Eukaryotic Gene Expression: Basics & Benefits

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Lecture No. # 40

Metabolic Engineering & Synthetic Biology

Welcome to this last lecture on eukaryotic gene expression basics and benefits.

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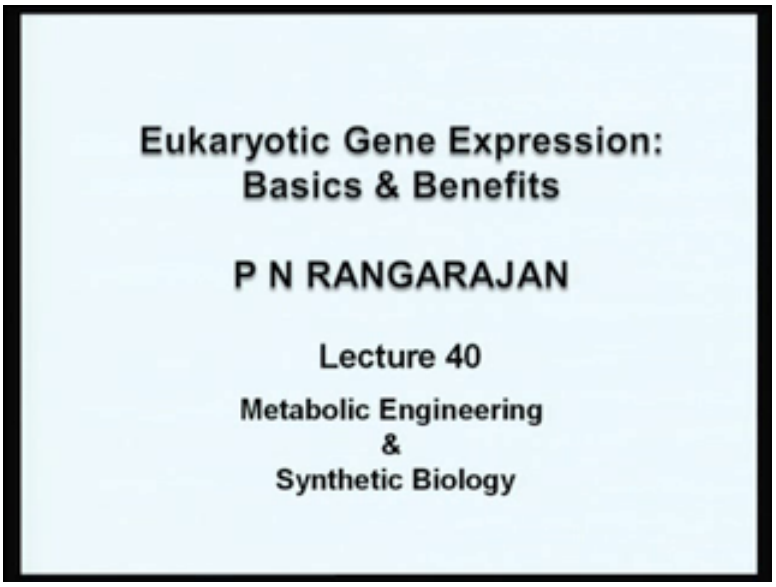

In this lecture, I am going to introduce to a very new and exciting area of research in biology and medicine known as metabolic engineering and synthetic biology. Now, what is metabolic engineering?

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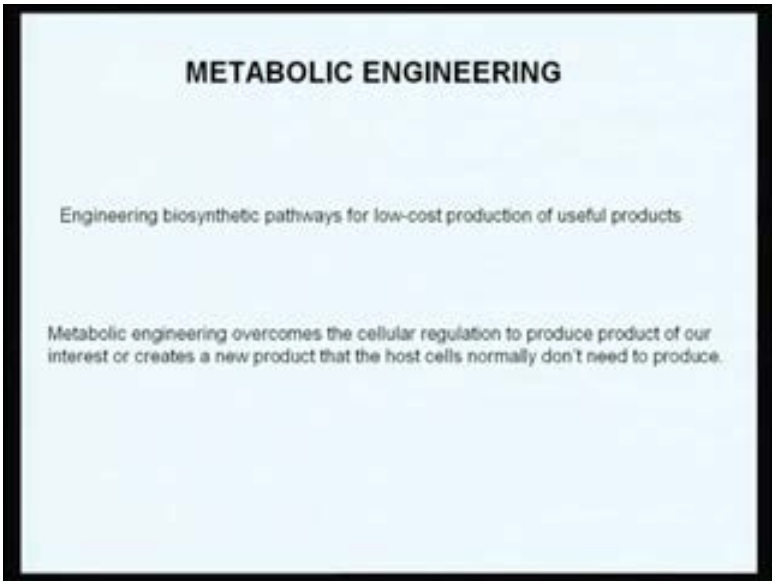

Engineering biosynthetic pathways for production of low cost production of useful products is a very simple definition of metabolic engineering that means you alter the metabolic pathways of any organism, and as a result, the (( )) out of engineering these metabolic pathways, you can now synthesize an useful products. It can be a small molecule or it can be an amino acid or it can be a flavonoid it can be a terpene or it can even be a protein.

So, by engineering certain specific metabolic pathways, so if you can ultimately produce a useful product that can be known as metabolic engineering. Now, metabolic engineering overcomes the cellular regulation to produce product of our interest or creates a new product that host cells normally do not need to produce.

Two important aspects in metabolic engineering, one is usually all these metabolic pathways are very finely regulated, and therefore, the intermediates which accumulate in these metabolic pathways are often of limited amounts, but, by engineering or by tinkering with this regulation of these various metabolic pathways, it is possible to overproduce certain components of this metabolic pathways. We will discuss specific examples later.

So, a molecule which is already being produced by a particular organism can be overproduced by engineering some of these metabolic pathways, but in another case, a molecule may not be produced at all by that organism by introducing certain new genes,

and by expressing certain new enzymes you can now force that organism or you can force that particular metabolic pathway to produce a compound of your interest.

So, either you can overproduce a compound that is already being produced by the organism by tinkering with the metabolic pathways or you can force the organism to alter certain metabolic pathway so that it can now synthesize a new compound, which is not normally synthesized by this organism. So, both examples are possible and both are being carried out using metabolic engineering.

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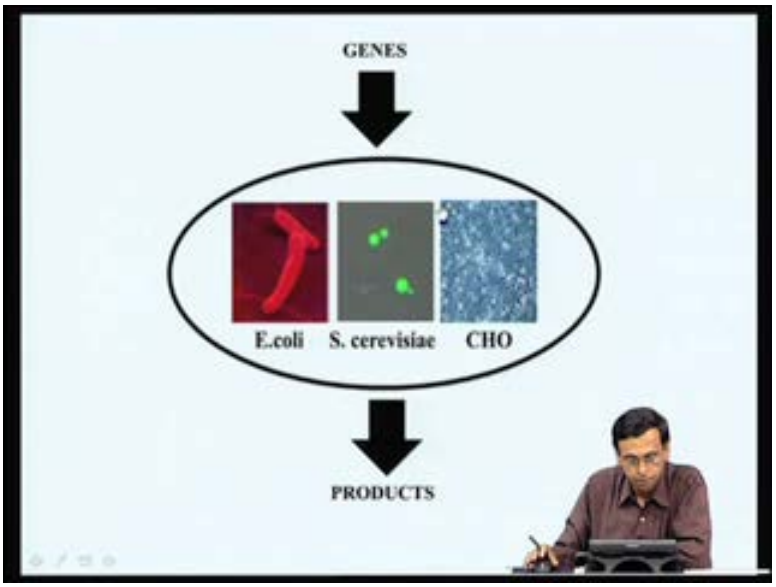

So, let us now try to understand how by introducing certain genes, which actually code for enzymes of certain specific metabolic pathways you can force these organisms, it can be an E. coli, it can be Saccharomyces cerevisiae, or it can be chinese hamster ovary cells that is bacterial, yeast, or mammalian cell systems to express certain enzymes or certain molecules of your choice, which can be now sold as products.

So, by expressing a group of genes, you can now alter metabolic pathways so that you can get a desired product of your interest. That is what is going to be the focus of today is talk.

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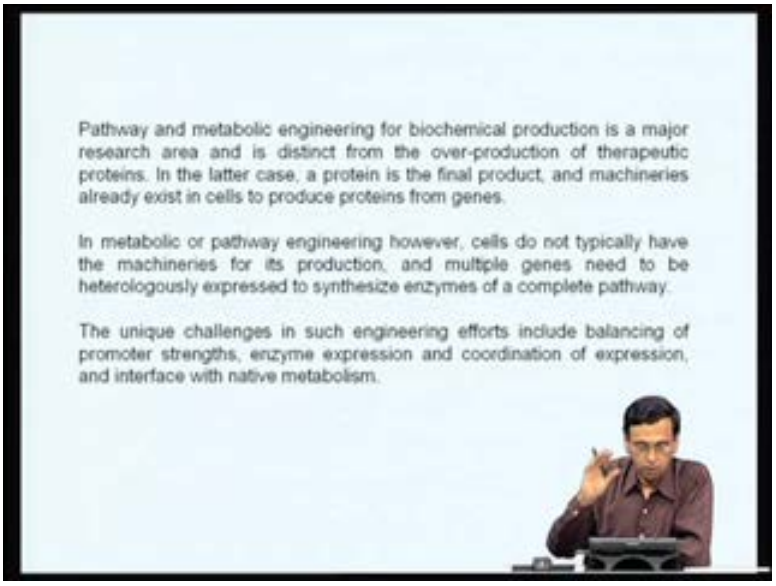

Now, pathway or metabolic engineering for biochemical production is a major research area and is quite distinct from the over production of therapeutic proteins. In the last few classes, we have extensively discussed about over expression of expression systems, prokaryotic expression systems, eukaryotic expression systems, gene therapy, and so and so forth, but of all forth, the focus has been on making proteins.

How you can overproduce a protein or how you can knockout the expression of a gene so that a particular protein is not made or so on and so forth. So, one might ask the question in what way metabolic engineering is different. Here also, ultimately you are making a product that can be protein or an enzyme, but the major difference between a metabolic engineering or a pathway engineering is different from a recombinant protein production is that in the case of recombinant protein production the protein is the final product and machineries already exist in the cells to produce proteins from this genes.

You are not tinkering with any pathways to over produce a protein of your interest. Basically, you are putting a promoter in front of the gene of your choice, and by transcription factors, bind to this and a protein of your interest is produced, so you are not tinkering with any pathway whereas in the case of a metabolic engineering or a pathway engineering, cells do not typically have the machineries for its production and multiple genes need to be heterologously expressed to synthesize the enzymes to

complete the pathway, so this is the major difference now. For over producing proteins, you do not have to engineer any pathway because the machinery already exists.

You just have to introduce your gene under the control of a particular promoter and your protein will be made by the organism, but in the case of metabolic organisms, many a times you are not happy with the pathway that exists in the cell. Therefore, you alter the pathway by either tinkering with the enzymes which are existing in the cell or by introducing new genes so that certain new enzymes can be made so that your desired product can be produced in large amounts.

The unique challenges in such engineering efforts include balancing the promoter strengths, enzyme expression, coordinate expression, and interface with the native metabolism. So why metabolic engineering has become a new discipline or an independent discipline of its own because it offers a many challenges because you have to express a number of genes coding for a number of enzymes of a pathway and many a times all these enzymes are under very tight control.

They have what is called as a feedback regulation or feedback inhibition, so if you over produce or under produce a particular compound or intermediate in that particular pathway the reactions do not proceed further. So, a lot of balancing efforts has to be done. The kind of promoter that you use for one particular enzyme of a gene coding for an enzyme pathway should be different from the promoter that you have to use for another gene coding for another enzyme pathway.

So, one has to do a lot of balancing act in order to make sure that this metabolic pathway functions and ultimately you get the desired product of your interest; that is why, metabolic engineering or pathway engineering became an independent discipline of it is own and is quite distinct from producing recombinant proteins of your interest.

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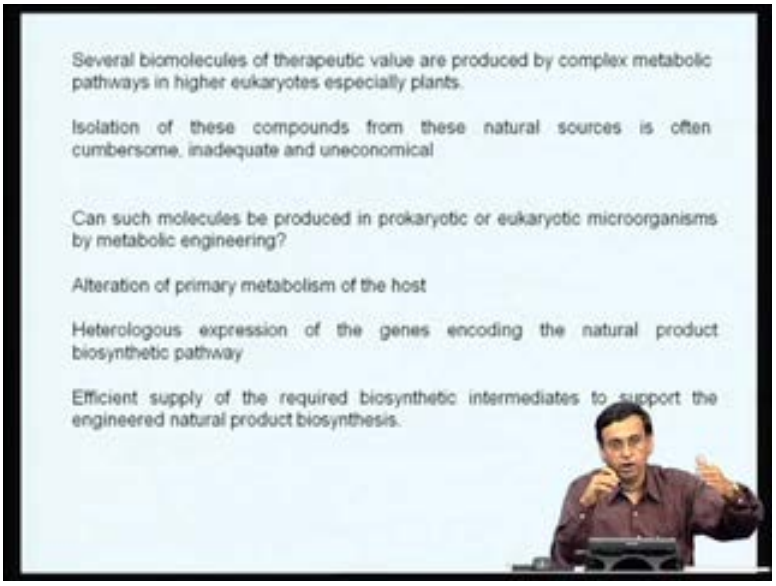

Several biomolecules of therapeutic value are produced by complex metabolic pathways in higher eukaryotes, especially plants. The medicinal plants produce a number of compounds of medicinal value and they also produce a number of other exotic compounds like flavonoids which give like aromatic compounds, aromatic plants which all have a lot of commercial value, so not only plants are products of medical importance, they also products of commercial importance which are of non-medical in nature, but, the major problem is isolation of these compounds from these natural resources is often cumbersome, inadequate, and uneconomical.

Most of the times, these compounds which these plants produce are often known as secondary metabolites and they are often produced in small amounts so if you really want these compounds in large amounts it becomes very, very difficult to isolate this compound.

We have to really kill a large number of plants and extract these compounds from these natural sources, which becomes uneconomical and often the demand of such products is very high. Therefore, what you isolate from the natural sources is not adequate and the process is very cumbersome.

So, that is why, you need to find out alternate ways by which these commercially important compounds or compounds of commercial importance can be generated from alternate sources. So, the challenge in metabolic engineering is can such molecules

which are produced in very small amounts in some of these plants or in fungal organisms can they be produced in other prokaryotic or eukaryotic microorganisms by metabolic engineering. That is the challenge that we are addressing now.

So, if you have to do these things or many a times what you have to do is you have to alter the primary metabolism of the host. Many at times, you find that the plant which is synthesizing this molecule probably has a primary metabolic pathway, which is common to many organisms, but at some point during this metabolic pathway, it branches out leading to the production of specific secondary metabolites.

So, if you want to now produce these secondary metabolites in organism that normally do not produce, you have now alter this primary metabolism of that host organism in such a way that instead of producing a compound which the organism normally produce you have to divert the pathway in a different direction so that the compound of your interest can be produced.

So, you may have to alter the primary metabolism of the host, heterologous expression of genes encoding natural product biosynthetic pathway which is because many of the enzymes which are required for making that particular compound is missing in this host organism. so, you may have to introduce these genes so that those enzymes can be now made, and then they divert the metabolic pathway in a direction that you want and efficient supply of required biosynthetic intermediates to support the engineered natural product synthesis. Many a times, the intermediates which are normally produced in that particular plant may not be produced here, so often you may have to supplement such intermediates to these organisms.

So, that now these intermediates now go and join in this metabolic pathway and along with the endogenous compounds can now produce the compound of your interest. There are a number of challenges in doing metabolic engineering. So, it is not simply taking genes for about 4 or 5 enzymes in a pathway, just put together, and you get your product. It is lot more complicated than just expressing a bunch of genes.

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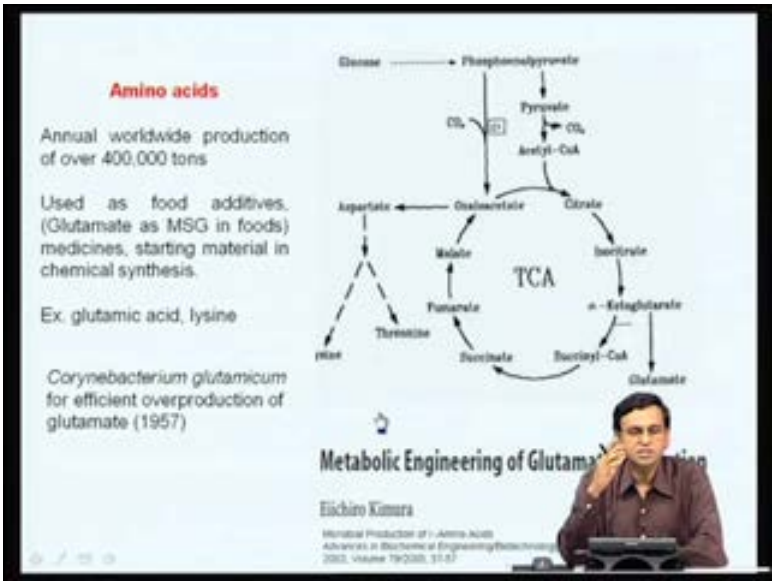

Metabolic engineering is not new. People have been exploiting microorganisms especially bacteria, yeast, and fungi for producing a number of compounds that have either medical or other non-medical commercial use. For example, one of the very successful compounds which have been produced in large amounts by pathway engineering or metabolic engineering is production of amino acids.

Annual worldwide production of amino acids is about 400,000 tons; just imagine the market for these amino acids. Especially, two of the amino acids glutamate and lysine are one of the most abundantly produced microorganisms using metabolic engineering or pathway engineering. Glutamate, for example, glutamic acid is actually used as preservative in foods called as monosodium glutamate and it is also used in medicines.Many a times, these amino acids are starting materials for synthesizing a number of other commercially important compounds.

They become the starting material for making a number of other compounds.One of the most well-characterized amino acid biosynthetic pathways, which are commercially exploited, is that of Corynebacterium glutamicum.As early as1957, this microorganism has been exploited for the overproduction of glutamic acid.

There is a very nice review on metabolic engineering of glutamate production. I will not really go in detail because this is primarily based on bacteria.I would like to go to some of the recent developments in the area of metabolic engineering. so, I have just given

here for example glutamate actually is produced as a byproduct of the Kreb cycle where alpha-ketoglutarate is a ketoacid can be converted into glutamate by transamination reactions, so if you want the organisms to produce glutamatein large amounts, you can actually introduce certain genes or tinker with certain enzymes of this pathway. So, that now Kreb cycle is required for energy, soalong with the generation of the energy, you should also be able to produce sufficient amount of glutamate so a lot of engineering is done to make sure that while running Kreb cycle, the organism can also produce a large amount of this glutamate.

So, this is an excellent review which actually tells you the historical aspects of how microorganisms were modified and exploited for producing large amounts of glutamic acid. The other example is citric acid.

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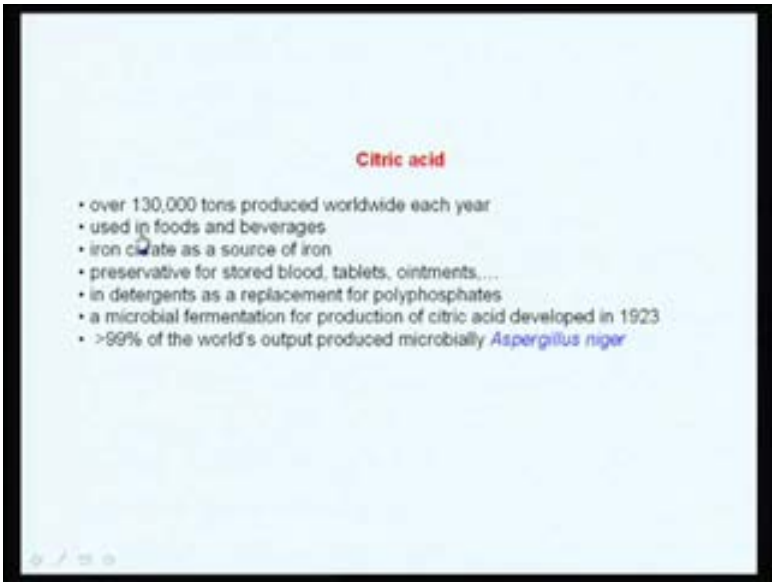

Over 130,000 tons of citric acid is produced worldwide every year and citric acid is used in foods, beverages, and iron citrate is a source of iron. It is a preservative for stored blood, tablets, and ointments, and it is also used in detergents as replacements of polyphosphate. Microbial fermentation for production of citric acid was developed as early as 1923 and 99% of the world is output is actually produced using microorganism. Aspergillus niger which is a fungus. so, you can see bacteria as well as fungi have been exploited for producing some of these very important molecules like glutamic acid, lysine, or citric acid and so on and so forth.

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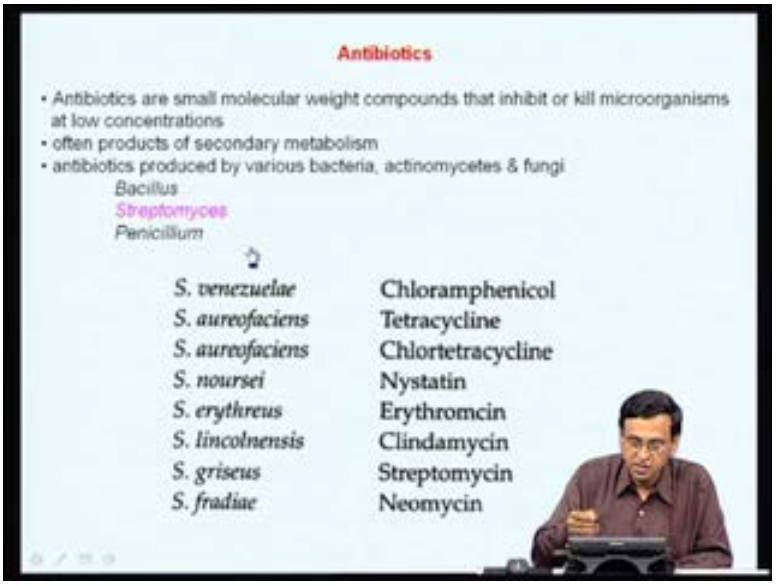

Antibiotics again are a fantastic example. Antibiotics are small molecules compounds that inhibit or kill microorganisms at very low concentrations, and once you demonstrate that a particular fungus is producing a particular antibioticor particular microorganism producing an antibiotic, then you do what is called as a metabolic engineering or a pathway engineering to see how we can force that organism to produce large amounts of this antibiotic, or once you understand how this antibiotic is being produced by the organism. you can actually take these genes or enzymes and express the genesin heterologous organisms.

So, that this organism can now produce this antibiotic in large amounts. So, a lot of metabolic engineering has been done to exploit enzymes of this pathway leading to production of high levels of antibiotics.

These antibiotics are products of secondary metabolism and antibiotics are produced by various bacteria, actinomycetes, and fungi like bacillus, streptomyces, penicillium, and so on and so forth. For example, just from streptomyces alone you can see so many number of antibiotics are being produced, chloramphenicol, tetracycline, chlortetracycline, nystatin, erythromycin, clindamycin, streptomycin, and neomycin.

So, from different species of streptomyces, a number of these antibiotics are produced. In all these cases, general principle is that once you know that a particular bacterium or a particular fungus is producing a particular antibiotic, but not at a concentration that you

would like, then you do biochemical engineering or a pathway engineering to see how we can exploit this pathway so that this particular antibiotic is produced in large amounts.

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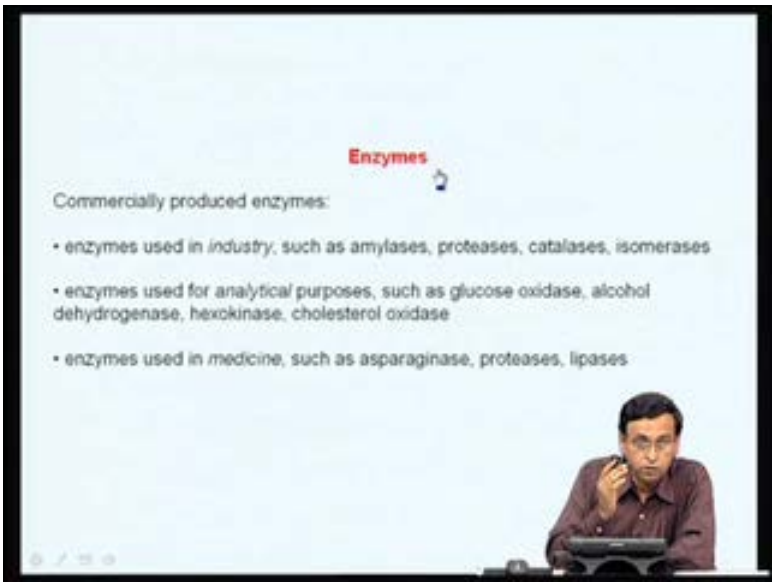

In addition to amino acids and antibiotics, a number of enzymes of commercial importance are also produced using microorganisms. Many enzymes are used in industries, which include amylases, proteases, catalases, and isomerases. They are all have lot of commercial value.

Enzymes are used for analytical purposes such as glucose oxidase which is extensively used in glucometers for determining blood glucose, alcohol dehydrogenase, hexokinase, cholesterol oxidase all of them again have lot of commercial importance so you need microorganisms which can produce these enzymes in large amounts.

Many enzymes are used in medicines such as asparaginase, proteases, lipases, and so on so forth. All these can be produced, overproduced, or produced in commercially viable proportions by metabolic engineering.

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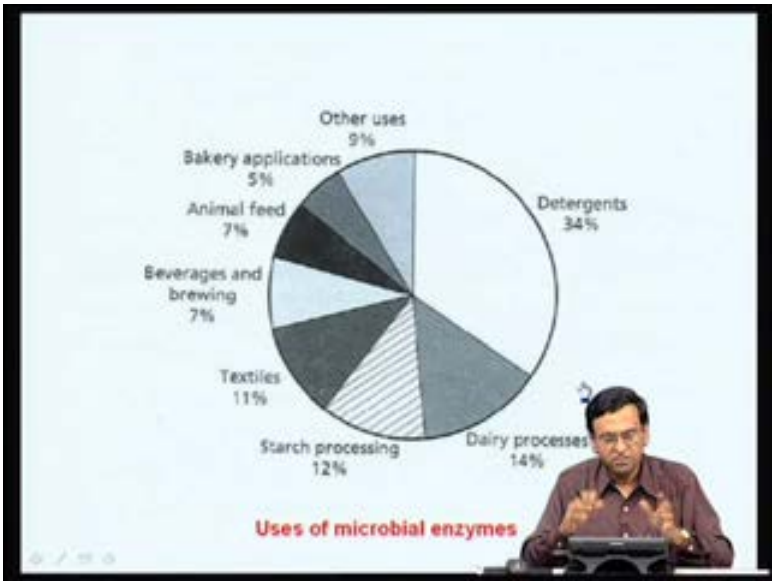

This just tells you how microbial enzymes have tremendous commercial applications,34 percent of the microbial enzymes are used in detergents, lipases and proteases to remove the dirt in your clothes, 14 percent are used in dairy processes for making curd and for making cheese and so on and so forth.

Starch processing, textile industry, beverages and brewing, animal feed, bakeries, and number of other applications, so producing enzymes by manipulating metabolic pathway so that these enzymes are over produced in large amounts is one of the major commercial activities and metabolic engineering has been a very very important player in this area.

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So, just a list of the various enzymes and their applications, bacterial proteases are being used in detergents, asparaginase is an antitumor agent, glucoamyalases and bacterial amylases can be used in fructose syrup production, starch liquefaction, brewing, baking, etc. Glucose isomerase is used in sweeteners. Rennin is used in cheese manufacture. Pectinase is used to clarify fruit juices. Lipaseis used for cheese production. Penicillin acylase, semisynthetic penicillin, tag polymerase of coursemost of you know is actually used in polymerase chain reaction.

So, you can see producing these enzymes in commercially viable amounts has tremendous applications, either you can use a recombinant route or actually what is called as a strain improvement or strain modification you can engineer these strains to over produce these enzymes so that you can isolate these enzymes either from the native organism or in the recombinant form.

Having looked at the importance of metabolic engineering and understood what kind of molecules have been produced by metabolic engineering for a number of years right from 1930, let us now try to understand how doyou go about doing metabolic engineering. Suppose you want to produce a molecule of your interest by metabolic engineering in prokaryotes or eukaryotes, how do you go about Now, you can do metabolic engineering in prokaryotes as well as eukaryotes, in fact the Escherichia coli,

Bacillus subtilis, these organisms have been extensively exploited for metabolic engineering of number of compounds of commercial importance.

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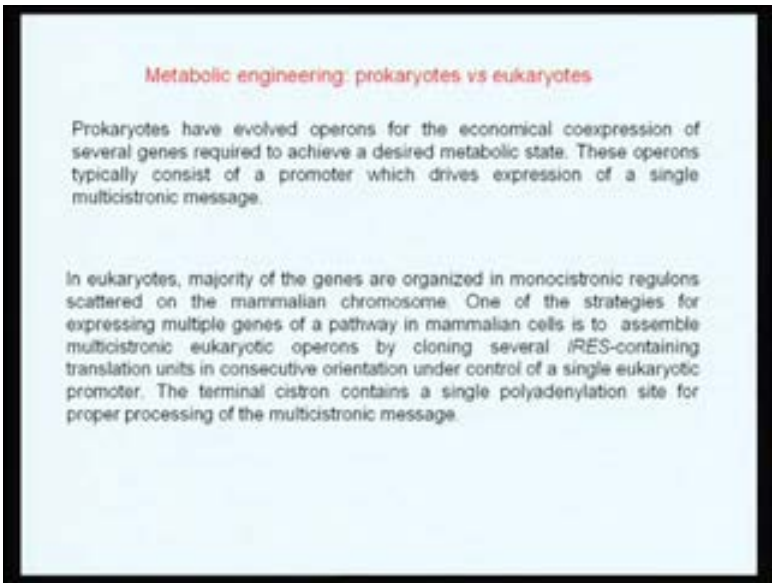

The ease or the advantage of using prokaryotic host for metabolic engineering is that prokaryotes have evolved operons, which we studied in the first lecture of this series.

Prokaryotes have evolved operons for the economical coexpression of several genes required to achieve a desired metabolic state. So you already know for example you have lac operon, trp operon, so on and so forth.

The prokaryotes are already tuned to expressing all the genes required, which code for enzymes of a particular metabolic pathway, so if you want to now over express enzymes of particular metabolic pathway, all that you have to do you have to take the genes code of this particular pathway, put them in operon, and see whether you can get one single polycistronic messenger RNA and all the enzymes can be made in one shot.

So, because genes coding for metabolic enzymes are grouped into operons, it is often possible to engineer metabolic pathways much more easily in prokaryotes because by manipulating a single operon you can produce all the enzymes of a particular pathway. So, these operons as we all know typically consist of a promoter, which does the expression of a single multicistronic message, which we studied long back about how lac

operon, trp operon, and so on they all work. However, when you come to eukaryotes, there is no operon concept in the eukaryotes.

Majority of the genes are organized in monocistronic regulons scattered across the mammalian chromosome, so if you have 5 enzymes that are required in a particular metabolic pathway the gene coding for each of the enzyme may be present in a different chromosome, so unlike in prokaryotes where all of them are present in a (( )) arrangement under the coordinate regulation of a single operon. So, One of the strategies for expressing multiple genes of a pathway in mammalian cells is to assemble a multicistronic eukaryotic operon by cloning several internal ribosome entry sites containing translation units in consecutive orientation under the control of a single eukaryotic promoter.

Now when we talked about eukaryote expression plasmid, we discussed extensively about the use of internal ribosome entry sites, how it promotes cap independent translation initiation and how by using internal ribosome entry sites you can express multiple genes under a single promoter, so by creating vectors containing such internal ribosome entry sites, one can often produce up to 3 to 6 proteins in a single plasmid or in a single regulon. The terminal cistron usually contains a single polyadenylation site for proper processing of this multi cistronic messenger.

So, by using this concept by generating this kind of artificial constructs containing a promoter gene on 1 followed by internal ribosome intercedes gene 2 internal ribosome intercedes gene 3 and so on and so forth. and the final cassette ultimately containing polyrelation signal one can produce a multi cistronic message in eukaryotic messenger R N A in eukaryotic cells which ultimately gets translated the first gene gets first R N A gets translated in a cap dependent fashion whereas, the subsequent R N A's in this multicistronic message get translated in a cap independent fashion and you can get all the proteins or enzymes of the particular pathway.

This is one strategy. Now, one of the, what kind of compound that you'd like to produce by metabolic engineering the compound that usually are produced in large amounts or you those which are actually produced normally in plants. Now the plants produce such useful compounds both as a result of for primary metabolism or as a result of secondary metabolism.

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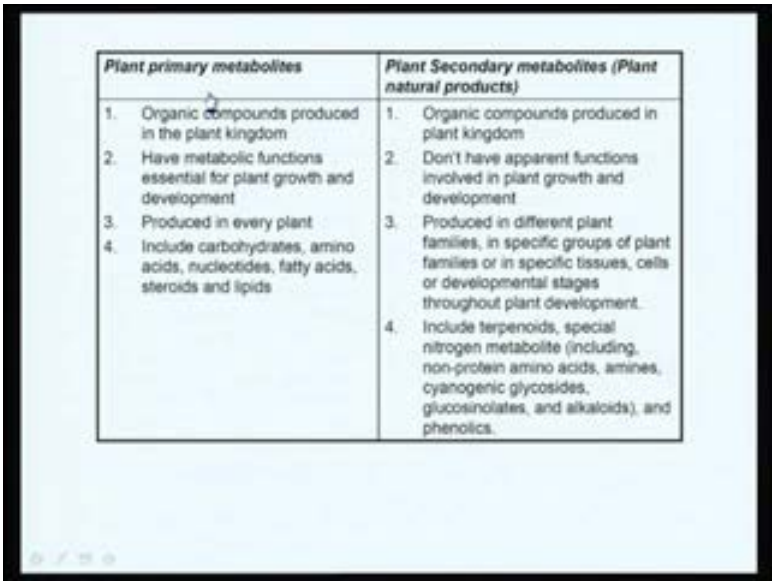

Now, what are the plant primary metabolites? These are organic compounds produced by the plants they've metabolic functions essential for the plant growth and development they are produced almost in every plant because these are primary metabolic pathways so they must be present is all the pathways and these include metabolic pathways involving carbohydrate metabolism of amino acid metabolism, nucleotides, fatty acid steroids and lipids.

These pathways are common almost all plants or even for animals kingdom. However the secondary metabolites are organic compounds they will also produce in a number of plants, but, they usually they do not have apparent functions involved in plant growth and development. They are produced in different plant families in specific groups of plants families or in specific tissues of a plant or either throughout the development or in specific stages of the development. Such organic such secondary metabolites include terpenoids special nitrogen metabolites such as amines.

Cyanogenic, glycosides glucosinolates, alkalidesphenolics and so on and so forth. So, you can see the secondary metabolites which are actually of great commercial importance are present not present in all the plants and they usually do not have a primary role or in primary metabolic pathways and they are only produce the hand full of plants and even a plant they may produce only certain tissues or certain cells.

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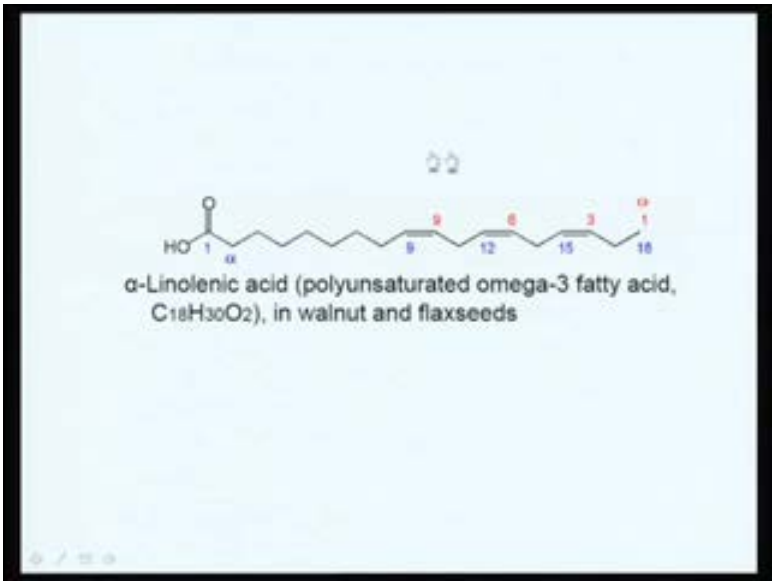

Some of the examples of such secondary metabolites which have a extensive commercial importance are for example, alpha linolenic acid which is a pufa or a polyunsaturated fatty acid which is actually present in walnut and flaxseeds. Polyunsaturated acids plays a very important role for human nutrition especially for the normal development of a child you required this polyunsaturated fatty acid.

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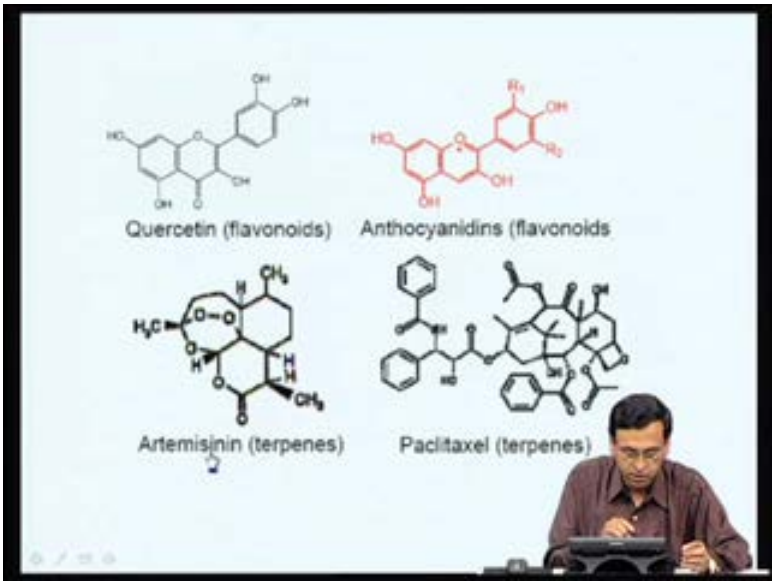

Flavonoids, terpenes by such as paclitaxel artemisinin is a very important anti-malarial compound paclitaxel is a precursor of a taxol which is a very important anti-cancer

compound you have flavonoids which are used in R M I industry. These are secondary metabolites produced by selected group of plants, but, not produce the all the plants.

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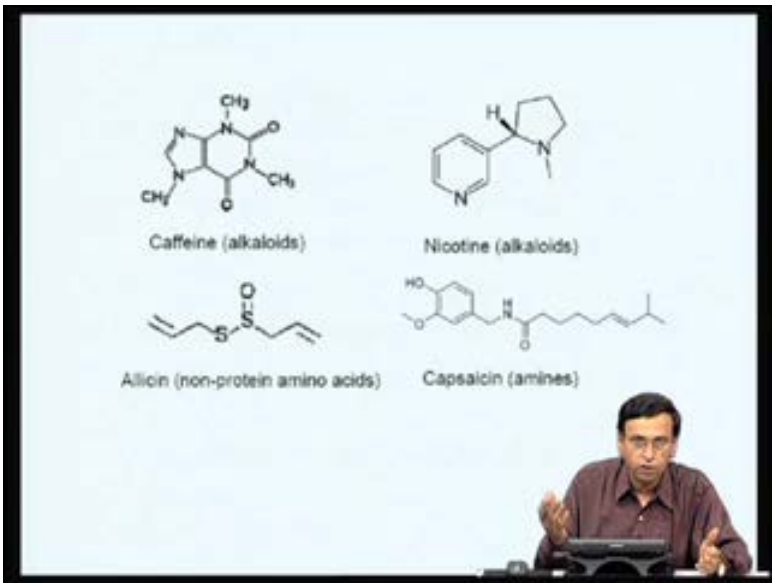

Caffeine for example, is a alkaline again nicotine produced by nicotine and tobacco alicion which is a non-protein amino acid capsaicin produced by capsicum these are all examples of secondary metabolites which have tremendous commercial importance, but, is not produced by all the plants.

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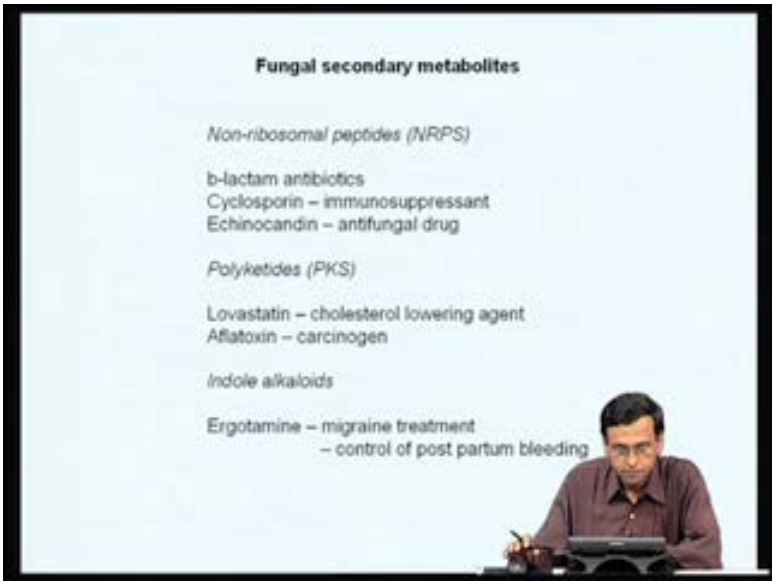

Just as the plants produce many of the secondary metabolites which have important commercial value many fungi also produced number of secondary metabolites like non-ribosome peptides which have lot of medical importance, beta-lactam antibiotics, cyclosporine, immunosuppressant, echnocandin and antifungal drug polykitides lovastatin a important cholesterol lowering drug, aflotoxin ergotamine are used in migraine treatment and so on and so forth. So not only plants even the fungi have the secondary metabolites metabolic pathways and these secondary metabolites which are produce by plants or fungi have a enormous commercial importance.

Now, has I said once we understand what kind of compounds are being produced by the primary metabolism secondary metabolism and these secondary metabolites produced by plants or fungi the question comes what you have to do metabolic engineering? As I explained above all of these plants or fungi producing these compounds these compounds are usually not produced in a commercial viable prepositions so if want to make kilogram amounts or tons of these compounds these natural sources are inadequate.

So, unless you do metabolic engineering, unless you make this organism to over produce this molecules, or unless you transfer these secondary metabolic pathways to a heterologous organism so the this organism now can produce this kind of metabolism in large amounts this will not be a commercial viable proportion. That is way the metabolic engineering becomes very, very important. And as I said many of these compounds are produced by complex metabolic pathways involving several enzymes and therefore,

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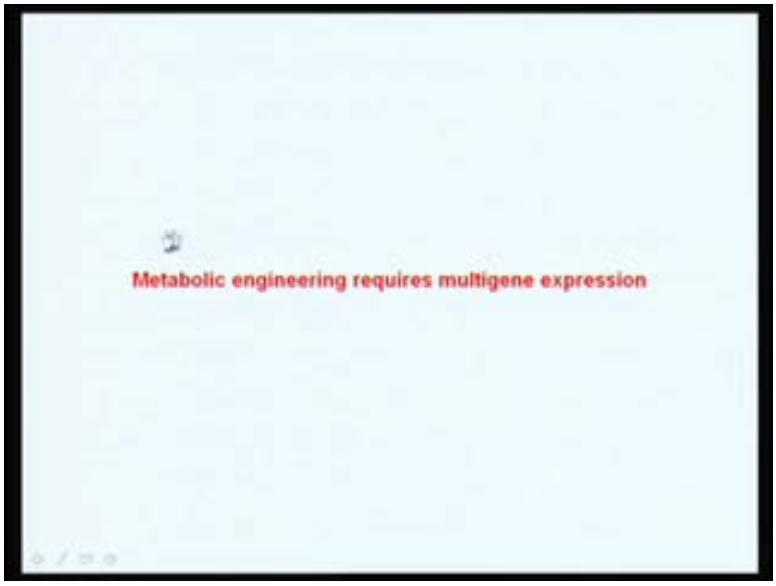

If we have to do metabolic engineering you have to do what is called as a multi gene expression just by expressing one gene you cannot get say secondary metabolites of your interest often a at least half a dozen to one dozen enzymes have to be over expressed or has to be engineered so that you get do you can alter the metabolic pathways and over produce the compound of your interest

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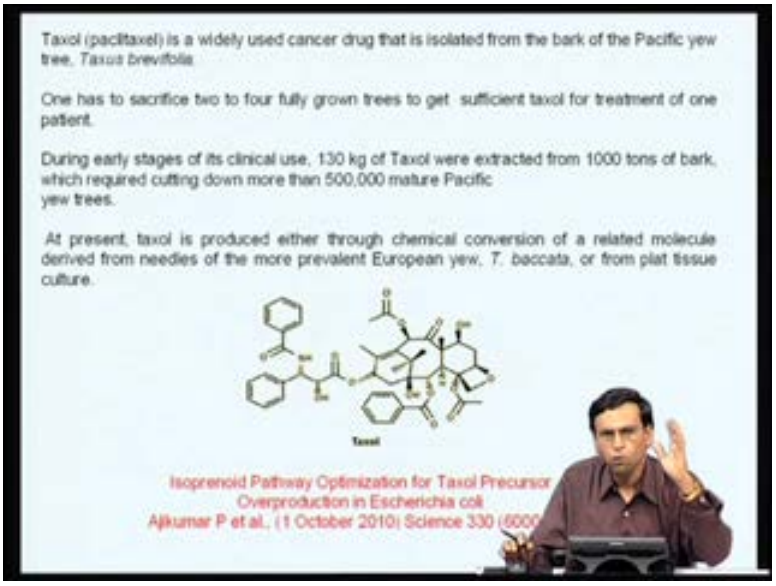

So, I will give one particular example taxcol now, taxol is a widely used anti-cancer drug that is usually isolate from the bark of pacific yew tree called taxes brevefolia. Now let

us see the the logistics here now one has to sacrifice 2 to 4 fully grown trees to get sufficient taxol for the treatment of just one patient.

Just imagine although taxol is an anti-cancer compound if you want to treat one patient with taxol you have to cut 2 to 4 fully grown trees and then isolate this compound from this bark in fact during the early stages of its clinical use 130 k g of taxol was extracted from 1000 tons of the bark which recurred cutting down more than 500000 mature trees so you can see what an ecological disaster it is because you cannot just now suppose you have we have global needs it is impossible for you to make taxol by going on cutting trees like this and isolating this compound form the bark. Whereas, at present nobody is cutting these trees to make taxol any more at present taxol is being produced either through chemical conversion of a related molecule derived from the needles of a more prevalent european yew that is eh taxes baccata or by plant tissue culture. So there are alternate methods now to produce this taxol in large amounts and you have do not have to go and cut these trees in large scale.

I want now just spend some time to explain a very recent paper that actually came in science just last month in october 2010 about how you can engineering this taxol by synthetic pathway to produce a taxol precursor using E. coli so initially when once taxol was discovered as a anti-cancer compound it was being isolated from the bark of this huge trees so have to cut down large number of trees, but, then people went and then started producing other fungi or they started producing by plant tissue culture.

But, now people are trying to see whether microorganisms can produce taxol can you engineer this taxol by synthetic pathway into microorganism like E. coli e r e's so that this microorganisms can now produce taxol in large amounts and is a recent paper so i'll just spend some time explaining what is the highlights of this particular paper. Now, as I said if you have to understand how metabolic engineering can be done for taxol we need to very briefly understand how taxol is biosynthesized.

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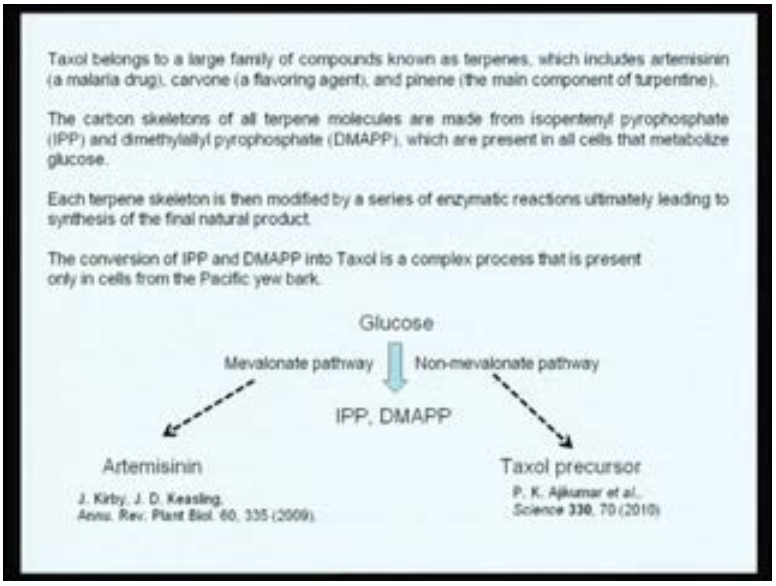

Now, taxol belongs to a large family of compound known as terepenes which include artimisinin which is a proponent anti-malarial carvone a flavoring agent pinyin it is a main component of turpentine.

So, all these very, very commercial important terpenes are all produced from a common metabolic pathway. Now, the carbons skeletons of all these terepenes molecules are made form a compound called isopentenyl pyrophosphate and dimethylallyl pyrophosphate I P P and D M A P P. So, these are common precursor for a synthase of not only taxol, but, also many other commercial important compounds like artemisinin carbon or pineine and so on and so forth.

Each terepene up to a up to a up to a certain steps the pathway is common for the production of all these terepenes after than they branch out and these different branches now produce different kind of this terepenes. Each terepene skeleton is then modified by a series of a enzymatic reactions ultimately leading to this of the final natural product. Now the conversion of this I p p and d m a p p into taxol is a very complex process that is present in the cells of only in that particular tree that is the specific yew bark.

That is why only this particular tree produces taxol now let us understand and see how you can now engineering this pathway or taxol production into a non-producing organism like E. coli or yeast. So, to put in nutshell glucose as we know is the carbon source form all organisms and this glucose during normal metabolism is converted into

this I p p or d m a p p in most of the organisms there are 2 pathways by which this glucose is converted into I p p or d m a p p one is called as a mevalonate pathway another one is called as a non mevalonate pathway now what I am going tell you in the next few ah minutes is to see how you can engineer or how you can tinker with both these mevalonate pathway and non mevalonate pathway so that you can now make microorganisms produce either taxol or artemisinin in large amounts that is what is the so let us now spend some time to understand how this particular group ajitkumaretal in the just about one month back.

Have engineered in equalize strained so that it can now start producing a taxol precursor using this particular pathway. So, what exactly this people have done let us now understand what the the reality and these people have achieved.

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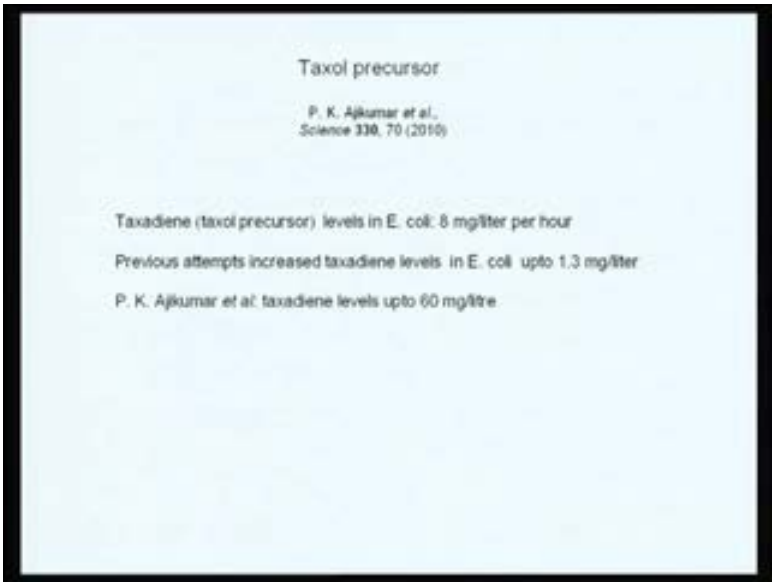

Now, the precursor for taxol is called taxodine usually nuclei. So, for the maximum that what could achieved by metabolic engineering is about 8 milligram per liter per hour that is the level that they could produce. In this particular paper these people have been able to demonstrate that by doing metabolic engineering they can actually produce the do 60 milligram per liter of this taxol so several fold increase has been done will lets now just understand how this was done.

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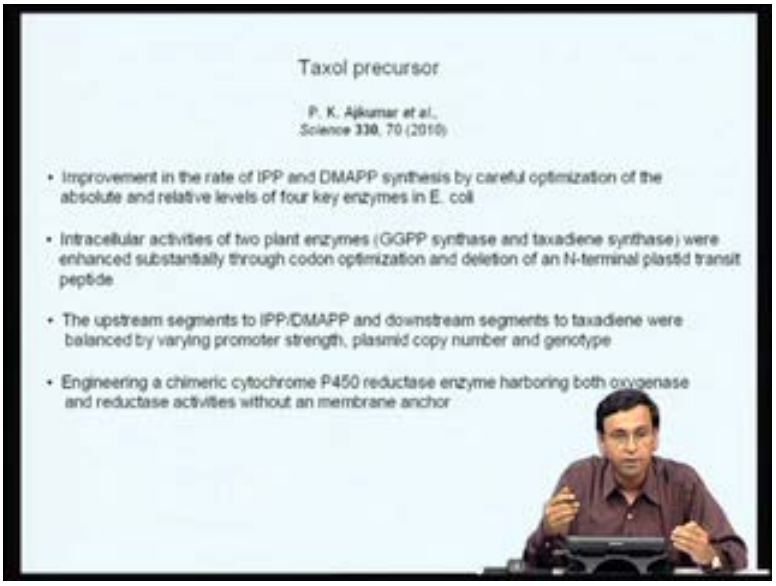

This was done by a number of series of very very innovative steps first what they did is that improvement in the rate of I p p and d m a p p in the synthesis by careful optimization of the absolute and relative level of this 4 key enzymes in E. coli.

As I told you when we do this metabolic engineering or when you do this pathway engineering one has to be extremely careful about the levels at which various intermediates in pathways accumulating. So, if you randomly over express all the enzymes of the pathway ultimately you'll not get the result because you require what is called as a metabolic flux many of this intermediates are retained in specific ratios in order of this pathway to drive in a particular direction.

So, what this people actually did is that they actually carefully optimized by using of appropriate promoters they make sure 4 of the key enzymes pathways is expressed in appropriate levels that will drive the reaction in a favorable direction. The intracellular activity of 2 plant enzymes called g g g p synthase and taxadiene syntheses were enhanced substantially by codon optimization and deletion of an n terminal plasma transit peptide. So, is typical genetic engineering.

So, they optimize this codon of this so that you use codon which have very highly express in E. coli so that E. coli optimize codon were used for making artificial genes so that they can be expressed in high amounts. The upstream segments to the I p p d m a p p and down streams segments taxodine were balanced by varying the promoters strength

plasmid copy number and genotype. As I said you cannot put the same promoter and same regulatory elements for all the genes of the pathway depending upon the need if you know for the example enzyme one is required in high levels and enzyme 2 is required lower levels you need to put the appropriate promoter so that this kind of a ratio is maintained so all these things were taken care and of coarsely the last thing is engineering a chimeric cytochrome p450 reductase enzyme harboring both oxygenase and reductase activity without membrane anchor. Usually these cytochrome p450 is reductases you they are membrane proteins without a membrane anchor they'll not function, but, they have been able to engineer a cytochrome p450 reductase enzyme which has both oxygenase and reductase activity even without such membrane anchor.

So, I can see a number of modifications were done to their expression system and by doing all these things they were able to engineer and E. coli which now produces very high levels of a precursor for taxol and this precursor then chemically reconverted into taxol.

So many of these things are called are the semi synthesis of taxol that means you have not engineered the entire bio synthetic pathway but, up to a point you produce an organism and once the precursor comes by just another 2 or 3 steps you can make the final product so it is called has semi synthetic method.

So, this is how these reasons study has actually shown that by modify by taking care of this parameters that I just explained one can now make a eh bacterium like E. coli to produce a taxol precursor in very very high amounts and these precursor can then be converted into taxol by chemical means.

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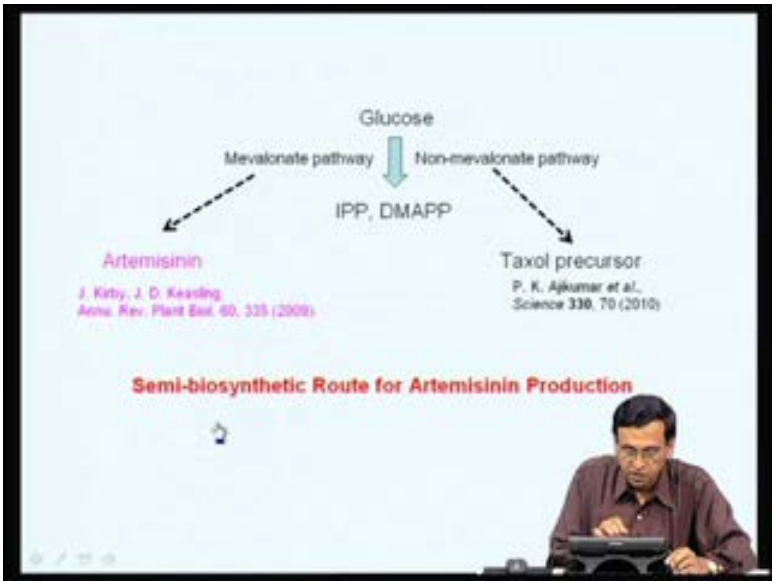

Let us now spend some time to understand another very important paper that again came just last year about how yeast cells actually engineered produce artimisinin is again is a very potent anti malarial and is in a very high demand in fact one of the most potent anti-malarial known today. So, how did people engineer semi synthetic route for artemisinin production? That is what will going to discuss in couple of minutes.

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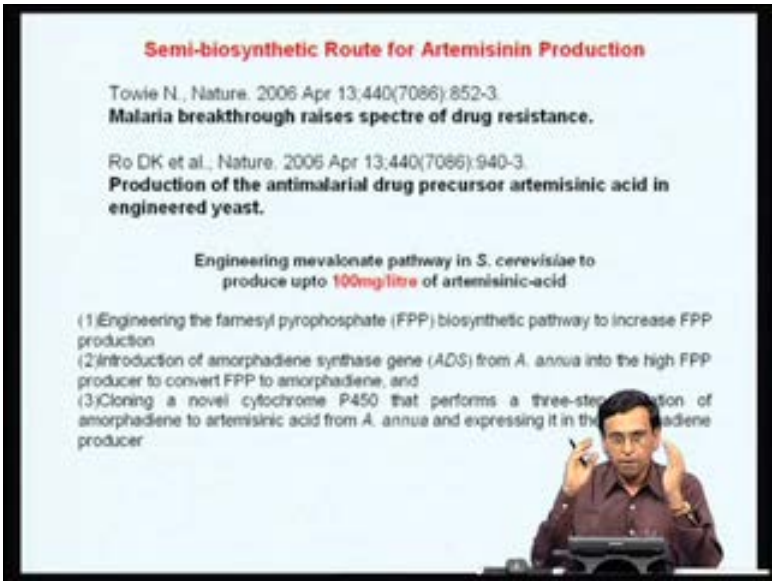

The 2 papers which show made a created lot of noise or which attracted lot of attention among the researcher is the one that published in 2006 in nature entitled a production of

anti-malarial drug precursor optimistic acid in engineered yeast. So, yeast cells were actually engineered to produce artemisinic acid in large amounts and these artemisinic acid another 2 or 3 steps can be converted the final artimisinin by chemical rights. And there is a very very nice news and views in nature the that particular issues saying malaria break through raises spectre of drug resistance.

What they actually did is that there is a primary metabolic pathway in almost all organisms sarcomas called as mevalonate pathway this actually produces mevalonic acid and is actually involved in cholesterol by synthesis and this pathway is common for all organisms. So, what these people actually did is that they engineered this mevalonate pathway in this saccharomyces cerevisiae in such a way that after a certain step this mevalonate pathway branches of and instead of producing the normal end products which are seen in the saccharomyces cerevisiae it now produce a artemisnic acid.

So, what did they do I am just going to highlight I am not going to give all the details because pretty complex paper and one has to spend lot of time to understand how a exactly they did, but, the purpose is not to confuse you just to give you the highlight so that you can appreciate how metabolic engineering is done. What these people did first they did is they engineered the farnesyl phosphate bio syntactic pathway to increase farnesyl phosphate pyrophosphate production this is one of the one of the very important intermediates in the synthesis of this artemisinin.

Then they introduce what is called has a amrophedene synthesis gene from artemisia annua now this is the plant form which artemisnic normally produced which is actually chinese herb which is grown only in specific geographical region and you this was first discovered has an anti-malarial by chinese and is present in this chinese herb. So, they took this gene from the artimisica annua which is gene called amrophedene synthase gene into the high f p p producer so that this f p p can now be converted into amorphadiene.

So, the enzyme amorphadiene synthase which is not normally percent in saccharomyces ,but, which is percent only in artemisinin artemisia annua has been now engineered into the saccharomyces cerevisiae so when you now express this enzyme f p p now gets converted into amorphadiene. Next they cloned a novel cytochrome p 4 fifty that performs a 3 step oxidation of the amorphadiene into artemisinic acid from annua and

expressing it in the amorphadiene producer. So they took another cytochrome p 4 fifty enzyme from this artemisia annua so that you can now convert in a series of steps this amorphadiene can be now converted into artimisinic acid.

So, you can see first they produce the saccharomyces which can produce large amounts of this farnesyl ferrous phosphate then they took a gene which is normally present in the artemisia annua and put in the in the saccharomyces cerevisiae so that this f p p can converted into amorphadiene, then they put another enzyme so that this amorphadiene can converted into hot artemisnic acid.

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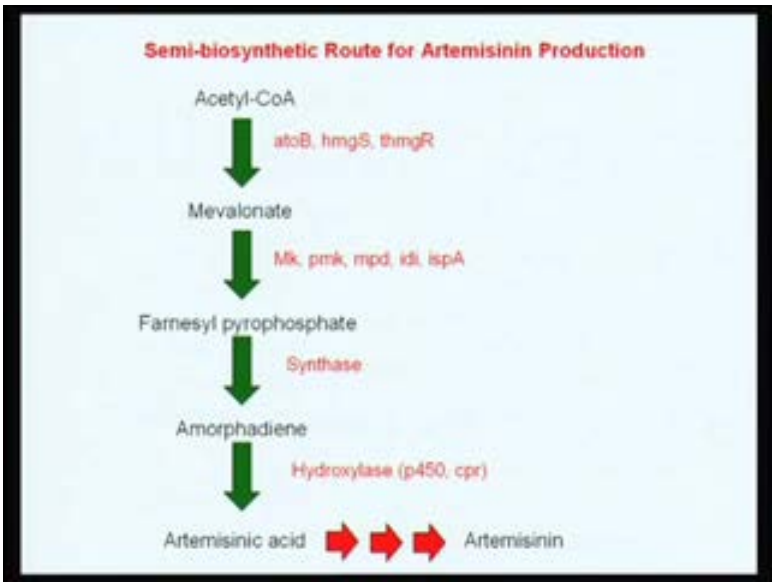

So, this is basically the juggling they did to make this artemisnin known as the semi synthetic bio bio syntactic artemisnic production this the normal pathway acetyl co a up to mavolante farnesyl phosphate is the primary metabolic pathway which is already present in saccharomyces cerevisiaea, what they did is to they tinkered here little bit so that this farnesyl phosphate can be produce in larger amounts then they introduced 2 enzymes from artimisia annua.

So, that farnesyl phosphate can now become converted in to artemisnic acid and a green arrows indicates biological synthesis, the red arrows indicates the chemical synthesis. So, once you have artemisnic acid produce in large amount saccharomyces cerevisiae a you isolate it and by about 2 or 3 steps in vitro in by using chemistry you can convert into final artemisnic.

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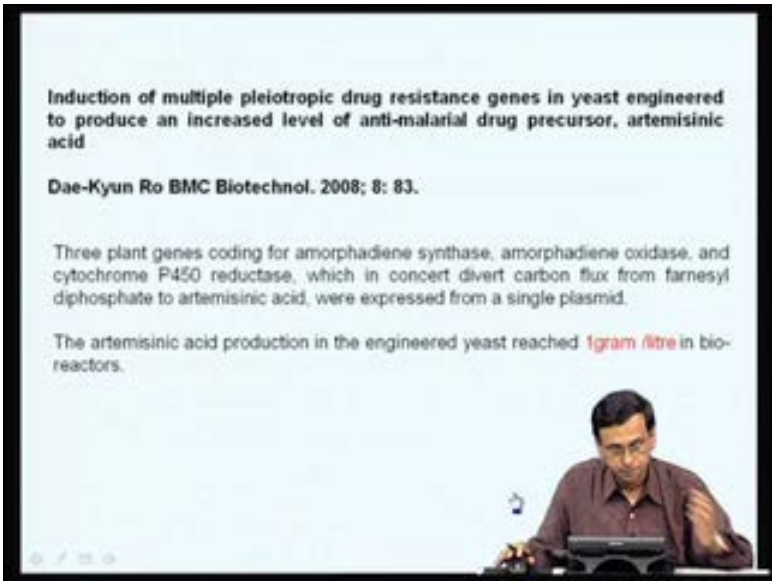

The in fact other 2008 these people have worked again on this organisms and finally, now in this in this particular paper they actually show that you can even produce up to one gram of artimisinin per liter by doing this kind of a bio engineering business.

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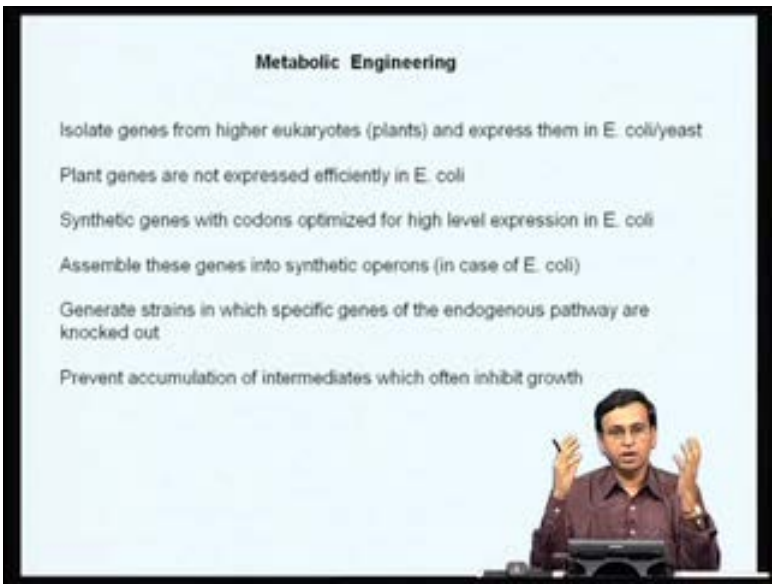

So, to put it in a nutshell what metabolic engineering basically is it involves isolating genes from the higher eukaryotes it can be a plant or it can be a fungus and then express them using appropriate promoters in your host organism of your choice it can be either E. coli or it can be yeast, but, since plant genes are not a very efficiently expressed in E.

coli you have to do what is called as codon optimization so you synthesize genes which contain codon that are very highly used in that particular organism for example, what is expressed in E. coli you optimize the codons for high level expression E. coli or use those codons which are highly expressed in yeast if you want to express in yeast in large amounts. So, synthetic genes with codon optimized for high level expression E. coli has to be used and then you assemble these genes into synthetic operons if you are if you engineering pathway E. coli generates strains with specific genes of the endogenous pathway or knocked out and prevent the accumulation of intermediates which often inhabit growth.

So, a serious of modifications has to be done so that this strain this bacterium or yeast now produces the compound of your choice. I want to just spend 2 minutes to explain this particular aspect which is very, very important fact is the one of the major bottle necks when you do metabolic engineering that is how to prevent accumulation of intermediates which are often growth inhibitory.

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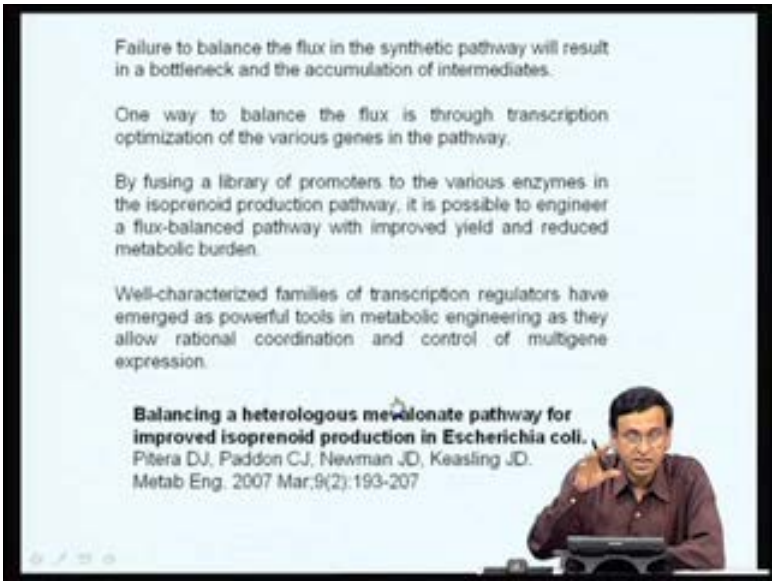

Now, as I said many of this metabolites which are many of the intermediates which are synthetize during these metabolic pathways they have to be present in appropriate amounts if you either produce the these intermediates accumulated either in low amounts or high amounts it is detrimental to the organisms, the organism may not grow at all so you can produce high amounts of your end product if the organism does not grow so you

have to do lot of what I called as the balance the flux balancing the flux of the synthetic pathway has to be very, very important and if you do not balance this flux these accumulates intermediates will accumulates inappropriate amounts and then your engineering will not be successful. So, one way to balance this flux is through transcription optimization of various genes of the pathway is what I told you, cannot.

If the same promoter for expressing all the genes of the enzymes of this pathway if a particular intermediate has to be present large amounts you have to use a strong promoter if a particular enzyme has to be express lower amount so you have to use a low promoter all these balancing has to be done so that this flux is maintained. Or by using a library of promoters to various enzymes and isoprenoid production pathway it is possible to engineer a flux balanced pathway with improved yield and reduce metabolic burden. So, this operon contain often should contain a combination of promoters and enhancers.

So, that you have to carry this flux is maintained in fact well characterized families of transcription regulators have emerged has very powerful tools in metabolic engineering they ask they allow rational coordination and control of this multiple gene expression so unless you understand how this various promoters were and how you can manipulate these various promoter it is not possible to metabolic engineering and that is why understanding this regulation of gene expression very very important you need to have a hand bunch of promoters in your hands some of them are expressed in high levels some of them expressed in low levels and by putting them all together one can do this pathway engineering and prevent this balancing the and you can balance the flux so that this intermediates do not accumulating in large amounts.

I think you can just recommending this particular paper balancing a heterologous mevalonate pathway for improve isoprenoid production E. coli came into (( )) keasling is one of the major authorities in metabolic engineering is a very nice review one can read and then try to understand how this is done. The other example which I will not discuss in detail is about producing ethanol in microorganisms as you, know today everybody is talking about non-conventional energy people are trying to see can you find alternates for petroleum so people are now trying to see can you now produce bio diesel, can you produce ethanol which all can have when we used as bio fuels can you produce from corn, can you produce from microorganisms like bacteria, can you produce from fungi so

one of the very attractive choices for metabolic engineering or producing ethanol is yeast.

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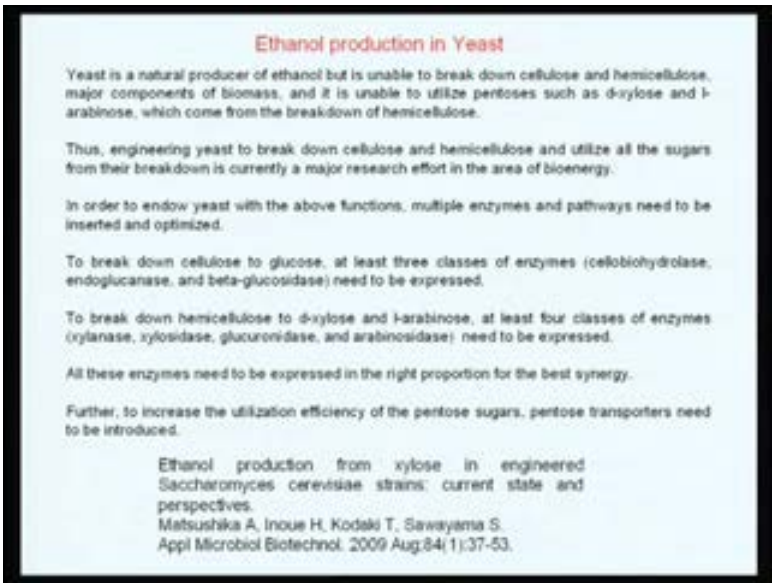

Why, because yeast is a natural producer of ethanol, but, it is unable to break down cellulose and hemi cellulose major components of the biomass. As you can see we have large number of plants now and to so have a large amount of waste material with all derived from cellulose and hemi cellulose which is actually called biomass, but, unfortunately, if you can now convert- somehow convert these cellulose by enzyme enzymatic redaction pathway ultimate to ethanol you have solved energy problem of the world. So, one of the major focus that is happening in the area of bio technology is to see how you can degrade this biomass, which is present in large amounts in the form of cellulose and hemicellulose and how you can break down these glucose from these cellulose and hemi cellulose and ultimately convert into ethanol.

So, although yeast as been known doing used for conversion of ethanol, yeast can only produce ethanol from glucose, but, it does not have the machinery to break down this cellulose and hemi cellulose. So, engineering is to breakdown cellulose and hemi cellulose and utilize all the sugars from there breakdown is currently a major research area in the area of bio energy. In order to endow yeast with these sub of functions multiple enzymes and pathways needs to be inserted and optimated it is not it is not just easy for example, I have saccharmyces cerevisiae which can only convert glucose to

ethanol, but, you want cellulose and hemi cellulose to be broken down to glucose and then the glucose to be converted into ethanol so you need to now introduce those enzymes which can breakdown cellulose and hemicelluloses can be converted into glucose the so that it can now then converted into ethanol.

For example, to breakdown cellulose you need to introduce all these genes into saccharomyces cerevisiae similarly, if you want to breakdown hemi cellulose to zylose or arabinose at least 4 class of enzymes are required zylenace zylosidase glucunosidase and Arabinosidase. So, you can see unless you engineer this pathway unless you introduce this cellulose or hemi cellulose degradation pathway into yeast like saccharmocyses cerevisiae you cannot break down cellulose or hemi cellulose and convert them into ethanol so a major area of research in the area of bio energy is focused on how to degrade this abundant biological waste so much of biomass is generated every year by the decay of this plant material and if you can somehow convert all these bio waste into ethanol the energy problem can be solved.

So, this is a I do not I will not go into the details of this I suggest that you read this very nice review ethanol production from xylose and engineered saccharomyces cerevisiae strains current state and perspectives which actually gives you a very modern perspective of what kind of efforts are actually going on to do metabolic engineering so that yeast like saccharomyces can engineered to breakdown cellulose and hemi cellulose so you can generate ethanol from these sources.

So, having emphasizing the importance of pathway engineering for producing useful molecules like taxol artemisinin ethanol and so on and so forth.

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I want to now spend another may be about another 5 10 minutes explaining another very, very important area of metabolic engineering namely engineering glycosylation pathways for the production of humanized glyco proteins another very fascinating area of research that is opening in the area of metabolic engineering.

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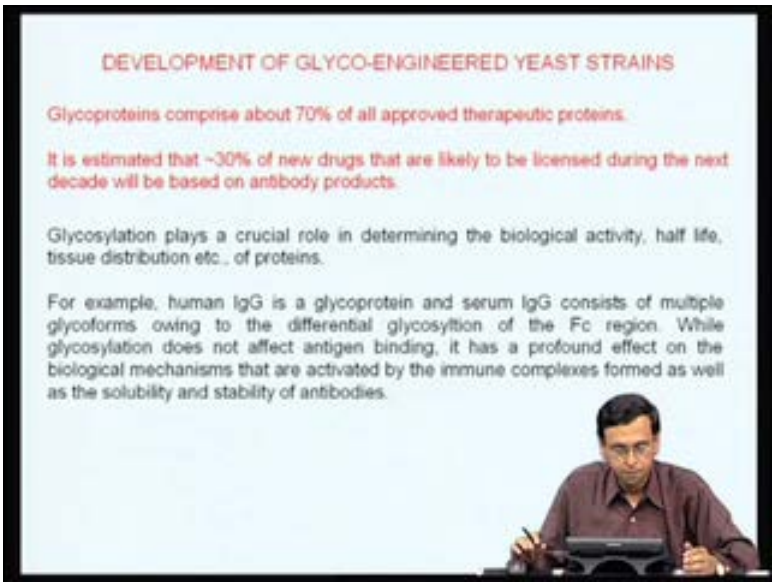

Now, let us for understand why you have to do this, what we are now going to discuss is how we can develop glyco engineered yeast strains. Now, let us understand importance of glyco proteins now glycol proteins that is these are proteins in which carbohydrate

moiety are attached glycol proteins comprises about 70percent of all approved therapeutic proteins you take many proteins like tissue plasmogenic activator many of these growth factors many of these cyto kinds they are all glyco proteins and is estimated that approximately thirty percent of the new drugs that are lightly to be licensed during the next decade will all be based on antibodies. Again antibodies are glycoproteins glycosylation plays a crucial role in determining biological activity half-life distribution of these proteins so if you have if you have a properly glycosylated protein you have hit the right thing because that is what you want human I g g.

If you take for example, it is a glycol protein and serum I g g consists of multiple glyco forms going to the differential glyco oscillation of the f c region of the antibody while glycosylation does not affect the antigen binding of the antibody molecule it has profound effect on the biological mechanisms that are activate by immune complexes formed as well as the solubility and stability of the antibodies.

So, although the glycosylation is not important for antigen and antibody resection it as very other important functions.

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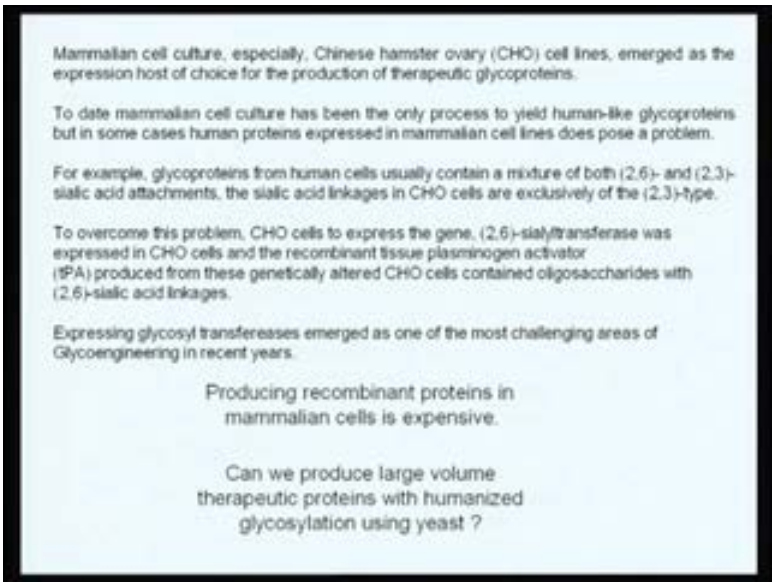

Now for producing this kind of a glyco proteins including antibodies the most preferred expression system as been the mammalian expression system mammalian cell culture especially the Chinese hamster ovary of the cho cells emerged as the expression host of choice for production of therapeutic glycol proteins. To date mammalian cell culture as

been the only process to yield human like glyco proteins, but, in some cases human proteins expect the mammalian cells and also process a problem, because the glycosylation pattern is different in different species it is even within within mammals it is different from humans to other mammals. For example, glyco proteins produce from human cells usually contain contain a mixture of both 2 6 and 2 3 siallic acid whereas, the siallic acid linkage is cho cells is exclusive of 2 3 type so you can see the way these siallic acid is used or added the protein moiety is different in cho cells versus in humans. So, to overcome these problems cho cells have been engineered to express this 2 6 sialyltranferase gene so if we now engineered introduce this 2 6 sialyltranferase gene so that cho cells alnow can do this.

So, that you can get a protein which as both 2 3 as well as 2 6 siallic acid linkages. So, expressing these glyco cell transverses that is the enzymes which add this carbohydrate moiety to proteins are called as glycol cell transverses expression of these glyco cell transverses is now as become one of the most important emerging areas of biotechnology and this is actually known as the glyco engineering, but, one of the problems in producing these kind of glyco proteins in mammalian systems is that, as I told you in long back mammalians expression systems are very very expensive maintaining cho cells and producing proteins in cho cells is very very expensive so question is can we produce this large proteins in a yeast can you do this kind of a human glycosylation of proteins in each system so that glycol proteins can be now produced in economical manner in yeast cells that is the challenge.

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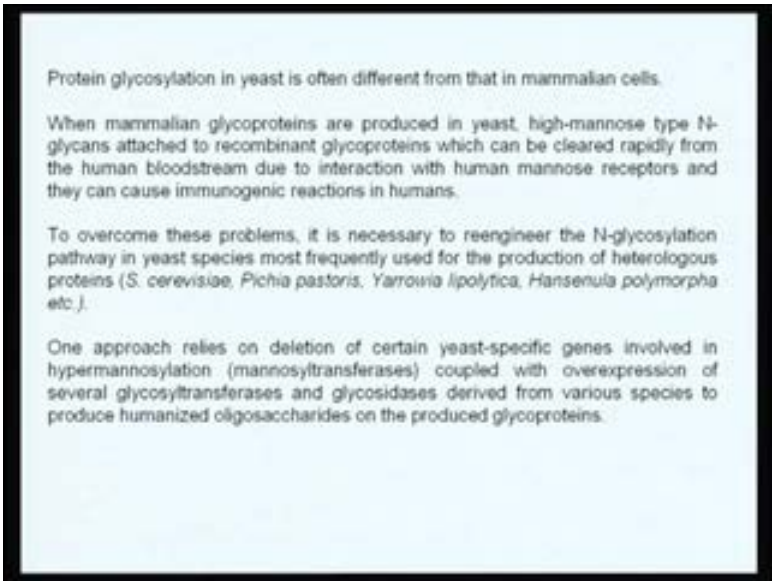

Protein glycosylation in yeast is also very different although yeast does glycosylation the glycosylation that happens in yeast is very different from mammalian cells so when mammalian glyco proteins are produced in yeast high mannose type n glycans are attached to the recombinant glycol proteins which can be cleared rapidly from the human blood stream due to interaction of the human mannose receptors and they can cause immunogenic reactions in humans. So, to overcome these problems it is necessary to re-engineer the glycosylation pathway in the yeast.

So, that it now produces glycosylation pattern which is very similar to that in humans. So, how do you do that one approach is to delete some yeast specific genes involved in hyper mannosylation that is delete certain genes coding for this mannosylation transverses and then introduce genes of other enzymes which are normally present in the humans. So, I am going to give one example to illustrate this point here is an example, as you know glycosylation takes place in 2 cellular compartments one is endoplasmic reticulum another is golgi.

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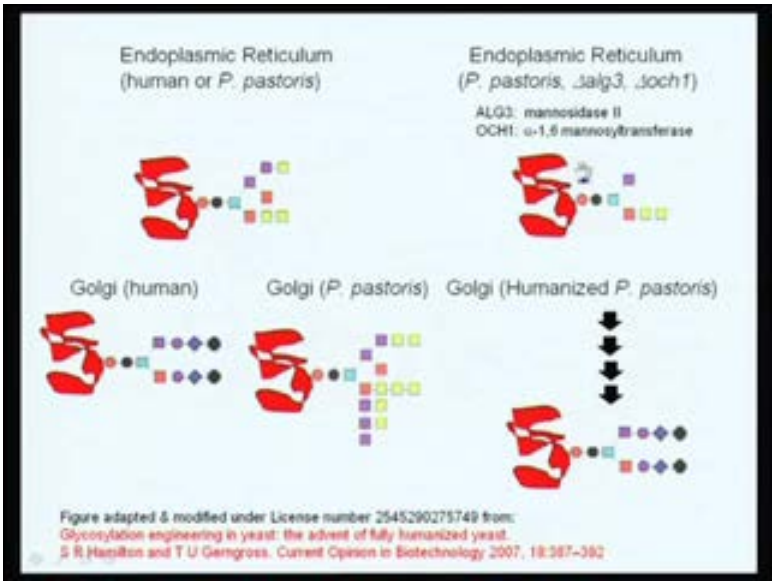

Now, when we look at the glycoproteins in a either humans or pastoris pichia pastoris which is the yeast in the endoplasmic reticulum the glycosylation pattern of the protein is more or less are the same the red here is the protein and this is the carbohydrate point that this different colors shows different carbohydrate molecules so in the endoplasmic reticulum where n glycosylation takes place the pattern is very similar to that of human and pastoris, but, after that when the protein enters the golgi compartment the final product that comes out of the golgi in human you can see the glycosylation is quite different from that of the pichia pastoris. So, although the glycosylation pattern is very similar in e endoplasmic reticulum after golgi the product that comes out in pichia is quite different from the human.

So, the challenge now is the glycoprotein that is produced in pichia pastoris is golgi should now look like the protein that is produced in the human golgi how do you do that this is what you do you delete 2 genes called as mannosidase 2 and one 6 mannosidase transverses in this pichia pastoris so in the endoplasmic reticulum you now end up with a glycosylation pattern like this rather than like this and you can see the glycosylation pattern is different here then you express a series of glycocell transverses which are normally will do human type of glycosylation.

So, that you now ultimately end up with a glycoprotein you can see now this looks very similar to that made in the golgi of human beings and not the same as that is being made

in the pichia pastoris. so by removing certain pichia pastoris vglyco transverses and by introducing certain new glyco transverses you have now ended up with you have now ended up with a glyco protein which now looks very similar to that made in the human golgi and then the yeast golgi.

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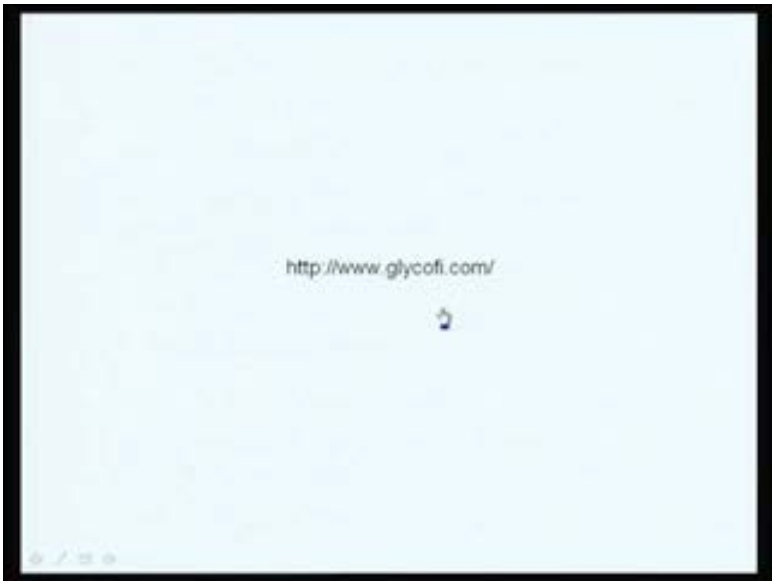

I suggest you read this very nice article glycosylation engineering in the yeast, the advent of fully humanized, is in current opinion bio technology which gives the entire details of how this metabolic engineering has been done there is actually a company called glycofi which as pioneered this kind of a engineering yeast cells for producing humanized glycosylated proteins and if you go to this company website you can understand much more about how this humanized glyco proteins can be made in yeast cells.

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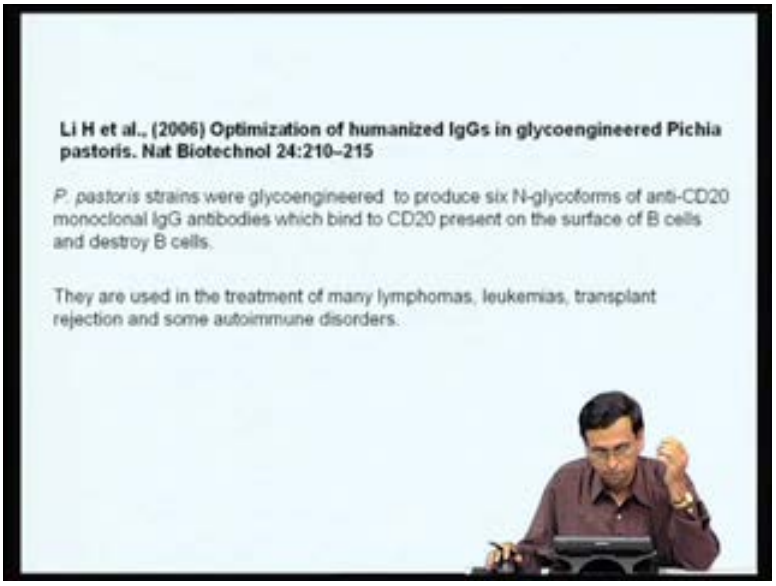

I think I will skip some of these things.

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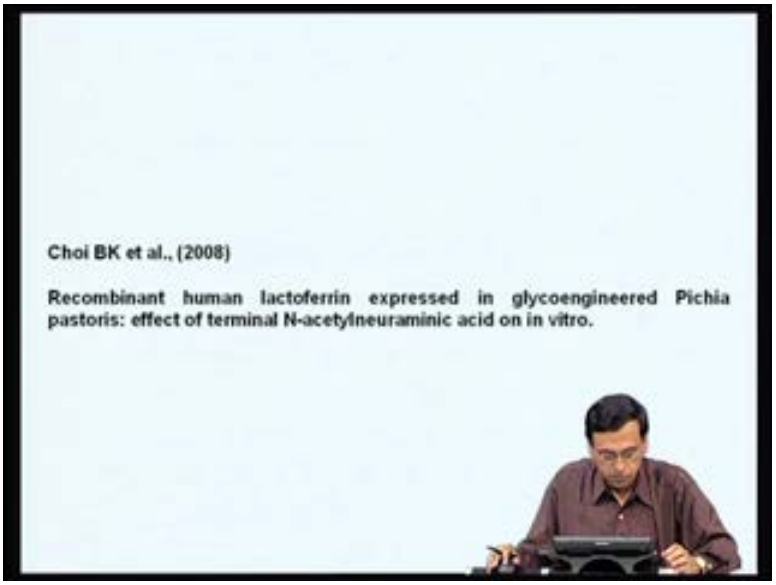

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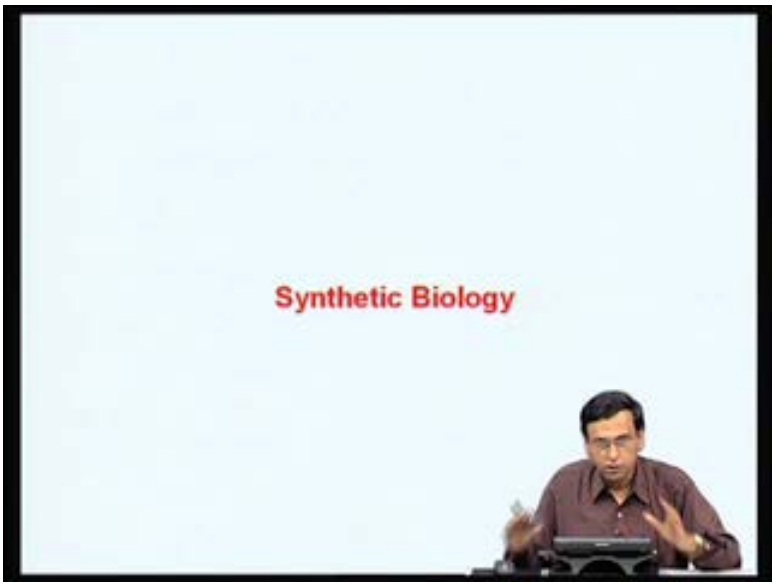

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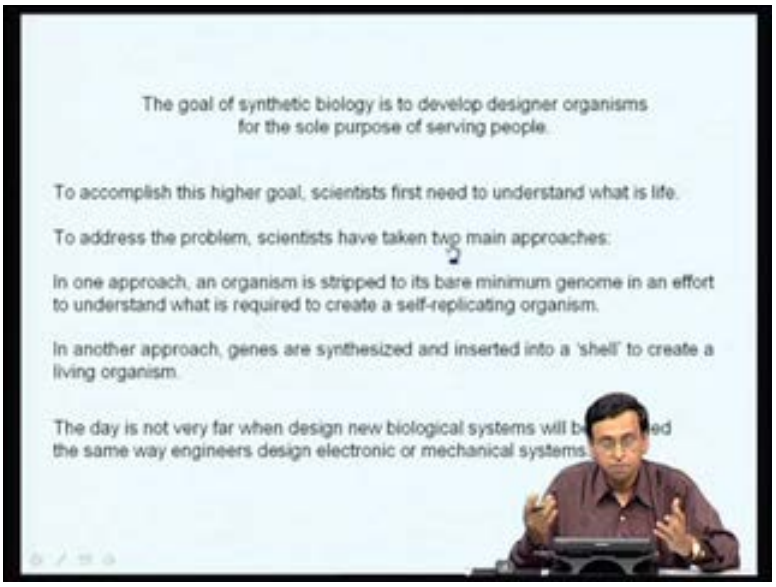

I would like now to spend about 5 minutes expressing in another fascinating area called synthetic biology which is actually in extension of metabolic engineering. what is synthetic biology? The goal of synthetic biology is to develop designer organisms for the sole purpose of serving people to accomplish this goal, scientists first need to understand what is life? To address this problem scientists have taken 2 main approaches.

In one approach the organisms is stripped to its bare minimum genome in effort to understand what is required to create self replicating organisms what is the minimal number of gene that is required for an organism to self replicate that is the first challenge the second once you understand this can you now actually synthesize this genes and

make an artificial organism which contains genes which have been artificially synthesized and if you now put this genes in to a shell way of a leaving organisms you created a artificial life this is the challenge of synthetic biology can you create artificial life. Now in fact many things have been achieved so i'm just going to take 5 minutes to explain what has been achieved in the area of synthetic biology.

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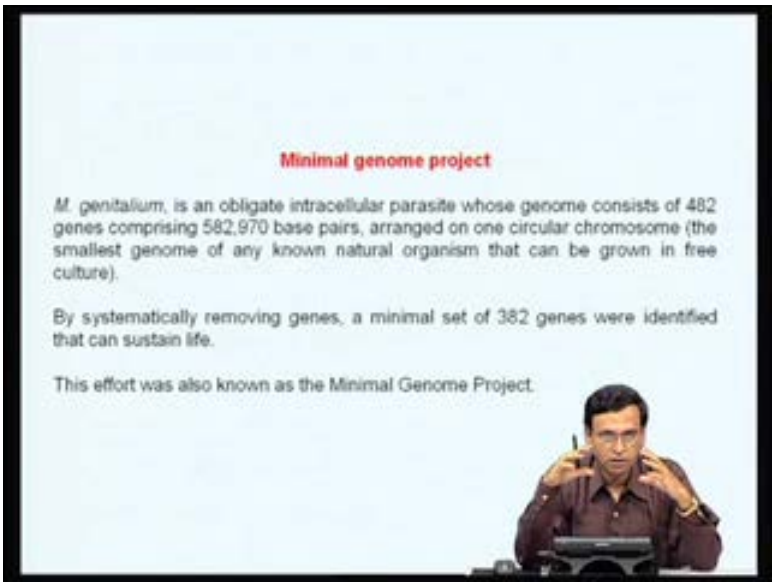

There is one thing that is start the one of the first challenges of the synthetic biology is what is called as a minimum genome project. Micro bacterial genitalium this is an obligate intracellular parasite whose genome consists of about only of 482 of genes now how much genome consists thirty thousand genes so this is a much smaller organism and this arranged in just one chromosome and this is the smallest genome of any known natural organism.

So, they took this organism which is an independent leaving which is a self-replicating organism and ask the question among this 482 genes how many genes are actually required what is the minimum amount of genes required for this organism to self-replicate and they found out a minimal set of 382 of genes is required that can sustain life tosustain life we require just 382 genes that is what came out of this so this is known as the minimal genome so this minimal genome containing 382 genes if you put it in organism it can replicate itself.

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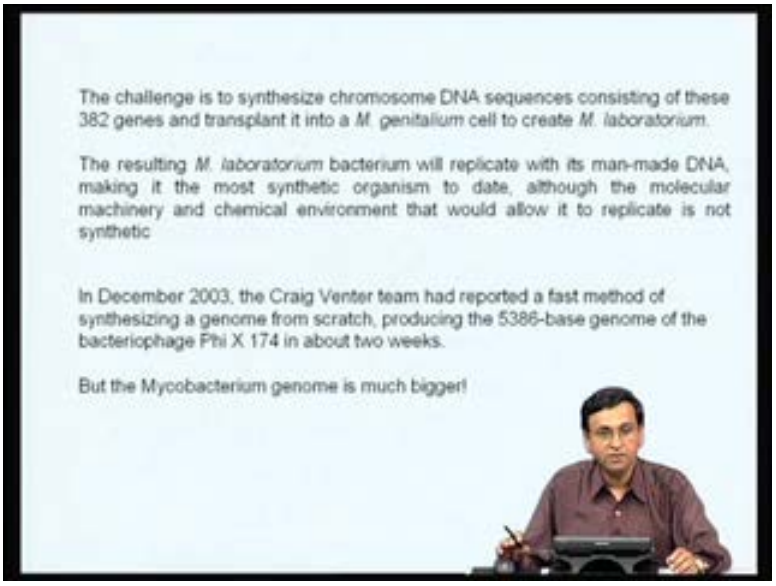

Then, the challenge is can you synthesize chromosomal D N A sequences consisting of 382 genes and if we now them put it them in to a another micro bacterium tissue which does not remove which does not have its own D N A now this artificial D N A can now replicate and you have created basically artificial life. So, can you now generate what is called as a micro bacterium laboratorium which contains which now replicates with a man made D N A that has been the excitement.

So to achieve this you cannot go and actually synthesize 382 genes straight away because it is too large amount of D N A so in the december 2003 craig venter one of the pioneers in the area of synthetic biology actually synthesized about a 5000 base genome of a bacteriophage called 5 x 174 in about 2 weeks time so you can actually synthesized about 5005 base pairs of D N A in about 2 weeks time. But, the micro bacterium genome is much bigger

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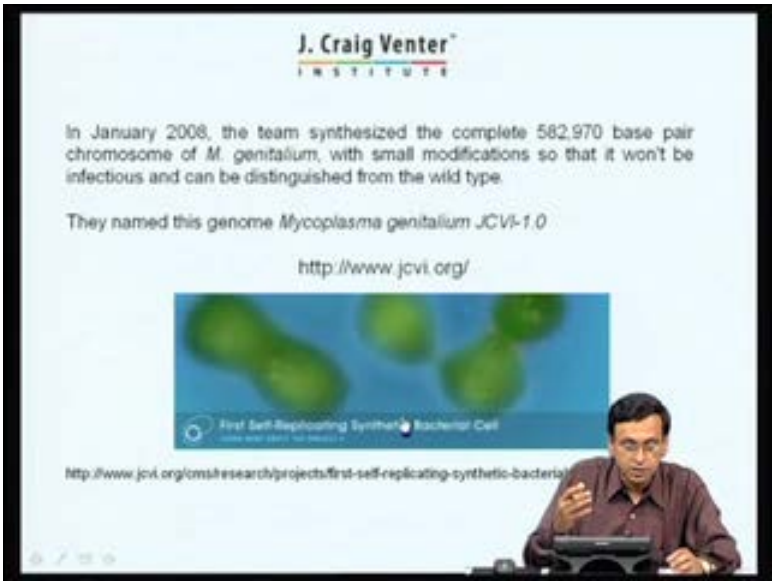

So, it took at least another 5 years before they can synthesize the entire 5582000 base pair gene chromosome of micro bacterium genitalium and then they put this in to another micro bacterium species and created a new bacterium which as a man made genetic material and this was named as mycoplasma genitalium J C I this synthetic biology extensively being done in institute called craig venter institute in san diego and california and if you actually go and then click on this website it gives a very nice video and extensive literature on on the synthesis of the first cell replicating synthetic bacterial cells how they went about and how they created artificial life.

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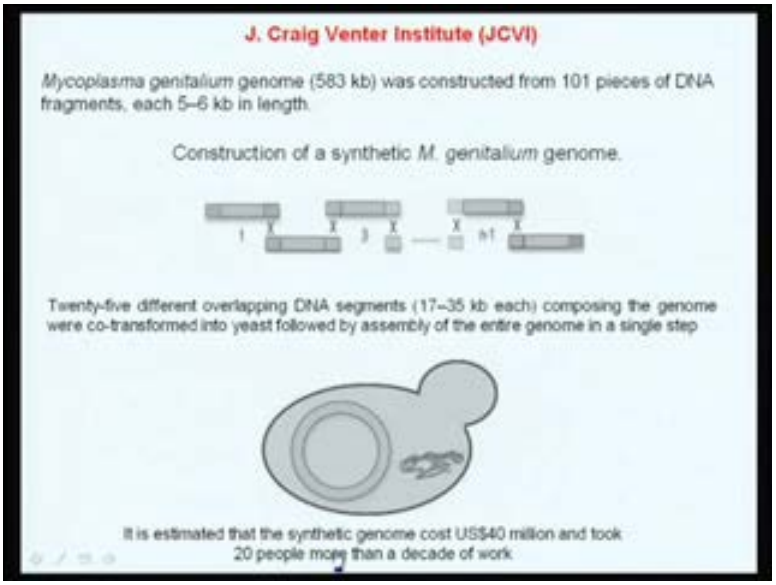

So, this is basically in the nutshell what they did they constructed a series of D N A pieces D N A fragmentation of 5 to 6 k b length about hundred such pieces and then in vitro by recombination they assembled all of them and then 25 such overlapping D N A fragments of about 17to 35 k b basically each were then put into yeast cells and once you put these pieces of D N A in cells by invival homologous recombination they got assembled into a huge chromosome and you start it replicating and you have basically created what is called as a artificial life. And was estimated that this synthetic genome caused about forty million u s dollars and took about twenty people and they work for about 10 years to create this artificial life.

This is the smallest genome a microorganism containing a smallest artificial genome. So, what I would like to say is that metabolic engineering and synthetic biology are very exciting areas of research fantastic things are being out and we are almost very close to creating what is called as an artificial life. And I can tell you this lecture series on eukaryotic gene expression in all these lectures in all these topics are made sure I have stressed how important is understanding gene expression without understanding gene expression, without understanding how gene expression is regulated you cannot achieve any of these things.

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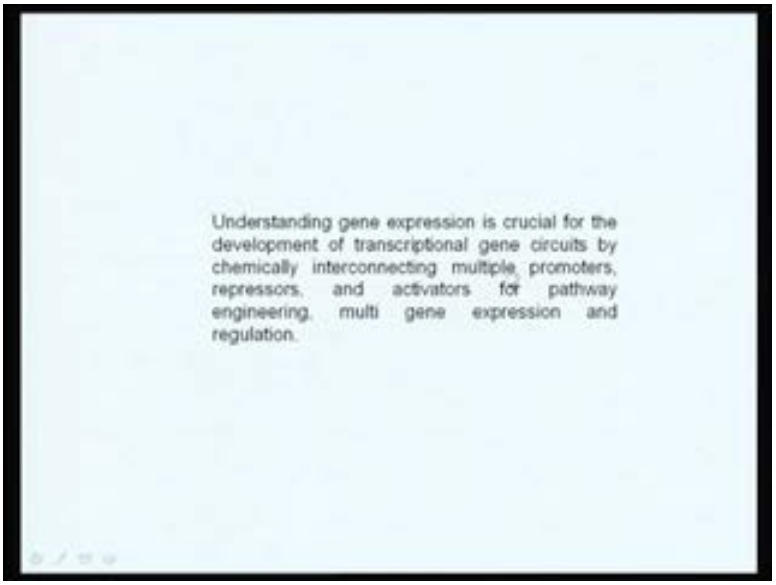

So, understanding gene expression is crucial for the development of transcription gene circuits by chemical interconnecting multiple promoters, repressors, activators for

pathway engineering as well as multi gene expression pathways in metabolic engineering as well as for synthetic biology

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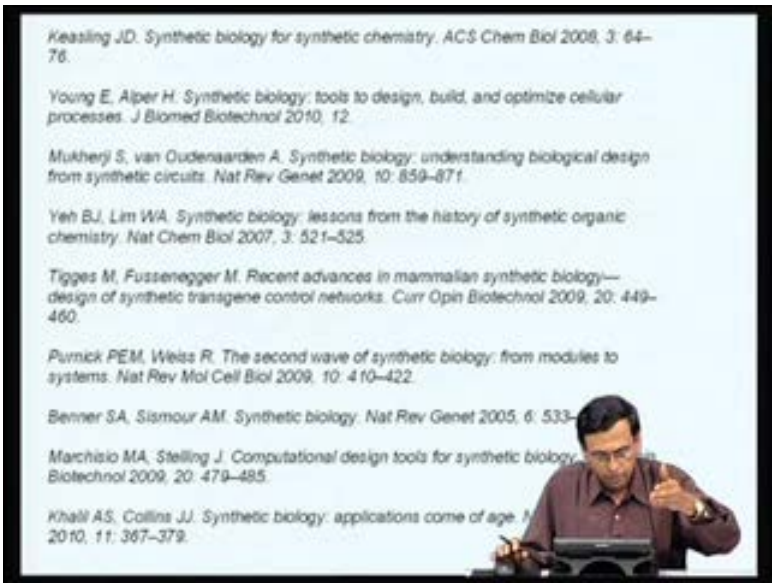

So, I given a series of references here one can go through there is a tremendous excitement in the area of metabolic engineering as well as synthetic biology and we are not very far away of creating designer organisms there is a designer microorganisms that will actually make any compound of engineer any metabolic pathway into microorganism you can produce any compound of your interest and you can actually make what is called as design organisms that will produce what you want by doing all these as synthetic biology.

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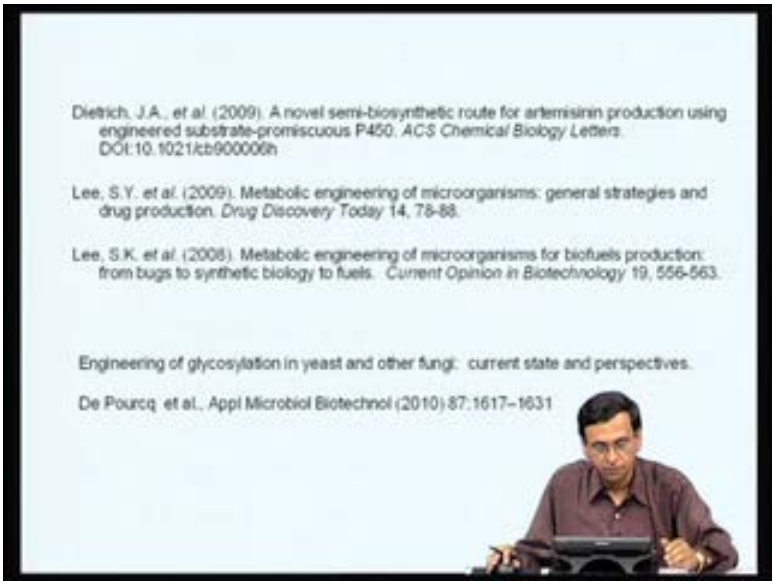

Again a series of references one can go through.

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And I would like to conclude that this is the last lecture in this forty lecture series and I hope that you enjoyed this forty lecture series on eukaryotic gene expression basics and benefits we began by discussing very basic aspects of gene regulation and I have taken you through a journey of about forty lectures of explaining not only many basic aspects of gene regulation, but, wherever possible I have explained to you what are the applications that is coming out and that hopefully will come out lot more exciting

applications are being awaited in the near future and this twenty first century actually belongs to biology and biotechnology lot of exciting things are happening in the area of eukaryotic gene expression and many more interesting things are likely to come out of eukaryotic gene expression and if you have any questions of any clarifications you can e mail to me at this e mail address pnr@biochem.iise.ernet.in .Thank you and good bye .