeutic drugs.

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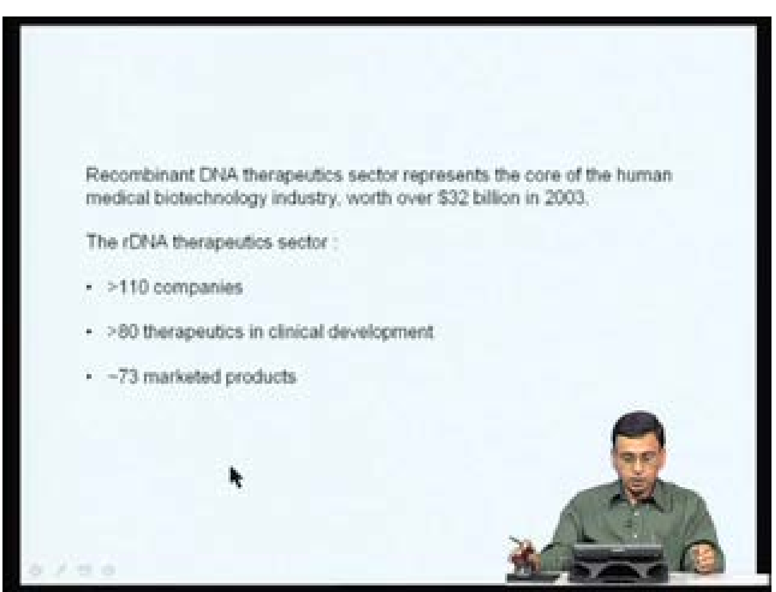

So, the Recombinant DNA therapeutics or bio therapeutics, as they are called today, is a huge industry by itself worth over 32 billion in the year 2003, and it is one or much more in the year 2010. That it is estimated, that there are more than about hundred and ten companies which specializing in making this kind of Recombinant DNA therapeutic protein therapeutics, for human as well as animal health. More than 80 therapeutics are in various stages of clinical development, and at least 73 biotech drugs have been

approved for by various regulatory agencies and are being marketed for human news. So this has tremendous implications. That is why, we need to understand how today, for example, we want to clone a gene and express a gene of your interest. How will you go about , will you choose a prokaryotic system, will you choose a Eukaryotic system. And if you choose an Eukaryotic system, what kind of Eukaryotic cost, you will choose for your purpose.

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Top ten recombinant therapeutic proteins and their global sales in 2003

Product /marketing company	2003 ($million)
Procrit (epoetin alfa)/Johnson & Johnson	3,986
Epogen (epoetin alfa)/Amgen	2,435
Neupogen (filgrastim)/Amgen	1,268
Neulasta (pegfilgrastim)/Amgen	1,255
Novolin (insulin systemic)/Novo Nordisk	2,235
Avonex (interferon beta-1a)/Biogen IDEC	1,170
PEG-Intron A franchise (pegylated interferon alpha)/Schering Plough	1,851
Enbrel (etanercept)/Amgen	1,300
Aranesp (darbepoetin alfa)/Amgen	1,544
NeoRecormon (epoetin-beta)/Roche	1,318

The recombinant protein therapeutics market is valued at $52,150 million in 2010.

I have just listed some of the top 10 therapeutic proteins that have been sold in the market. Using this kind of a recombinant technology, and recombinant protein expression systems, I will not go through one by one, but a number of very useful proteins have been expressed by cloning these DNA top of expression vectors, and making these recombinant proteins in large amounts. And in fact, the recombinant protein, therapeutic market is estimated to be about 52, 000 million dollars in the year 2010. So, it is a huge industry has grown, thanks to the efforts to the people like Pal Berg, (( )) and Stanley Cohen. It has these people who initiated this, or developed this technology for cloning genes into expression vectors, and then express in these genes in large amounts.

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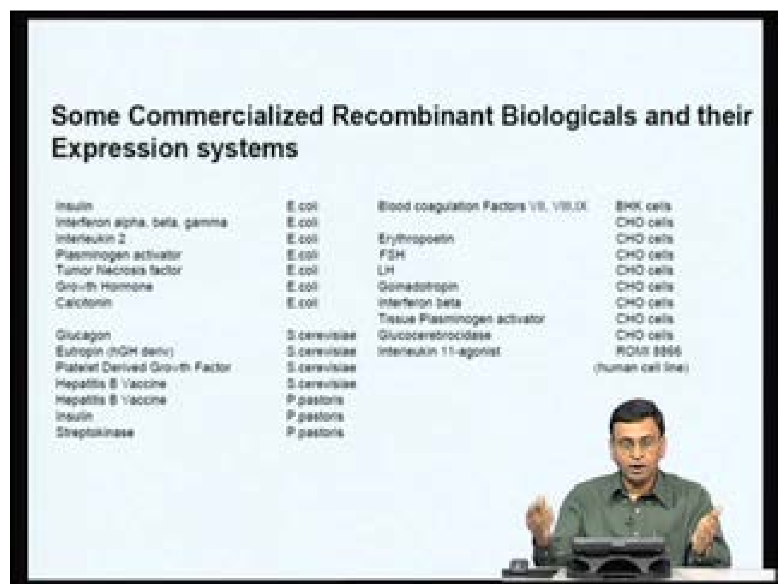

Again listed some of the Commercial Recombinant Biologicals which are available. today in the market. A number of proteins have been expressed in equal expression systems. A number of proteins have also been expressed in yeast either Saccharomyces cerevisiae pichia pastoris will discuss more about this today.

They have also been expressed a number of mammalian cell lines. So, you need to know, for example, if you want to make a protein large amounts, which of these expressions something you would choose. So, we will discuss some of the things in this class. Once you decide to make a recombinant protein your choice, will you choose a prokaryotic system, will you choose a yeast system, will you choose a insect expression system, or will you choose a mammalian expression system. How do you go about? What are the advantages and disadvantages of each one of these expression systems?

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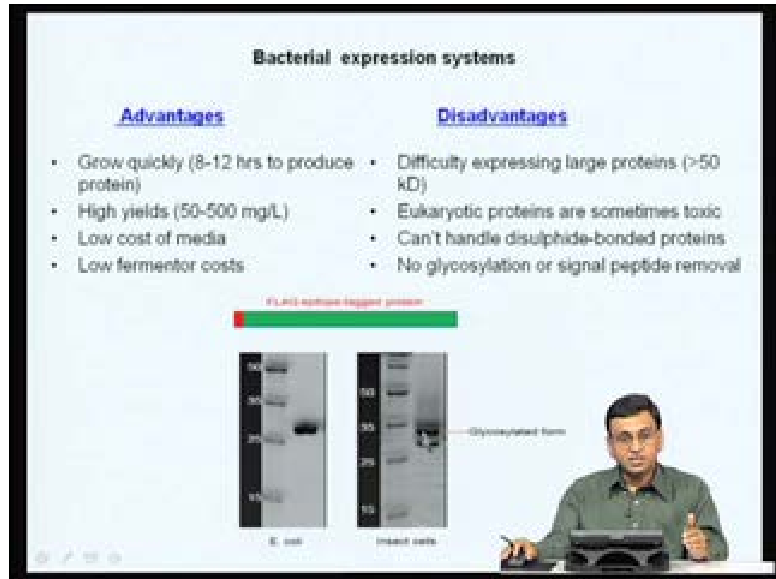

So, bacterial system expression systems which we have discussed very well in the last class has number of advantages. One of them is that these bacterial cells grow very quickly. The doubling time is less than 20 minutes or even less than that. So, it can this cells and large amount. So you can make recombinant protein in large amounts, in huge Bacterial fomenters. Their yields are very high. We have very powerful promote bacterial promoters. So, it can make your protein in large amounts. Usually something like 50-500 milligrams of a protein can be expressed per liter of the culture, and a media that are used for going to this bacterial cells are very very inexpensive.

So, the cost of production becomes very very low and as well as the ferment or costs. So, there are number of advantages in producing your recombinant protein, in bacterial expression systems, but there are also certain disadvantages. For example, very rarely you can express your protein in large amounts. If a protein is huge, usually if a protein sizes more than 50 Kilo Dolton, it is very very difficult to express these proteins in large amounts using a bacterial expression system, and many times when you express certain Eukaryotic proteins they are toxic in to the equalize cells of the Bacterial cells and therefore, they died. So certain Eukaryotic proteins can have been expressed in protein expression systems, and the prokaryotic machinery cannot have produced disulphide bonded proteins. So, if a Eukaryotic protein is highly disulphide disulphite bonded protein which has number of s bonds, then it is very very difficult to fold these proteins

appropriately, in prokaryotic holds and make a biologically active molecule which is properly folded, and properly bisulphide bonded proteins.

So, in such cases you have no other option, but, you go for a Eukaryotic expression system. The other very important drawback of the prokaryotic expression system is that prokaryotic systems cannot glycosylate proteins, and they also cannot do what is called let us say pro translate processing of proteins like. They cannot cleave the signal peptides which is requires for (( )) of the proteins like insulin for example. Insulin is made up as a pre pro insulin in our body, that the pre pro part cleaved and then you have the insulin right. This kind of a proteolysis processing cannot be done in a prokaryotic host. I will tell one example. For example, if you for example, have a protein which is supposed to be glycosylated, or we take this gene, and put it either in a prokaryotic gene expression vector and Eukaryotic expression vector, and in a prokaryotic expression vector, you will only see a non-glycosylated protein and if glycosylation is essential for the biological active of the protein, then this protein produce a prokaryotic host is of no use for you, because it will not have the therapeutic effect.

On the other hand, when you have express the same, and the insect cells which are Eukaryotic cells, you can actually see a glycosylatic form of the proteins is now present and which can be biologically active, and therefore, you have achieved a objective. So depending upon what is the end product that we are looking for whether it has a highly bisulphate bounded protein, whether proper bisulphate bound has to because formed for the protein to be biologically active, or does it how do we protologically process properly which does not take place well in the prokaryotic host, or if it require specific glycosylations and if this glycosylations very very important for the biological activity of the protein, then such proteins need to be expressed in a Eukaryotic host and not in prokaryotic host. So, let us now see what kind of Eukaryotic expression systems are available, and in this class, let us see how we can express these kinds of proteins in yeast. (( )) cells

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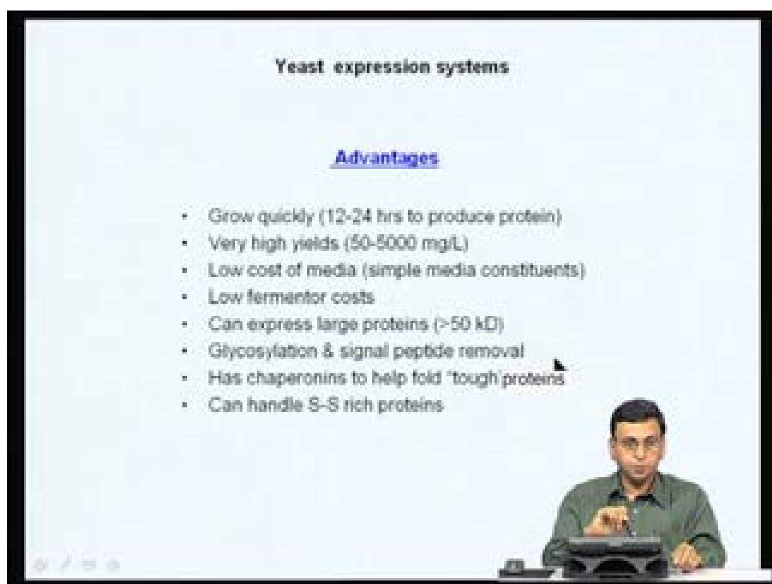

The Yeast expression system have a number of advantages, because just like bacteria they are also single cell organisms. So, they grow very quickly just like bacteria they also in a very high yields.

There are certain yeast species like pichia pastoris , which can go to very high cell densities. So, you can produce large amounts of the proteins per liter of the culture. Again just like bacteria culture media, the fermentation as well as the cost of media components are very very inexpensive. So, the cost production becomes very very low. You can express large proteins which is a major drawback in the prokaryotic expression systems. Many of the yeast species do have machinery for the Glycosylations of the proteins as well as protolytic processing. So, certain proteins if they require Glycosylation and the Glycosylations is important for the biological activity, such proteins can be expressed in yeast species like Saccharomyces cerevisiae pichia pastoris, and so on and so forth. They also have some chaperonins which help the protein to properly fold and therefore, they become biologically active and yeast systems can handle disulphide which bonded proteins. Suppose, we have huge number of disulphide bonds and therefore, they have to be properly folded, so compare to the equal expression systems.

Among the Eukaryotic expression systems the yeast expression system, have a number of advantages, because they are more or less similar like bacteria in terms of cost of production, and in terms of doubling time, and so on and so forth.

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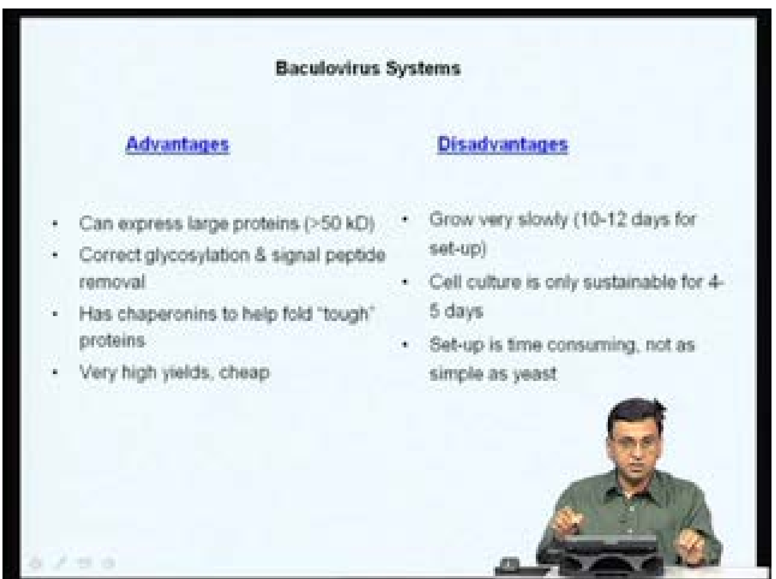

Suppose you made your expressed your gene in a yeast expression system, but still your proteins is not satisfied. For a number of reasons, or a protein is not biologically active then you have no other choice to try other Eukaryotic expression systems.

Next to yeast the most inexpensive of the Eukaryotic expression system is the insect cell expressing a proteins in insect cells for which you use a what is called a baculovirus expression system we will discuss in detail. The advantage of expressing your protein insect cells are just as yeast, they can also express large proteins this is not a problem, they can also do proper glycosylation and also they can properly cleave signal peptides and so on and so forth, so that your protein is properly processed of the translation. They also have chaperonins they help the four proteins to hold properly.

Once they have translated and insert expression systems also of a very high yields and the media components are relatively cheap compared to other mammalian expression systems. But, certain disadvantages of the Yeast expression systems are they cannot grow as rapidly as the yeast cells, so the growth is much slower, so takes about ten to twelve days for setup the entire system and a cell culture is only sustainable for 4 to 5

days and the setup time is time consuming and it is not as simple expression system, as that of yeast so there are disadvantages

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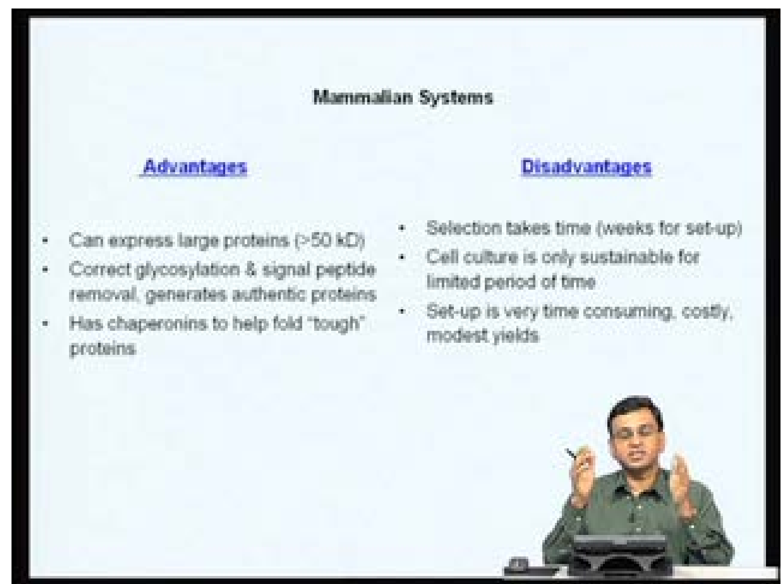

So if you have now, if your tide expressing your proteins yeast, your tide expressing proteins yeast cells, I mean insect cells nothing work, then you have no other option, but your expressive proteins in Mammalian cells. So the Mammalian systems also has advantages, because again as I said their size is not a problem, and since they are kind of homologous systems since the many of the cell lines used for Mammalian expression systems are divide from humans or Mammalian species.

So, they will have the right kind of glycosylation pattern. They will cleave all the protolytic cleavage of a translation will be an exactly as same as then in the mammalian cells, and also have the proper chaperonins and they fold proteins correctly after translation. But, the disadvantage is again it is much more difficult to setup compare each and expression systems they take weeks to setup. The cell culture is only sustainable for limited period of p time, and it is much more difficult if the mammalian cell culture is adherent type of cells much more expensive, and the setup time is very consuming. The media component, the kind of fermented that you require everything becomes more expensive. So, when it comes to the choice of Eukaryotic expression systems, you have to be extremely careful, and you need to make sure that you stop from a simplest Eukaryotic like yeast, and then clave slowly up and if nothing goes in yeast, it

does not work insect cells, then only go for the higher Eukaryotic systems, mammalian cell lines for your expression of a recombinant protein

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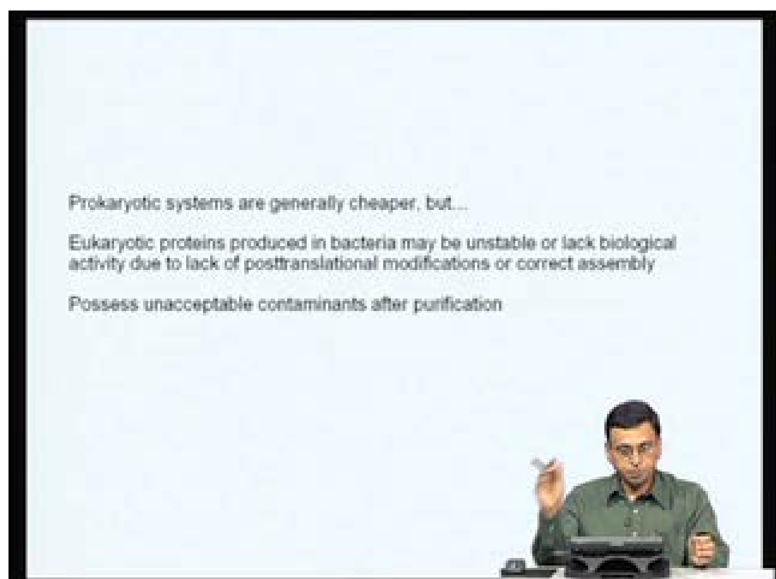

So proteins systems are generally cheaper, but Eukaryotic proteins produce the Bbacteria may be unstable or they may lack biological activity, due to lack of certain posttranslational modifications or they may not fold properly and therefore, you have no other option, but to go for Eukaryotic host. The other important problem in expressing Eukaryotic proteins in prokaryotic cost that the Bacterial cells that kind of a impurities like lipopolysaccharides certain indotoxins and which are not acceptable if you are going to use it for use in humans. So, you have to have a special mechanism to remove some of these impurities when you make this recombinant proteins in Bacterial cells.

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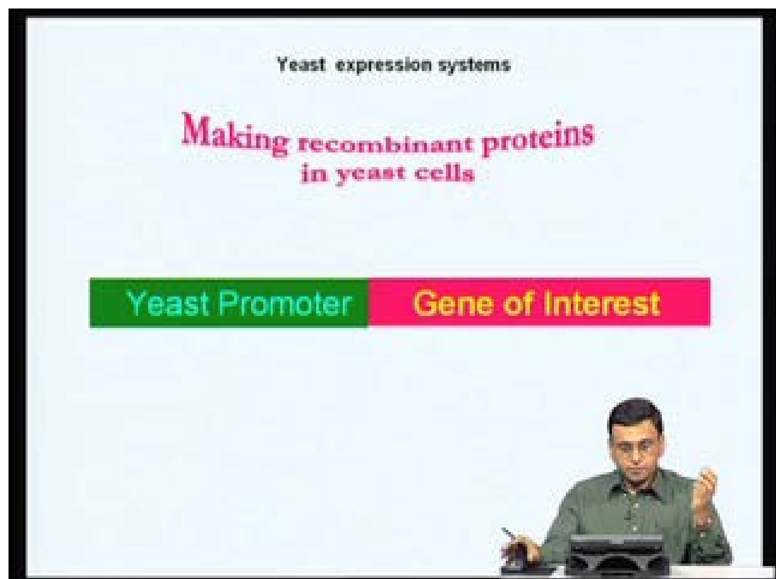

So having discussed what are the choices of expression systems that available to you, so you can either start from prokaryotic hosts and then gradually tried out in yeast, then tried out insect cells, then tried out in mammalian cells, and if even that does not work plant cells or they are also called as cell phase systems. We will discuss some of this things in the next few classes.

So let us now focus on what kind of yeast expression systems are currently being used for making recombinant proteins. So as I told in the many classes before, there are few to decide to express a protein express a gene in a Yeast expression system. The first thing you have to do, is to clone your gene of interest downstream of a Yeast promoter, that the first thing that you do. So, let us now see how what kind of a promoters are being used for expressing your genes of interest in yeast cells, and how do you a Yeast expression vector, and how do you introduce these genes, and how what kind expression are available for a recombinant protein production in yeast cells.

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The two most proper organisms are making recombinant proteins in yeast cells are Saccharomyces cerevisiae which is the common baker's yeast, as well as a methyler tropic yeast called Pichia pastoris. There are also methalotopic like hansenula polymorpha, yarrowia lipolytica etcetera, but today for simplest to say we will only discuss one methalotopic is Pichia pastoris is an example for recombinant protein production is such.

So, let us now take a look at what kind of expression systems are available for making recombinant proteins in yeast cells such as Saccharomyces cerevisiae and methylotropic is such as Pichia pastoris.

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Saccharomyces cerevisiae

A single cell

Well characterized genetically and physiologically

Can be readily grown in both small vessels and large scale bioreactors

Several strong promoters have been isolated and characterized

Carry out many post-translational modifications (phosphorylation, glycosylation and targeting)

Readily grown in small and large scale bioreactors

secretes few proteins, the product can easily be purified generally recognized as safe (GRAS)

As you know Saccharomyces cerevisiae is a very simple yeast, is a single cell organism is very well characterized genetically as well as physiologically. Saccharomyces cerevisiae became wonderful organism, model organisms for a number of Eukaryotic biological passes, the cell biology, gene expression, genetics Saccharomyces cerevisiae served as a model system for understanding many complex biological process in Eukaryotic systems. The Advantage of Saccharomyces cerevisiae is they can be grown very readily fermental vessals and also scaled up very nicely huge by reactors. So once you standardize your recombinant protein production to about something like 1 or 5 liter cultures, you can easily scale it up to thousands of liters of Biofermentors. this technologies are very very standardized

A number of strong promoters have been isolated, there very well characterized because each served as a model system for understanding Eukaryotic gene expression the rule of chromatin and so on and so forth. So, a number of promoters have been studied. The entire yeast genome has been sequenced. A number of powerful yeast promoters are today available. So, if we want to express a proteins large amounts, you can choose from any one of these strong promoters that are very well characterized in the Saccharomyces cerevisiae. The advantage of Saccharomyces cerevisiae is that it can carry out many posttranslational modification. So many signal transduction pathways like phosporylation, glycosylation, protein targeting myristoylation they are very well conserved in the yeast as well which happens in the higher higher carryout and therefore,

if your protein requires any of the post translational modifications it can be done in the Saccharomyces cerevisiae expression system.

As I said it can be readily grown both in large and small scale bioreactors or fermentors and more importantly, today, if you are making a recombinant protein in a micro organism or in a cell line of your choice. These particular micro organism or a cell line has to be certified by a regulator agencies such as the united states food and drug station as generally recognized as safe known as GRAS. So only those organisms which have been certified as GRAS are allowed for use or producing human therapeutics. So if we want to produce a human therapeutic, you need to first make sure that the cell line or the expression systems that are used has been certified as GRAS. Otherwise even if you express this protein, and even it is biologically active, you will not be given permission to bring it out as a product in the market. So, yeast such as Saccharomyces cerevisiae has already being certified by regulator genes like u s f d a as GRAS. So, if you make a recombinant protein in Saccharomyces cerevisiae, you will not have any problem in getting a regulatory approvals to it as a product in the market. So this is one advantage of the Saccharomyces cerevisiae.

Now, let us now look at how do you distinguish between once you made you expression construct and once you introduce this expression construct these cells. How do you eliminate cells which are not taken up your vector. How do you select for those cells which are taken up your expression vector and we know the equalize cells and in the prokaryotic expression systems usually antibiotic systems markers like (( )) tetracycline they are used as a selection markers, but these antibiotics do not kill yeast cells. They kill only Bacteria, therefore, the antibiotic gene resistance cannot used as selectable markers when it comes to yeast cells. Therefore people have developed what are called as auxoprotic yeast strains. These terminologies one has to understand yeast strain which can make its own aminoacids or which has a normal Purim or pyramiding bio synthetic pathway, metabolic pathways are known as prototroph's. So you do not have to provide any amino acids in the medium. If you just provide common, just this salts and carbon source it has its own amino acids synthetic pathways and therefore, you can synthesize all the amino acids by itself.

These are called as prototroph's, but when you take such prototrophic strains and when you introduce either deletions or mutations in one of the genes involved in any particular

amino acid biosynthetic pathway, then that particular strain becomes in auxotroph for the biosynthesis of that particular amino acids. For example, if I make mutation or deletion in the org 4 gene, then this particular yeast strain cannot synthesis origin n then therefore, it becomes auxotroph for origin n therefore, what you now do is you, now provide this org 4 gene in a plasmid vector or a vector, so and then put your gene of interest in particular vector.

So when you now introduce your gene into the auxotrophic strain which cannot make orgining all those cells are now taken up org 4 plasmid now becomes prototroph's. So they can now grow in a medium in the absence of origin whereas, those cells which are not taken up the plasmid cannot go in the option of the origin n therefore, you can select for recombinants by placing them in a media this is not containing origin.

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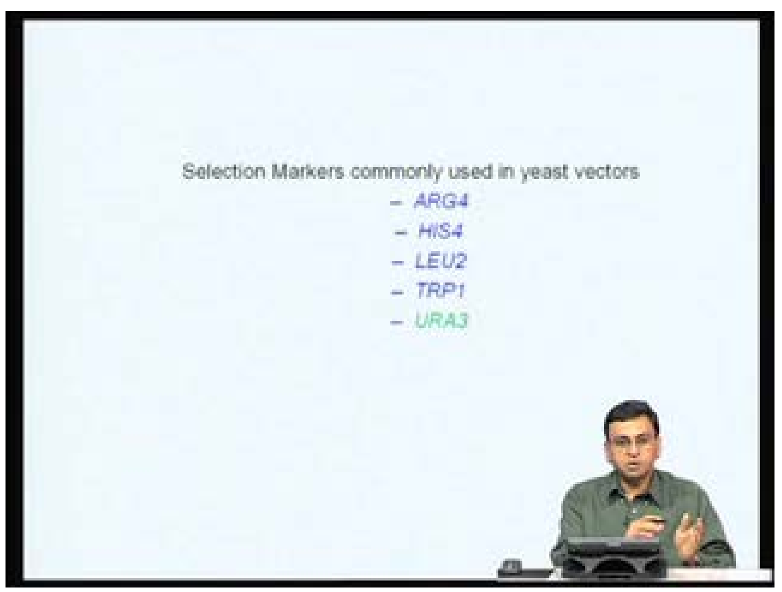

So all those cells which have taken up in the plasmid by which of the presence of org 4 and the plasmid can make origin and therefore, you can survive and grow even in the absence of the origin. In the same way mutations have been made a specific scenes like HIS4 is a gene involvement in historin biosynthesis 2 is a gene involved in a Leusin biosynthesis, TRP1 is a gene involved in tryptophan biosynthesis, URA 3 is a gene involved in uradium biosynthesis. So people have made certain mutations and these kinds of auxotrophic genes have been developed and is these auxotrophic strains that are actually used for making recombinant proteins. So you have corresponding plasmids

containing all these genes, and therefore you clone your gene of interest in these vectors which contain these genes and if now put in the appropriate auxotrophic strains, now you can select for recombinants which can now grow in the absence of that particular amino acid of that particular nucleotide. This is how you make a selection of recombinants in the case of yeast cells

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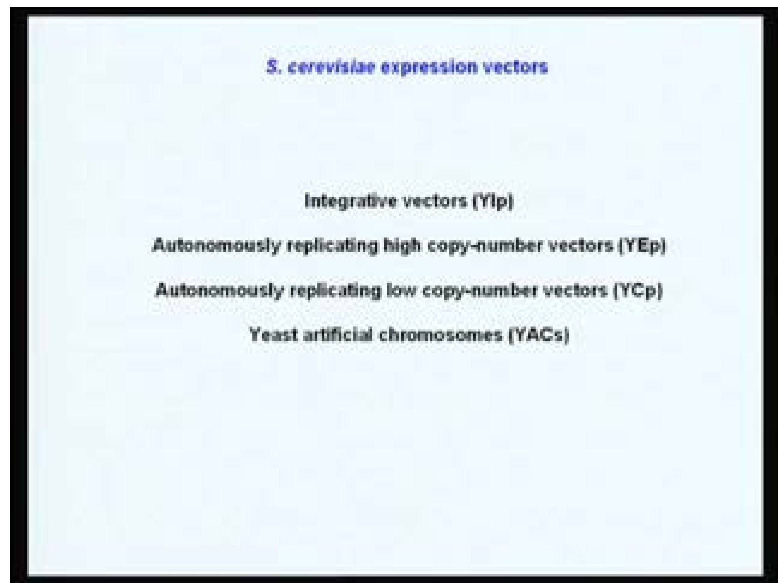

The most commonly used these expression vectors are what are called as integrative vectors or Y I p, autonomously replicating high copy number vectors are abbreviated as Y E p, Autonomously replicating Low copy number vectors are Y C p, and of course, yeast artificial chromosomes. We will not discuss about yeast artificial chromosomes because these are mostly used for cloning large chunks of DNA, and most commonly used for making something like genomic libraries C DNA libraries, especially genomic libraries. And we usually used for many of this mega sequencing projects, if we want to do sequence human genomes or other big genomes huge chunks of DNA are clone this vector, and when rarely yeast artificial chromosomes are used for making recombinant proteins. Sso we will not discuss this. So, let us now see what are the various vectors and what are their distinguishing features.

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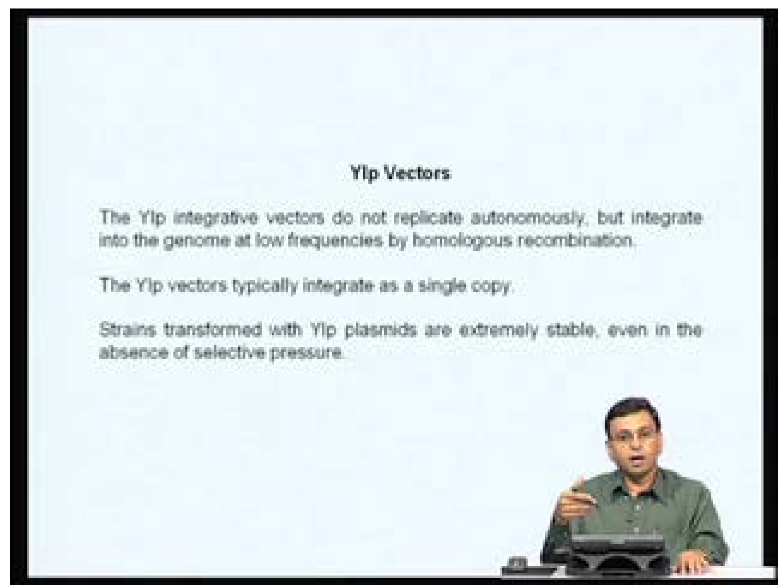

The Y I p vectors are the integrative vectors. They do not replicate autonomously. Remember plasmid vectors which we discussed in the prokaryotes they contain their own origin of replication. So they remain as extra chromosomal elements, and multiply autonomously independent of the chromosomes, whereas V is the integrating vectors they do not have a such a replication origins therefore, they cannot replicate autonomously. So once you clone your gene into you know Y I p vector, you have to introduce into the chromosomal. They go integrate with the chromosome therefore, they replicate replicate along with the chromosome. So, the the gene of your interest is introduced with the chromosome by homologous recombination, and therefore, your gene of interest becomes part of the chromosomes is chromosome. And along with the chromosome every time the cell divides the gene also divides so as a result when you clone your gene of interest to integrative vector, your gene will be present only in a single copy is very very important. The advantage of a Y I p vectors is that the strain transformed to the Y I p plasmids are extremely stable even in the absence of selective pressure, because your gene has gone integrate to the chromosome.

It is very stable every time the cell divides your gene also will divide along with the rest of the chromosome, therefore it is very stable. The disadvantage is that your gene is only present in a single copy and therefore, the expression levels are not going to very very high whereas, if you are present in a multiple copy and there may extra chromosomal,

since each cell containing large number of plasmid copies are extra chromosomal copies. The protein will because produce in the large amount in that particular yeast cell.

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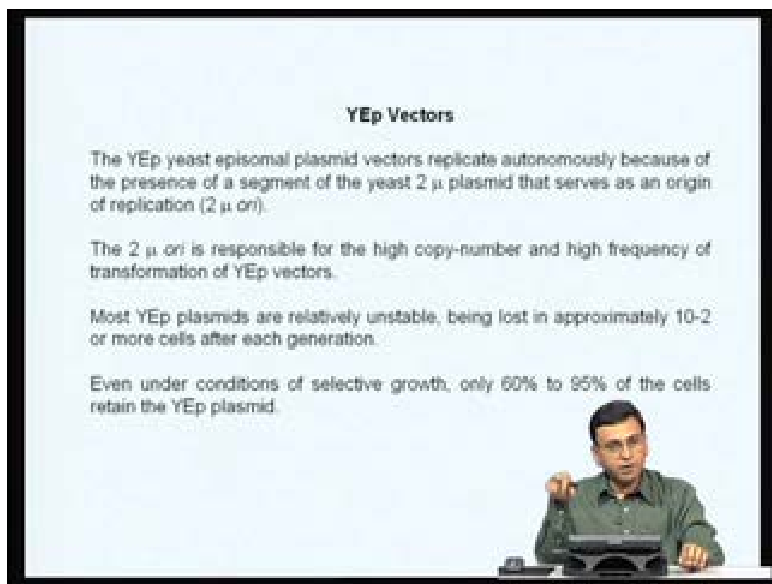

The Y E p vectors on the other hand or episomal plasmid vectors, they can replicate autonomously, because of the presence of a small 2 micron plasmid which serves as an origin of replication. So just like the E-coli expression plasmids or clonic vectors had a E-coli origin of replication, these episomal yeast vectors contain what are called as 2 micron plasmid, which serves as a yeast origin of replication. Therefore, they can replicate independently of the yeast chromosome

And because of the presence of autonomous replicating unit, these plasmid can be present in very high copy numbers per cell, and the most yep have the disadvantage is that many of this plasmids relatively unstable. And you have to maintain the auxotrophic the auxotropic marker pressure. Suppose if your 2 micron plasmid contains the Y E p vector contains a LEU 2 marker, you have to always maintain the strain in the absence of leusim, so that the plasmid is not lost and even under some selection pressures, sometimes you lose this plasmids. Therefore to 60-90 percent of the cells retain the plasmids, even on the pressure. So, you cannot maintain this plasmid for too many generations. After about 5 or 10 generations, we have to go back to the parent to the master cell bank, or original stock and then revive and then start again. So this one disadvantage of the Y E p vectors

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YCp Vectors

The YCp yeast centromere plasmid vectors are autonomously replicating vectors containing centromere sequences, CEN, and autonomously replicating sequences, ARS.

The YCp vectors are typically present at very low copy numbers, from 1 to 3 per cell, and possibly more, and are lost in approximately 10^{-2} cells per generation without selective pressure.

YRp vectors, containing *ARS* but lacking functional *CEN* elements, transform yeast at high frequencies, but are lost at too high a frequency, over 10% per generation, making them undesirable for general vectors.

The Y C p vectors are what are called yeast centromere plasmid vectors. They contain autonomously replicating sequences called ARS as well as centromere sequences, and therefore they replicate just like chromosomes, and Y C p vectors are typically present at very low copy numbers about 1 to 3 per number per cell, there also not as stable as the indicative plasmids. The other very rare kind of a yeast plasmids are used as Y R p vectors, they contain autonomous replication sequences, but they do not contain the centromier sequences , and they also transforms use higher frequencies. But they are also lost high frequencies, and usually they are highly unstable, and very rarely Y R p vectors are used. So normally people either use the Y C p vectors or the episomal vectors or the integrative vectors for most of the gene expression studies as well as come into making into recombinant protein, and among these the episomal vectors the integrated vectors are the vectors of choice for making recombinant proteins.

A number of yeast vectors have been developed by various researches across the world either for understanding basic aspects of gene regulation proteins trafficking cell biology for a number of regions, and also for making recombinant proteins in large amounts. Now what I do is not discuss all of them, but just give a flavor. I will just discuss 1 or 2 examples of commercial yeast vectors which are currently available with certain companies, and you can just go and by some of these vectors clone your gene of your interest with these vectors, and put them into yeast cells and make your recombinant protein in large amount .

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So what kind of commercial vectors are currently available for expressioning Saccharomyces cerevisiae, for example, one of the popular vectors which is applied by a company called invitrogen is vector called pYES2, which actually contains a promoter involved in galactose metabolism, namely the p galf galf 4 GAL 1 promoter, and as a result you can induce the expression of your gene by growing this, by adding galactose to the medium. So as long as you grow the yeast cells contains this particular plasmid in glucose, your gene of interest will not because expressed the movement. You change the carbon for some glucose, galactose the promoter will because turned on and your gene of interest will because expressed. So, it is a highly regulated inducible vector system that has been developed. So the expression of your gene can be induced by adding galactose to the medium, as long as the medium that glucose gene will not be expressed. So basically it has a multiple cloning site. So it containing a number of restrictions sites, you can clone your gene into any one of this restriction sites downstream of the GAL 1 promoter. It also have the sequences for the proper (( )) processing of the RNA, that is as a small a relation and so on and so forth. It also has an (( )) resistance gene and f 1 origin for going this plasmid first in E coli, and and making large amounts in E coli, and it has a 2 micron origin for replication in the yeast cells. So, it is in a episomal vector and it has a URA 3 as a selections. So, you can select the cells by plating this cells in URA minus medium, if there is no URA cell in the medium. Only those cells are taking other plasmids are survive.

Whereas, close cells which are nor or the non-recombinant will die. So, these galactose inducible Saccharomyces cerevisiae expression vector is a very powerful expression vector for inducing. It is a very popular inducible expression vector, both for basic research as well as for and producing number of proteins recombinant proteins.

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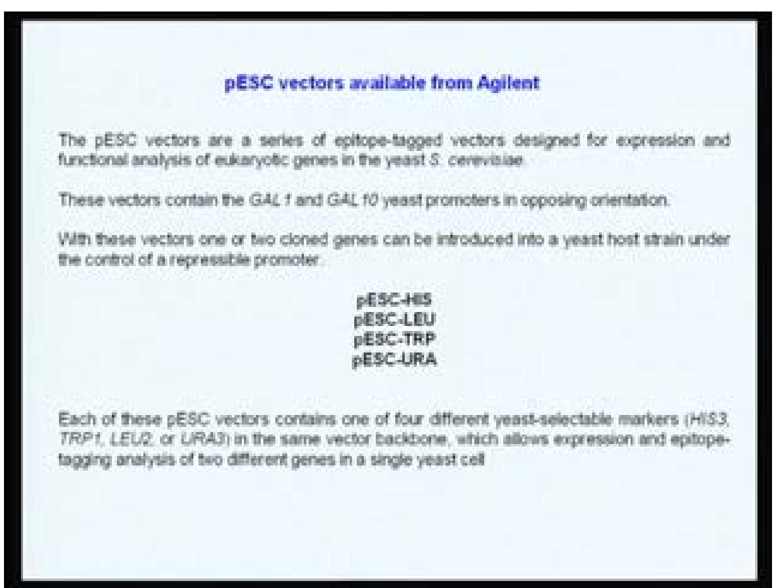

Number of other such vectors are also available for example, (( )) and cells what are called as p Esc ape vectors. They are a series of epitope tapped vectors designed for expression and functional analysis of Eukaryotic genes in Saccharomyces cerevisiae. They contain 2 promoters GAL 1 as well as GAL 10. So you can clone 2 gene simultaneously 1 downstream of GAL 1 promoter another downstream of GAL 10 promoter, and both can because simultaneous clustered. So, by introducing the 1 vector you can express 2 genes that the Advantage of the p Esc ape vectors the important the Advantage of p Esc ape is that they are available under a variety of auxotropic molders you have p Esc ape his LEU TRP or URA.

So, it can clone the genes under any of this vector and you can put this plasmids in all the 1 cell and you can select the 1 cell for example, if you put all these plasmids in auxotrophic strain which is his minus LEU minus TRP minus URA minus you can select for a cell which contains all the 4 plasmids that means 4 into 2 8 genes can because expressed in the simultaneously in a single yeast cell by using this kind of plasmids. So, it is a tremendous advantage, so each of this p Esc ape vectors contains 1 of 4 different

yeast selectable markers as attain histidin tripdofan lue or URA 3 in the same vector beck bone which allows expression epitope tagging analysis of two different genes in a single cell. So, if you now clone 8 genes with the 4 vectors, you can put all the 4 vectors in a single cell and you can express 8 genes in the single cell at a time .

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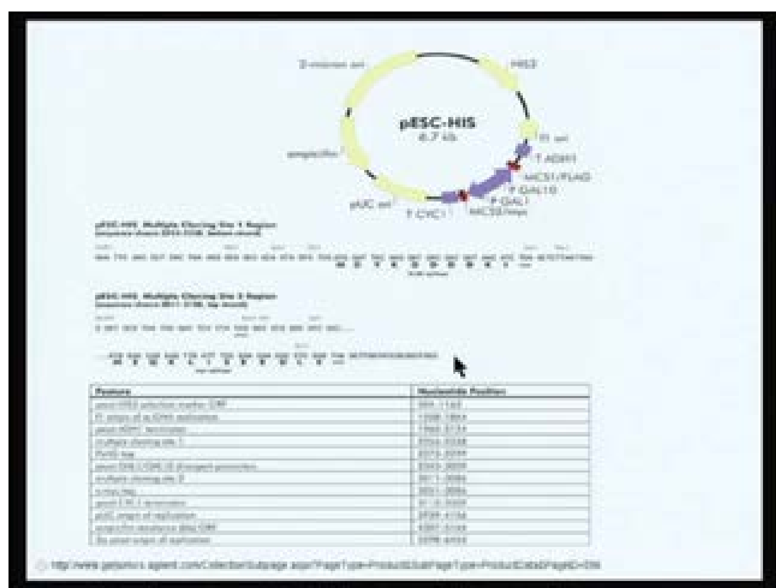

This is just the map of the vector again, taken from the company website which actually tells you, here is the p GAL 10 promoter contains a multi cloning site and you have the 3 prime processing signal.

So you can clone one of your genes downstream of the p GAL 10 promoter, and you can also clone your gene under the p GAL 1 promote here in the opposite direction. So this gene you will be transcribe this direction, this gene will be transcribe in the other direction. Therefore, two proteins can be made simultaneously number of other features including the multiple cloning sites are given. Here one can always go to the company website and can get more information about these things.

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Promoters for *S. cerevisiae* expression vectors

Promoter	Expression conditions	Status
Acid phosphatase (*PHO5*)	Phosphate-deficient medium	Inducible
Alcohol dehydrogenase I (*ADHI*)	2–5% Glucose	Constitutive
Alcohol dehydrogenase II (*ADHII*)	0.1–0.2% Glucose	Inducible
Cytochrome *c*, (*CYC1*)	Glucose	Repressible
Gal-1-P Glc-1-P uridyltransferase	Galactose	Inducible
Galactokinase (*GAL1*)	Galactose	Inducible
Glyceraldehyde-3-phosphate dehydrogenase (*GAPD, GAPDH*)	2–5% Glucose	Constitutive
Metallothionein (*CUP1*)	0.03–0.1 mM copper	Inducible
Phosphoglycerate kinase (*PGK*)	2–5% Glucose	Constitutive
Triose phosphate isomerase (*TPI*)	2–5% Glucose	Constitutive
UDP galactose epimerase (*GAL10*)	Galactose	Inducible

A Number of as I said equalize 1 of the I mean Saccharomyces cerevisiae is 1 of the well worked out Eukaryotic costs for gene expression as well as the biology aspects. So a number of equal Saccharomyces cerevisiae promoters have been very well characterized. I have (( )) given some of the very popular promoters which have been used a expression vectors have been constructed using many of these Promoters, such as the acid phosphatase gene promoter alcohol dehydrogenase 1 or Alcohol dehydrogenase 2 promoter, Cytochrome C 1 promoter the GAL 1 or the GAL 1 p uridyltransferase promoter, the Galactokinase promoter the glyceraldehydes 3 phosphate dehydrogenase promoter.

These are all constitutively expressed promoters. Some of them are inducible like all those the galaxy metabolism genes are induced galactose, and I have also shown here. Some of them are inducible. This is for example, the expression can be induced by removing the phosphate in the Medium for phosphate efficient condition. This promoter is turned on so as long as the phosphor in the medium, your gene will not be expressed similarly. You have Galactose inducible and so on and so forth. You have a Metallothionein promoter or the CUP 1 promoter. You can induce the transgenic expression by adding metals in the medium with a very small amounts of copper and again, you have Phosphoglycerate kinase triose phosphate isomerage UDP galactose epimerase of the GAL 10 gene. So, I have both constitutive as inducible promoters for expressing your genes is Saccharomyces cerevisiae.

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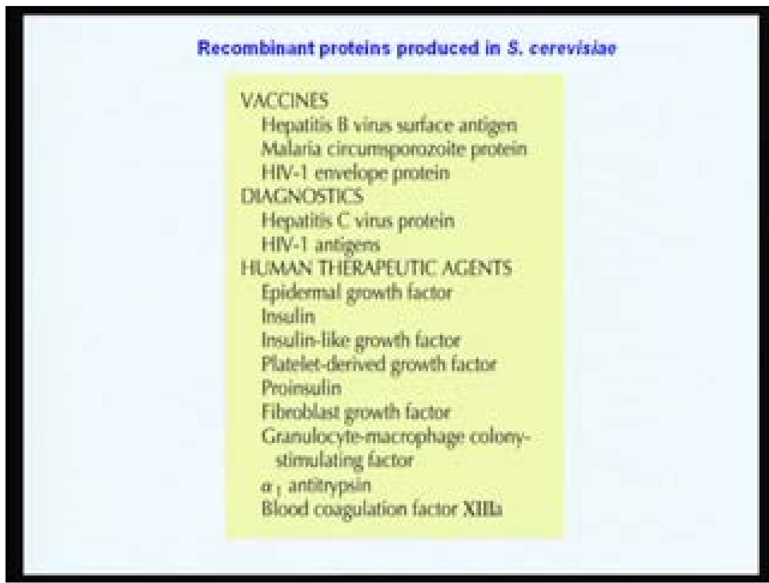

Number of recombinant proteins are expressed using any one of this Saccharomyces cerevisiae expression vectors. I just listed some of the proteins have been very successfully expressed, and some of them are actually available have been licensed for use for humans, and is available as products in the market.

Some of the VACCINES which have been very successfully expressed in the Saccharomyces cerevisiae or the Hepatitis b surface antigen, the malaria circumsporozoite protein H I 1 envelope proteins. They all been very successfully expressed and either they have approved for the Human news or a various cells of clinical trials diagnostics the hepatitis C virus protein many HIV 1 antigens have been expressed these recombinants can be used for things like elisa (( )) and so on. For diagnostic purposes, many human proteins which are very important therapeutic benefits such as Epidermal growth facto, r Insulin p DGA of pro G M C F S antitrypsin, many Blood coagulation factors like factorate have been very successfully expressed in Saccharomyces cerevisiae using the various expressions that have listed here.

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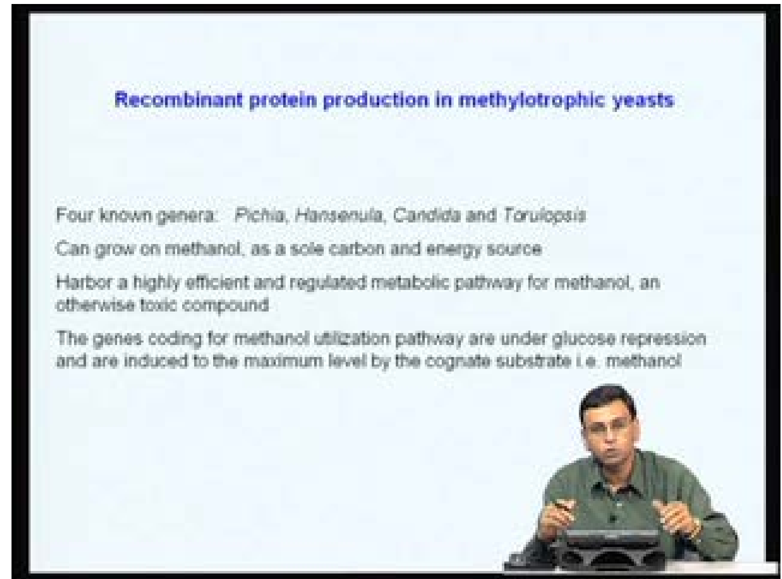

Now let us spend some time having looked at the Saccharomyces cerevisiae what kind of expressions are available. If you want to now choose methylotropic yeast as your choice of yeast cells, now what are methylotropic is there are at least 4 well characterized genera or pichia hansenula candida and torulopsis. These are called as methylotropic yeast because they can use methanol as a sole source of carbon whereas, Saccharomyces cerevisiae cannot use methanol as a carbon source. You can use a glucose or glycerol or fatty acids is a carbon source. It cannot metabolize methanol, because the proteins are the enzymes required for methanol metabolism are not present in Saccharomyces cerevisiae. So they harbor a very efficient and regulated metabolic pathway for methanol. Otherwise toxic compound if you had methanol Saccharomyces cerevisiae will die whereas, these organs something tolerate up to 1 to 2 percent of methanol concentrations, and I can actually metabolize methanol and use it as a carbon source. The genes coding for methanol utilization pathway are under glucose repression, so all the genes involves methanol metabolism are not expressed as long as you grow this methylotropic is in glucose as a carbon source .

The moment you change the carbon source from glucose to methanol, all these genes involved in the methanol metabolism turned on. So they often excellent inducible expression system, so your trans gene if you clone your gene in the promoter of genes coding for methanol utilization pathway. As long as you grow this cells in glucose, your trans gene will be turned on. The moment you change the carbon source of glucose to

methanol your gene of interest will have been expressed. So you can develop a very nice inducible vectors using this genes of promoters of gens involving methanol metabolism.

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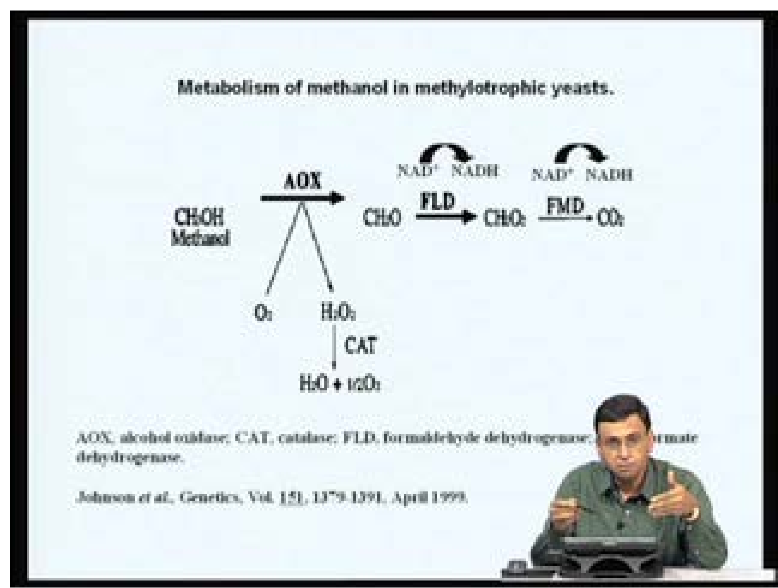

This is briefly the methanol Metabolism. The methanol is first metabolized to formaldehyde by the enzyme called alcohol oxidized, and then the formaldehyde is converted into formic Acid by formaldehyde the hydrogenise which is then converted into carbon dioxide by formaldehydrogenise, and there are many other enzymes which will not go into the key enzymes 3 k enzymes to the methanol metabolism, and the first reaction conversion of methanol to formaldehyde actually takes place in organ called paroxysms.

And therefore, when you change the carbon source to glucose methanol, the paroxysms also proliferating. The high amount in these Methylotropic is and therefore, these scenes also became very good model systems for understanding paroxysms biogenesis.

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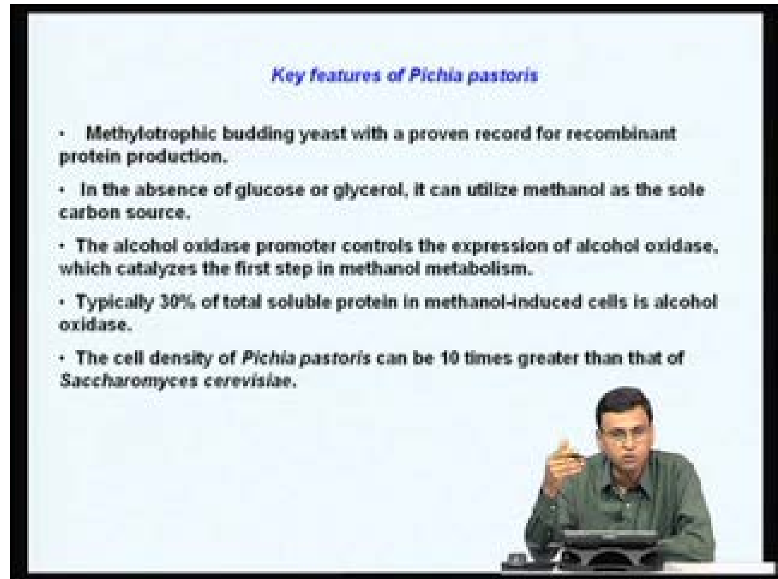

So what do you do? What are the Key features of some of this Methylotropic is? If you take pichia pastoris is a model system is a very valued or very widely used Methylotropic yeast for recombinant protein production. It is a mMethylotropic budding yeast with a proven record for recombinant proteins.

A number of proteins have been successfully expressed in particular organism in the absence of glucose or glycerol it can utilize methanol as the sole carbon source. I have already told you this, the alcohol oxides promoter which is the first enzyme in all the methanol metabolism which converts methanol to formaldehyde. It is a very powerful promoter. The promoter that controls the expression of alcohol is very powerful.

For example, when you grow this cells in methanol, almost thirty percent of the total soluble protein is accounted by alcohol oxidase. So the moment you change the carbon source glucose to methanol, thirty percent of the total protein in the methanol growing cells is accounted by 1 protein, which is alcohol oxidase. So that is a very powerful promoter and the other important Advantage of pichia pastoris is that the cello density of pichia pastoris cabe ten times more than that of Saccharomyces cerevisiae. So if you put YUR gene of interest in the Saccharomyces promoter and the pichia promoter introduce Saccharomyces in pichia and go then in identical conditions. The amount of cell density you will get pichia is ten times more compare to Saccharomyces. So per liter of the

culture you can make lot more recombinant protein, if you express gene in pichia pastoris compared to express same gene in Saccharomyces cerevisiae.

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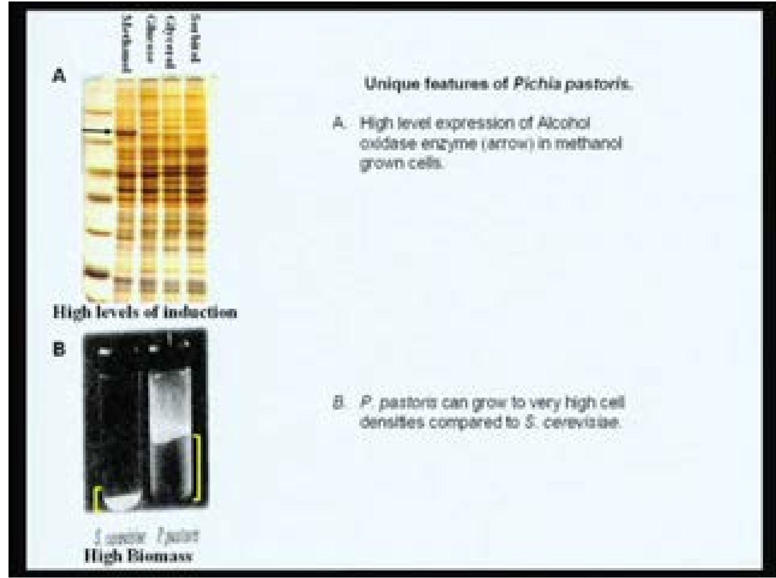

So pichia pastoris has two important advantages which is listed here. One is it has a very powerful promoter called the alcohol oxidase promoter. So your protein transient can be expressed very high levels here. I have just shown here if you. Grow the pichia pastoris under different carbon sources like sarbitol glycerol glucose or methanol and you can see this particular band which is actually the alcohol oxidase.

You do not see this band when you grow this yeast and other carbon sources only when going methanol this protein is induced in high levels and you can see it is one of the major proteins that are induced in almost thirty percent of proteins accounted for the one particular protein. So, it is a very powerful promoter and is a very abundant protein in the methanol growing cells. So, this is one advantage. So it has high level expression of alcohol oxidase enzyme in methanol grown cells. The other advantage is you can see the identical condition, the amount of cells that you can obtain using the pichia pastoris is ten times more than Saccharomyces cerevisiae. Here are the two flacks are actually you contained that shows this is the biomass generated from Saccharomyces cerevisiae is biomass generated from pichia pastoris. Because see this is the huge difference pichia pastoris can goes to very high densities compared to Saccharomyces cerevisiae. So the amount of recombinant protein that you can produce per liter of culture is much higher

when you use pichia pastoris other than Saccharomyces cerevisiae. So pichia pastoris has many advantages. compare to Saccharomyces cerevisiae.

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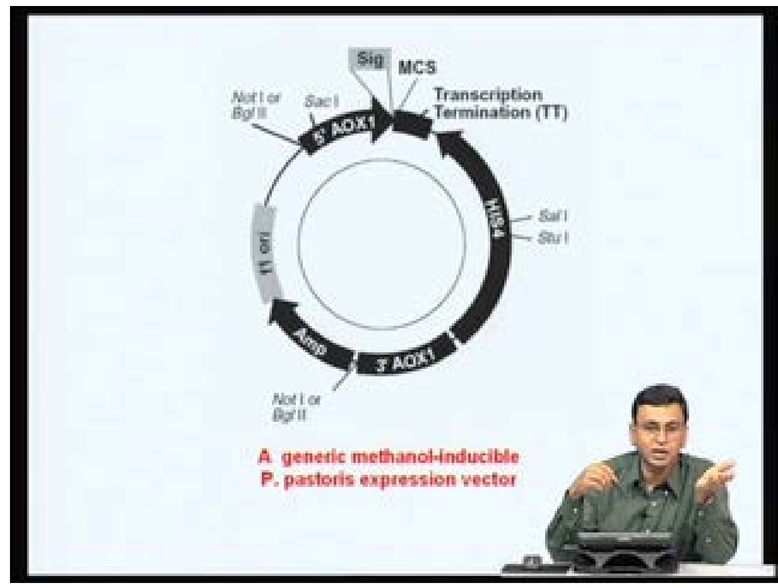

The kind of vector, the people normally use for expressing recombinant proteins in pichia pastoris are of integrative vector type. That is the gene goes into the integrating in the main chromosome, and what you normally do is that you have the 1000 base pairs of the alcohol oxidase promoter, and you clone your gene downstream of the oxidase promoter multi cloning cyclic here, and you also have the Transcription Termination sequences for the proper process of messenger RNA. You have a oxitrophic marker in t his case the, his 4 marker and you have the 3 prime ah (( )) AOX 1. This is for recombination I will come little later it also has (( )) system gene, and E coli (( )) of replication. So first you clone your gene into this vector. Make this vector in large amounts by Transform into E coli and then make this plasmid linearise plasmid and Transform into pichia pastoris. This is the general method of expressed recombinant proteins in pichia pastoris.

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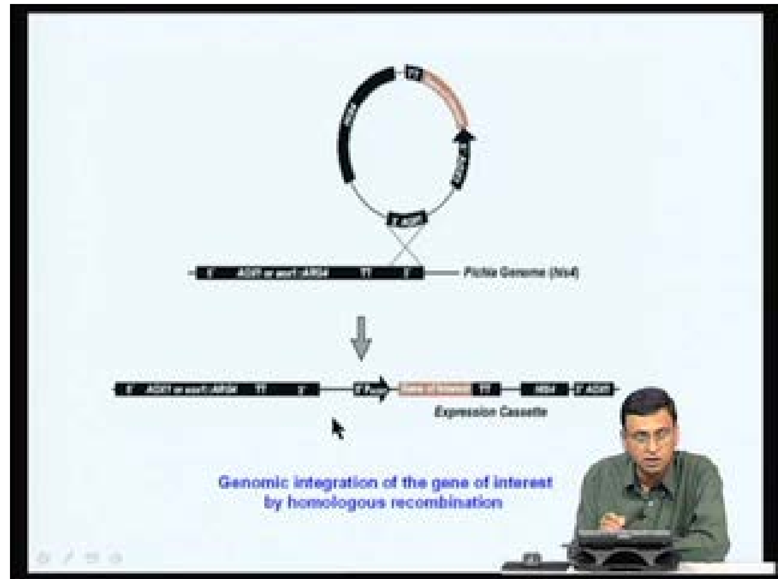

So this is what you normally do. The gene of interest is introduced downstream of the oxidase promoter, and then you line arise vector or and then by recombination it goes an into the oxidsase alcohol lotus of the chromosome of the pichia pastoris, and therefore, you get a integrate type of expression.

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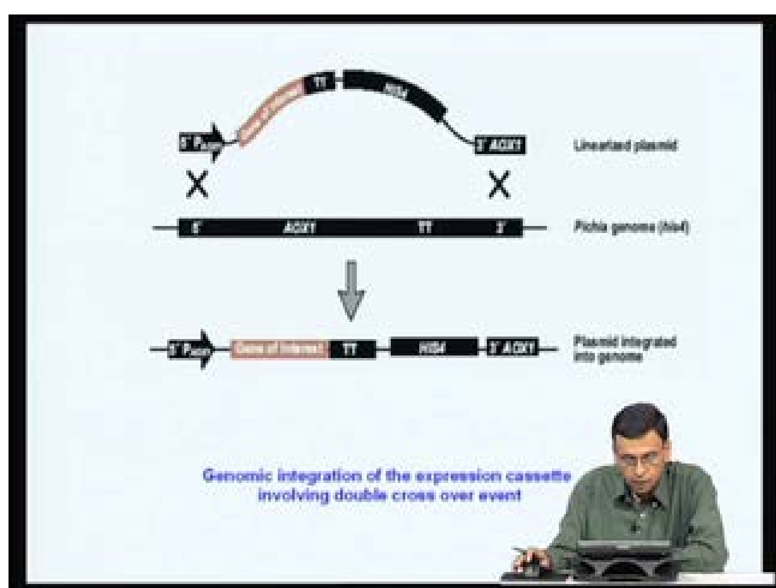

The gene can also get into the alcohol oxidase by reciprocal recombination involving a double cross over raiment where the endogenous alcoholic gene can replaced by your gene of interest, and such genes are called as MUT minus. That means these can no

longer utilize methanol because the endogenous alcohol oxidase gene has been lost. Whereas, in the previous case the endogenous alcohol oxidase gene is still interact. Therefore, they are called as MUT S. They can still utilize methanol where as if your trans gene has gone integrated by replacing the endogenous alcohol oxidase gene. When they are called MUT S prime that is they are methanol sensitive. Therefore, they can no longer utilize methanol very sufficiently and your trans gene actually replace the endogenous alcohol oxidase gene.

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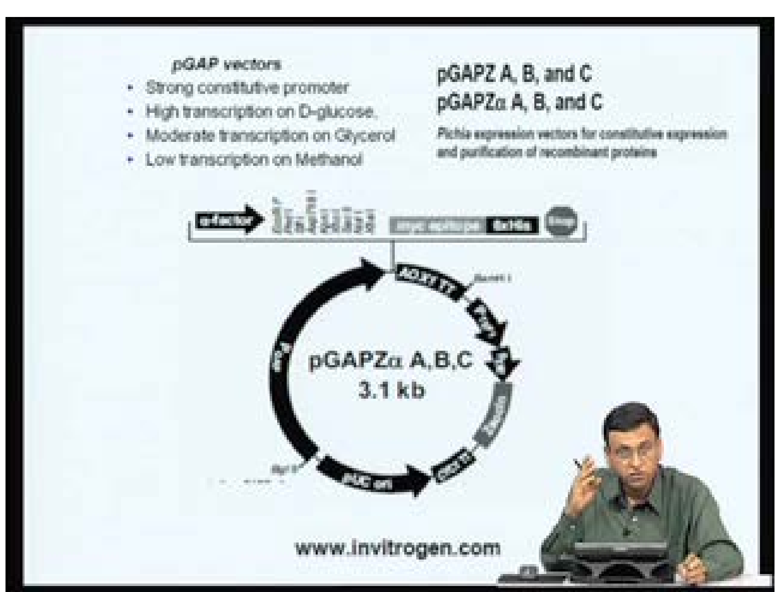

In addition to the powerful alhojas promoter, there are also constitutively expressed promoters available for proteins expression in pichia pastoris. For example, the p GAP vectors contain the glyceraldehyde 3 phosphate dehydrogenase promoter which is a constitutive promoter. It more or less opposite to that of the alhojas promoter, the strong constitutive promoter high expression in glucose moderate transcription on glycerol. Low transcription on methanol which is more or less opposite to what we assigned. In the case of alcohol oxidase, alcohol oxidase represents high levels in methanol not expressed in glucose here is highly expressed in glucose, but not expressed in methanol. So again the same strategy you can now clone the trans gene of your interest downstream of the p gap promoter. Again linearise you can introduce go and integrate to the gap glyceraldehyde 3 phosphate dehydrogenase promoter site, in either of these cases and also people have now come up the novel markers such as geos. You do not have to use oxotropic marker (( )) 4.

There are now geos, it can actually kill yeast cells which have now taken up the plasmid and you have geos in resistance those which have taken up the plasmids are now geos in the resistance and therefore, you can easily select the (( )) geos in resistance. You no longer require the oxotrphic marker stream the Advantages of things like the his 4 gene which is normally used in the previous vector and the geos is in that geos is much smaller gene his 4 is a much bigger gene and therefore, the size becomes a problem when you use the vector can oxotrophic markers but, here you get much much smaller size of the plasmids in sets only 3 point 1 Kb whereas, the original alhojas promoter based peak peak vectors or much bigger.

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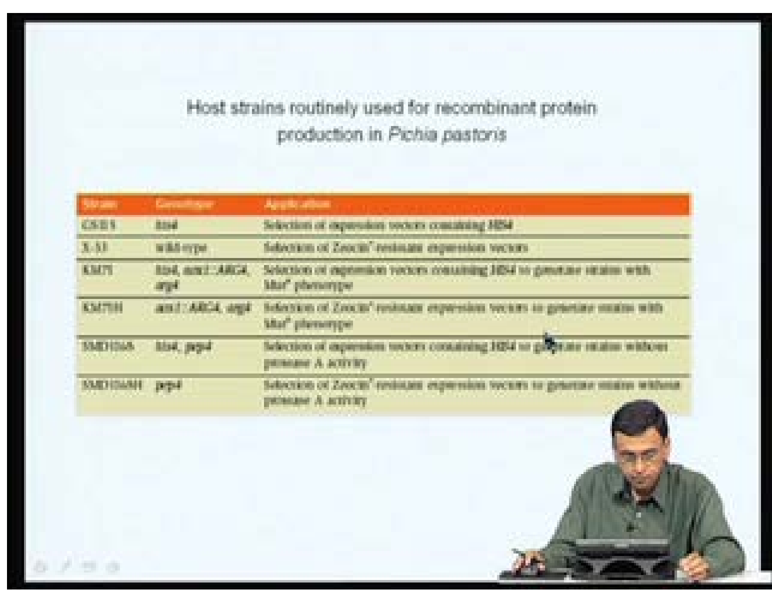

So a number of Host strains have been developed they are either oxotrophic markers. Some of them are also (( )) in specific proteases, so that recombinant can be successfully produced in the (( )) proteins, and then cleaved. I will not go into the details of it. So a number of pichia pastoris hostages are available for successful expression of various recombinant proteins

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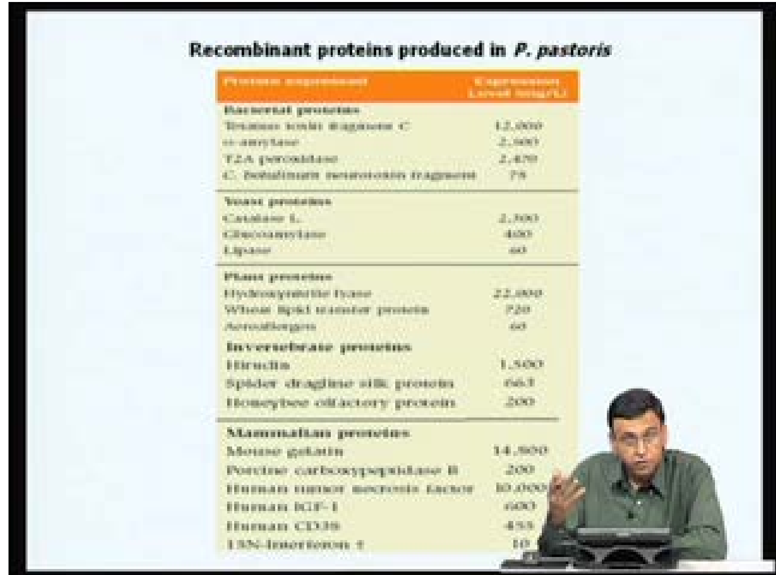

A number of Recombinant proteins have been expressed and at very high levels using the pichia pastoris as an expression system. Bacterial proteins have been expressed, yeast proteins have been expressed, land proteins have been expressed. Many like invertebrate proteins like hirudin which is a blood clotting protein, and many more mammalian proteins also have been successfully expressed using pichia pastoris has been expressed in system.

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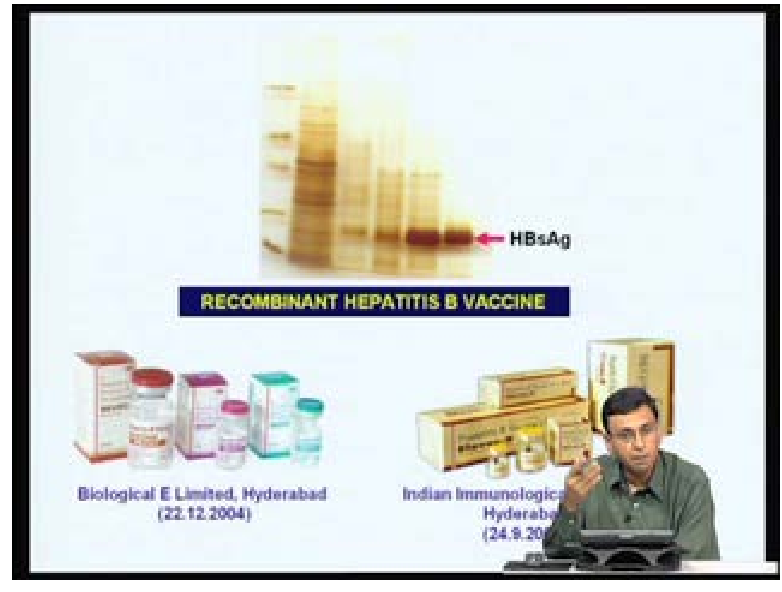

Own lab has been use this pichia pastoris expression system to make a Hepatitis B vaccine. We have very successfully expressed the Hepatitis B surface antigen in large amounts in this pichia pastoris results using the alcohol oxidase promoter purified, the antigen trigmogenity and this has given to two components. In India both of them brought out the vaccines in the market 1 is B VAC another silo VAC B.

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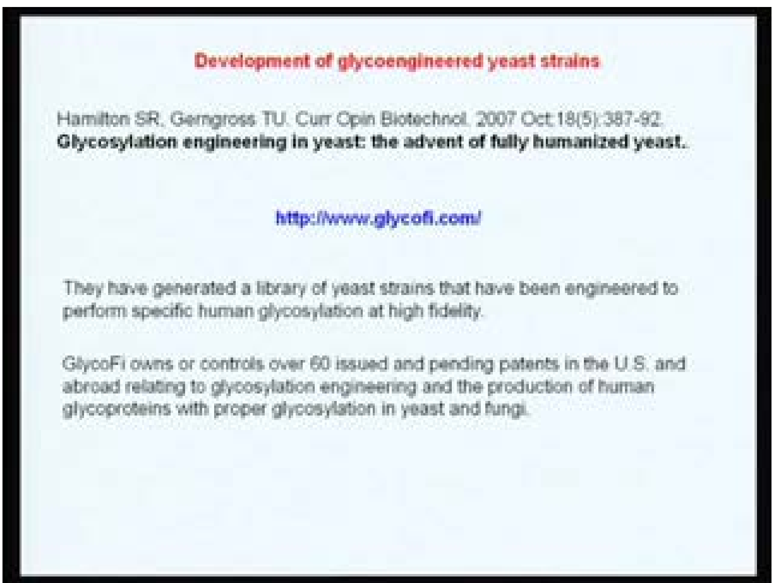

The other important development that took place in the case of pastorisis is that as I said yeast cells can also glycosylate, but many times the Glycosylation is not exactly the same as that happens in the human cells. So when even this fine differences are very very important, that means if you want to express a recombinant protein which has exactly the same kind of the Glycosylation pattern as in the mammalian cells. Then have now generated yeast strains which has the same kind of glycosylation pattern as that in the humans. These are called as Glycoengineered yeast strains is a strains have been developed by Gerngross and a company called Glycofi which is specializes in these kinds of strains. So this company has actually generated library of pichia pastoris yeast strains that have been to perform specific human Glycosylation at high fidelity. That means if you now express the proteins in these pichia pastoris cells, the Glycosylation of a protein is exactly the same as that happens in the humans. So, these are called Glycoengineered strains and this company actually controls on owns over sixty patterns on this

Glycosylation engineering pathway, engineering Glycosylation pathways in this is and therefore, you can actually produce human proteins with exactly more or less same kind of Glycosylation in yeast and fungi. So, this one major advancement that has took place in the case of recombinant protein expression yeast cells.

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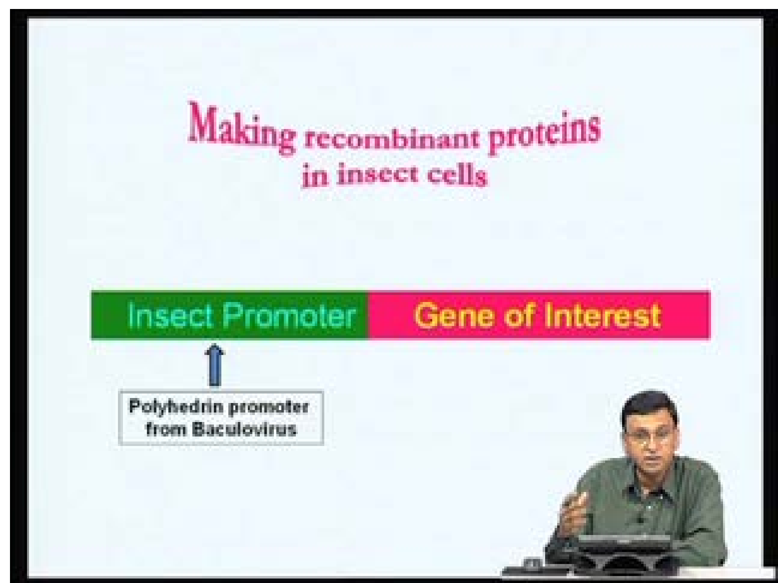

So having discussed at length at how you can express proteins in the yeast cells. Let us now spend some time to see, how you can make a recombinant proteins in insect cells. Again the principle is the same, you have to clone your gene of interest downstream of Insect Promoter. The most commonly used Insect Promoter for making a recombinant proteins in yeast called as a plyhedrin promoter from a virus called as baculovirus. This virus infects only the insect cells. So, let us now trying to understand what is this baculovirus and what is this polyhedron promoter.

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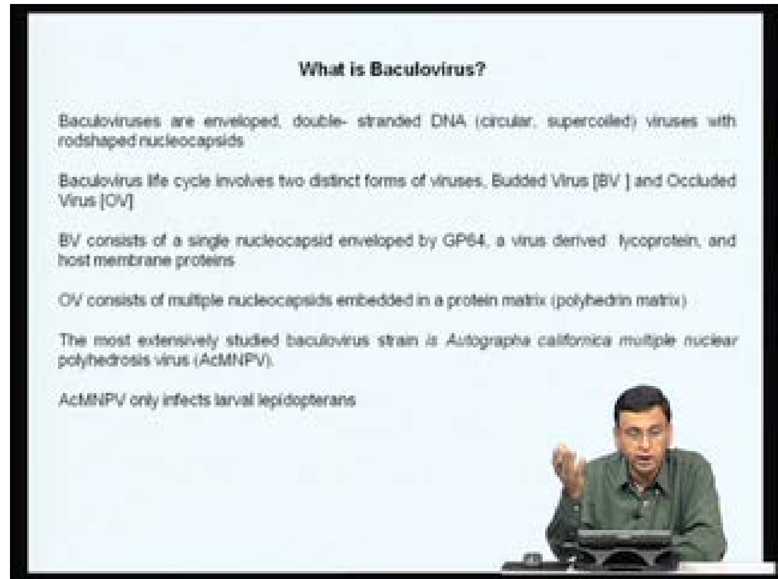

Now what is a Baculovirus? Baculovirus are envelope double standard DNA viruses with rod shaped nucleocapsids, the life cycles involves 2 distinct form of viruses budded virus as well as occluded virus.

The budded virus consists of a single nucleocapsid enveloped by the protein called GP64 a virus derived like protein, and host membrane proteins, whereas occluded virus consists of multiple nucleocapsids embedded in a protein matrix called as a polyhedrin matrix. The most extensively studied Baculovirus strain is called as the autographa calefornia multiple nuclear polyhedrosis polyhedrosis virus or abbreviated as ACMNP AMD. This ACMNP infects larval lepidopteron, so this is the basic phases of the Baculovirus which is based on which an expression system has been developed for making for recombinant proteins in yeast cells.

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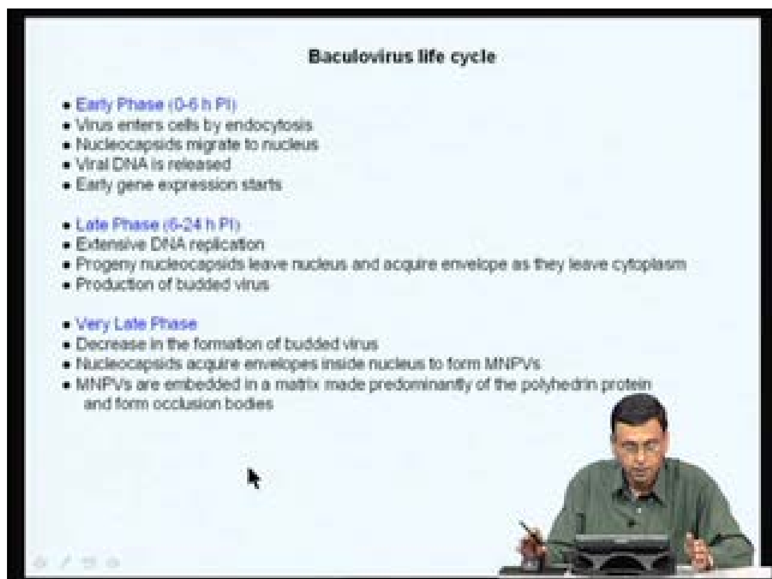

Now what is this life cycle of this Baculovirus it has 2, 3 phases life cycle, in Early Phase, late phase and a very late phase, in the early phase the virus enters cells by endocytosis, the Nucleocapsids migrate to the nucleus, the viral DNA is released, and early gene is expression starts the Late Phase that is 6-24 hours after infection.

The DNA viral, DNA replicates the progeny Nucleocapsids leave the nucleus and acquire envelop as they leave the cytoplasm and they produce the budded virus. The Very Late Phase the viral formation decreases the Nuclocapsids acquire envelops inside the nucleus to form what is called as the M N P V s or Nucleopolyhydrosis Virus and these are embedded in a matrix made predominantly of the Polyhydrin protein and forms occlusion bodies. So, I can see this Polyhedrin protein is actually made only in the vary late phase of the virus cycle. Virus life cycle which is very very important for the recombinant protein expression, so this ability or to make the large amount of this Polyhedrin. In the late phase of this Virus life cycle has been now taken Advantage and this feature has been use for making a recombinant protein using the Baculovirus expression system.

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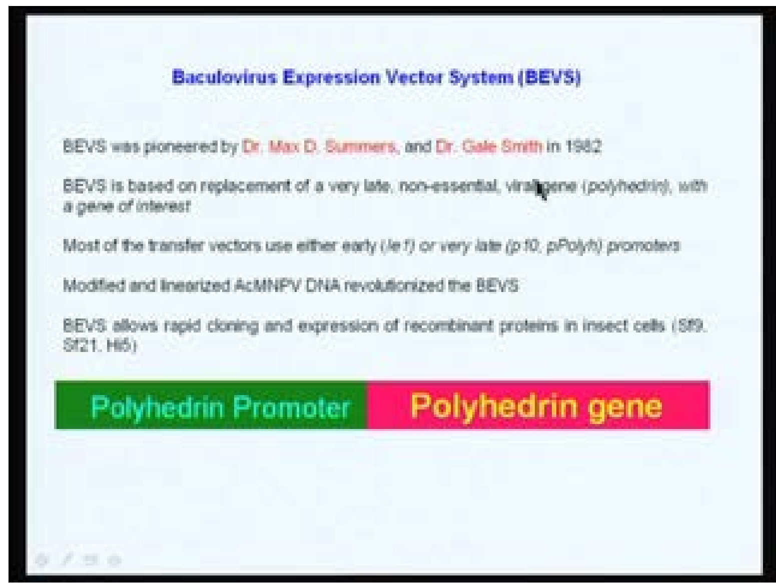

Now the Baculovirus Expression abbreviate as BVS was pioneered by Max summers and Gale Smith. In 1982, the expression system is based on replacement of the Polyhedrin gene with that of a gen of a gene of your interest.

So, this is the Poluhedrid gene is expressed in very very late phase of the virus life cycle, and is not essential for the virus life cycle. You basically remove the polyhedrin gene, and put your gene of your interest under the polyhedrin promoter. So instead of virus, now making the polyhedron, you will now make your protein of your interest. This is the principle of Baculovirus expression system. So, some of the key features have described here we will not go into the details.

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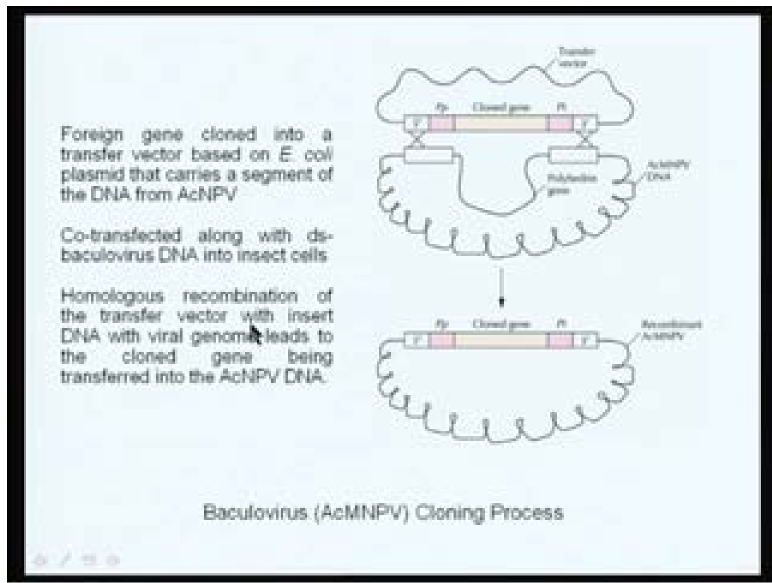

Basically what you do in the case of, if you want express your gene in insect cells in baculovirus expression system. The gene of interest is clone into a transfer vector based on e coli plasmid that carries a segment of the DNA from AcNPV. So you have a bacterial expression plasmid which contains two regions which are homologous to the polyhedrin gene at the insect. So first clone your gen between these homologous region and this is called as a transfer vector and making large amounts in the E coli cells, and then you co transfer this along the baculovirus gene into the insect cells. So homologous recombinant takes place between these regions in a transfer vector and the polyhedrin geneand as a result now you get a recombinant genome which now contain your cloned gene between the polyhedrin downstream of the polyhedron.

Instead of the polyhedrin gene, and this gene can now been introduced insect cells, and now the virus recombinant virus generated and then recombinant protein can be made in large amounts. This is the basic strategy of generating recombinant baculovirus.

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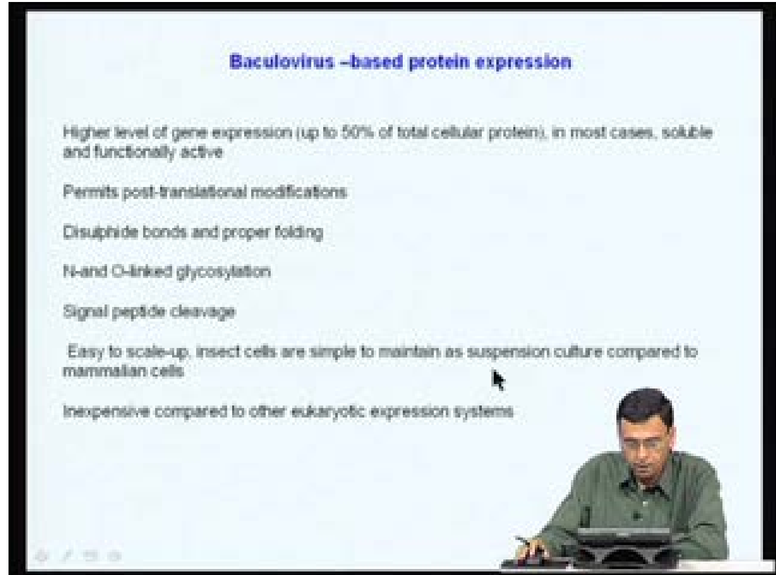

So the advantage of the Baculovirus expression system is that it can the polyhedrin promoter is a very highly active promoter highly inducible promoter. Almost 50 percent of total cellular protein can be your recombinant protein like the yeast. It also permits many posttranslational modification proper Disulphide bond will be formed, and it can also do signal peptide cleavage because a Eukaryotic expression system very easy to scale up, because insect cells can be grown in very large amounts in inexpensive media, and also is a very in inexpensive expression system compared to mammalian expression systems.

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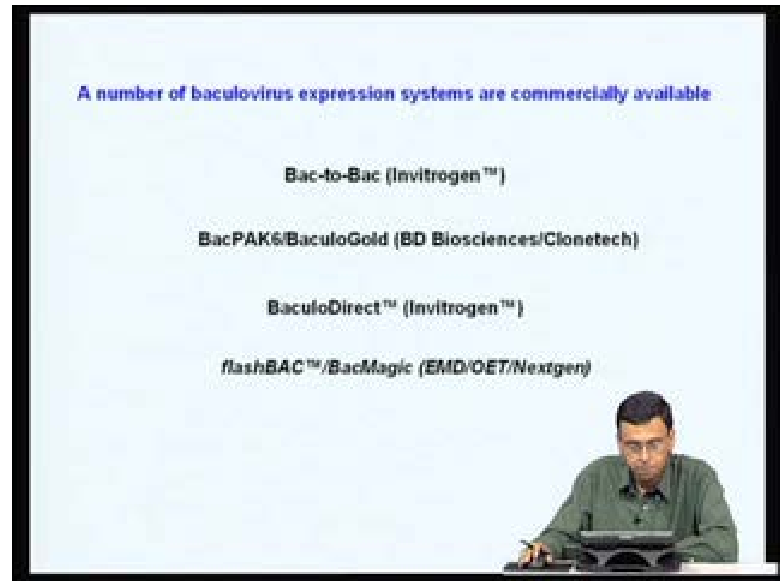

I will not go into the details of this a number of commercial baculovirus expression systems are now available from a number of companies like invitrogen cells. What is called as BAC-to-BAC Baculovirus expression system, the baculodensis and the biosciences of the clonetech cells. What is called as the BAC PAK 6 or a Baculogold system invitogen cells? Again baculodirect and another company cells flash BAC or a Bacmagic several variants. So basically it becomes very easy now using these commertial expression systems to express your gene of introducing a Baculovirus expression systems.

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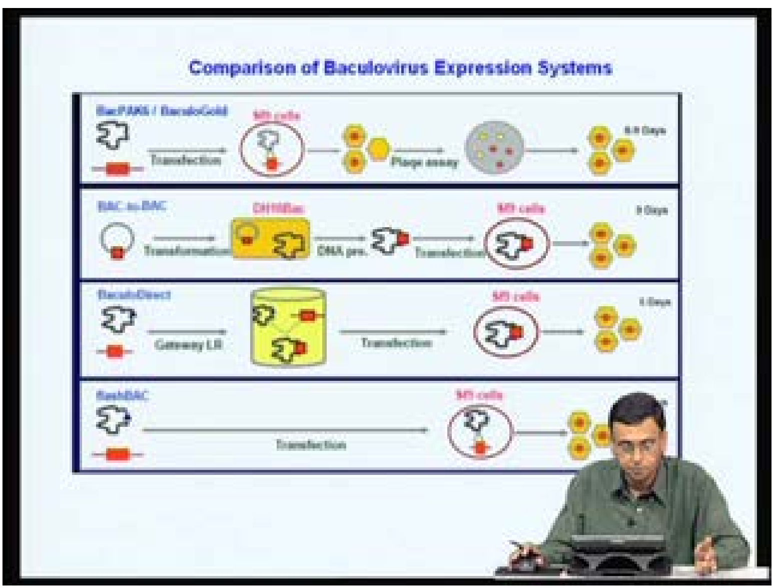

I have also given here the Advantages of all these commercial available Baculovirus Expression Systems. Again I will not go into the details if you are really interested in the recombinant protein Baculovirus. One can go through all these things see which one of them is to more suitable to you go to that particular company, buy the system clone your gen into the system make a recombinant baculovirus and make protein of your interest.

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Commonly used insect cells for protein production

Cell line	Origin	Use
Sf-9	*Spodoptera frugiperda* (Pupal ovarian tissue)	Recombinant baculovirus production Intracellular protein expression Plaque assay
Sf-21	*Spodoptera frugiperda* (Pupal ovarian tissue)	Intracellular protein production Secretion of recombinant protein
Hi-5	*Trichoplusia ni* (Ovarian cells)	Secretion of recombinant protein
Tri-Ex	Sf-9 derivative	Intracellular protein producti

These are also many insect cell lines actually which have been specifically developed for recombinant protein production in insect cells. For example, have S f 9 cells which has been produced from a inceptors protopterus.

(( )) it is basically used for recombinant baculovirus production, the intracellular protein expression and plaque assay, we have cell line called S f 20 1 again derived protopterus again used for intra cellular protein as well as secretion of recombinant proteins. What is called the H I 5 cells produced from tricoplusia n I primarily secretion of recombinant proteins, and how what is called as tri ex which is derivative of the S f 9 and primarily used for the intracellular protein production. Each one of them have its own advantages and disadvantages. So once you make made your recombinant baculovirus you can choose any one of these insect cell lines, introduce the recombinant virus and then produce a recombinant protein in large amounts.

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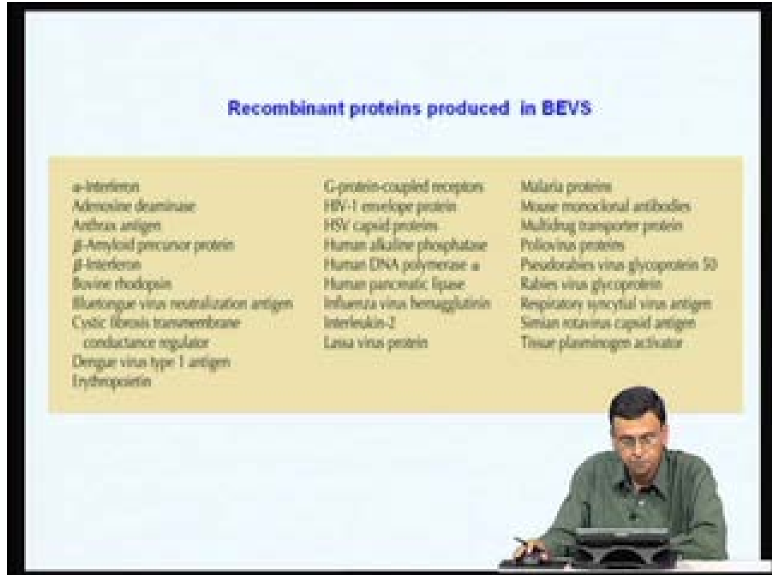

A number Recombinant proteins have been again expressed using the baculovirus expression systems have just listed. Some of them here you can see huge number of proteins of either parasite proteins or viral proteins human proteins have been made using the baculovirus expression systems.

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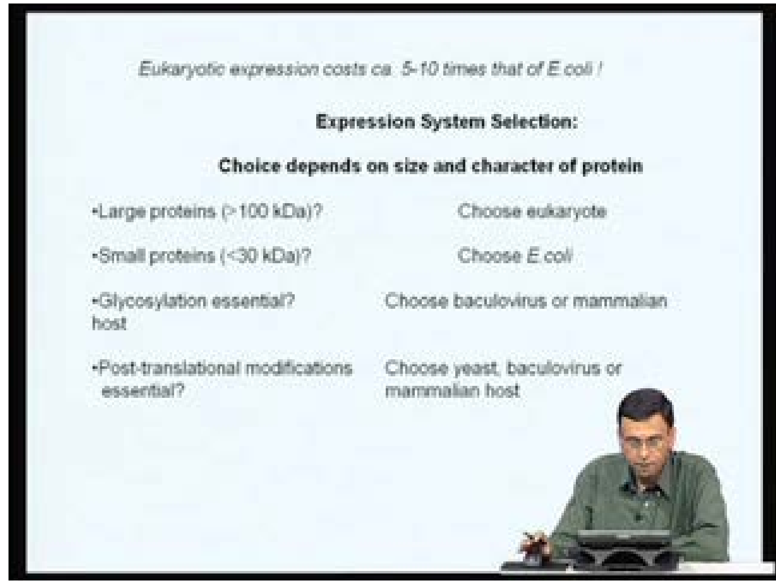

So, what we have discussed today it is two major Eukaryotic Expression Systems namely the insect Expression Systems as well as Yeast expression systems, and when it comes to the choice remember an Eukaryotic expression system always cause five to ten times that

of E coli, and usually whenever you want to make a recombinant protein your first Choice is a prokaryotic expression systems. Only if you are not happy with Eukaryotic expression system for a any reason, then only you start try Eukaryotic expression system. Even among the Eukaryotic expression system into the yeast and the expression systems are the least expensive. So, you try them first, and if you still does not work, then only you go for the mammalian expression systems which we will discuss in the next class.

So, what is it ? How do you decide and what expression system you choose, the choice depend on the size and character of protein. If you have expressed a very large protein you have no Choice, but you have use only Eukaryotic expression system. If you are expressing a small protein, go for a prokaryotic expression system, but if a protein Glycosylation is very very important for the protein. Then you cannot use a prokaryotic host, so either you have to choose a baculovirus or a yeast or a mammalian expression system again.

Certain specific posttranslational modifications are very very important for your protein. Again you cannot go for the prokaryotic expression system, and you have to choose one of the Eukaryotic expression system. So, the choice of the expression system depends on the kind of the protein that you are trying to express and the kind of modification that the protein require for its biological activity.

So, I just listed some of the important proteins which has a commercial interest have. I also strongly suggest you to go and visit the websites of some of these very important companies like, invitrogen, clontech, novagen, stratagene. They have been putting lot of effort to develop a number expression vectors for both prokaryotic as well as Eukaryotic expression. And if you go and then very useful presentations of how these vectors works, and one can go and educate yourself in these company websites, and then decide what kind of expression vector are suitable for your work, and then use it for your recombinant protein production, I think I will stop here .