Metabolic Engineering
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Lecture No-28
Application of Metabolic Flux Analysis

Welcome to metabolic engineering course today we will discuss about some of the application that have been extensively used in metabolic engineering where you can see that metabolic flux analysis have been used, where they have used a model driven technique for metabolic engineering. So, some of the example we will discuss without model and some of the example with model.

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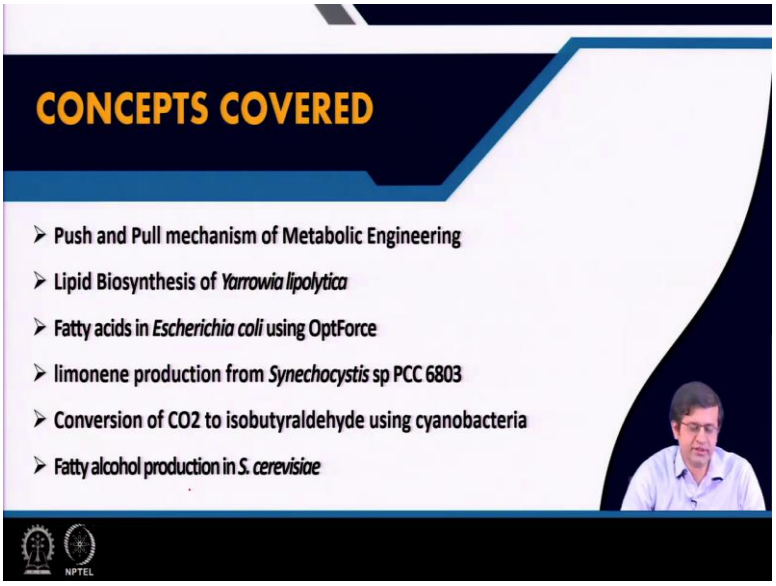

So these are the things covered in this lecture. First of all, we learn about the push pull mechanism of metabolic engineering. This is very well known concept in metabolic engineering. Using push pull mechanism many compounds have been manufactured in the lab as well as an industry where you do not need a model for pull push mechanism. Just an idea of metabolic network how the reactions are connected that idea itself will be helpful for push pull mechanism.

And then we have used push pull mechanism in lipid biosynthesis of Yarrowia Lipolytica. This is one of the oleaginous yeast, very well known where Yarrowia Lipolytica has been metabolically engineered for lipid biosynthesis. Also, we discuss about fatty acid production in E

coli using of Optforce. So the Optforces is another algorithm which is very similar to optknock. So you have learnt about optknock, so I think it gives multiple gene knockouts.

So similarly Optforce is also used to predict gene knockouts or genetic modification like up regulation and down regulation that can be predicted using Optforce and can be implemented in E.coli for fatty acid production. And also it will discuss about Limonene production from Synechocystis sp PCC 6803 and also how carbon dioxide can be converted into Isobutyraldehyde using cyanobacteria. And lastly, we will discuss about fatty alcohol production in saccharomyces cerevisiae.

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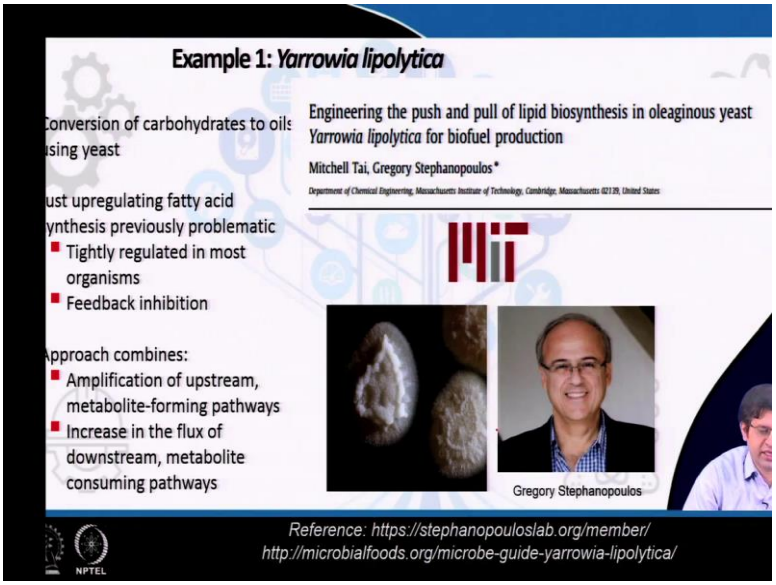

This metabolic engineering is very well known organism. Yarrowai lipolytica has been done by Gregory Stephanopoulos from MIT, he is well known as father of metabolic engineering. If you do not know you can visit his website which is given here. He works extensively in many area and metabolic engineering of yeast E.coli and also on 13 C metabolic flux analyses. He has developed many softwares, many strain working both on computational aspects of metabolic engineering and also at the metabolic Engineering on bench as well.

So here are his paper on which was published in metabolic engineering. He is also the editor of metabolic engineering is one of the best Journal in metabolic engineering where he engineered Yarrowai lipolytica for production of lipid. And how they have used carbohydrate like glucose;

cell consume carbohydrate in the form of glucose and produce oil that the yeast they have used Yarrowai lipolytica.

So they have up regulated of some gene when Lipid biosynthesis pathway, and there is a feedback mechanism which is tightly regulated in the organism and also they have looked for some genes in the upstream pathway and also in the downstream. So you have to guess like which are genes you need to be up regulated so that your pathway; see lipid synthesis pathway is already there indigenously in the Yarrowai lipolytica.

New genes have been added but only what you have to do, you up regulate the pathway so that the pathway becomes active. So when you look for metabolic pathway for lipid biosynthesis pathway there are many genes. Now you have to target which is the last gene that you want to up regulate. So if you up regulate the last gene of lipid biosynthesis pathway, you may not see that much improvement.

But that is basically the downstream pathway, so the gene which is close to the product that is known as downstream pathway and upstream is actually where the precursor molecules are coming. So these 2 pathways you can look in detail and see which are the genes, needs to be the targeted. So if you just target the downstream pathway, you may not get metabolite to that level which you are targeting.

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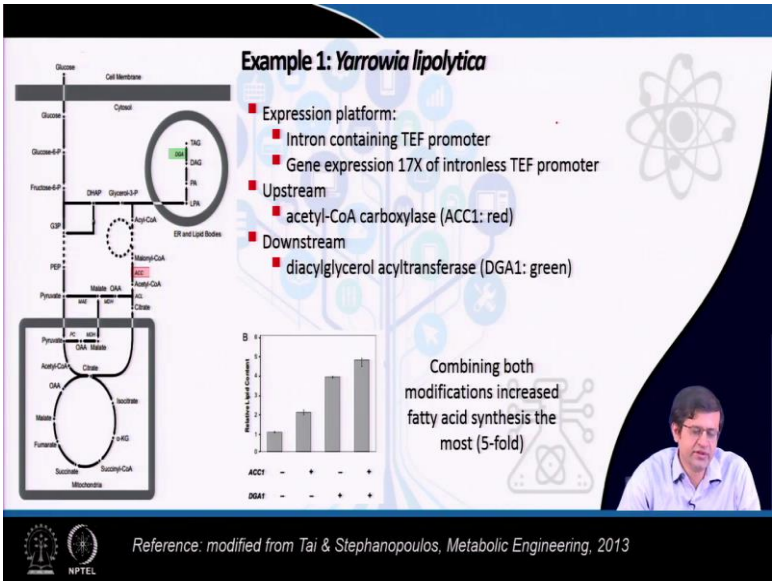

So for that he defined a technique that is push pull mechanism of lipid biosynthesis where they actually up regulated one of the gene that is ACC acetyl coenzyme A, which is convert into Malonyl Coenzyme A that pathway they have up regulated. This gene they have generated up regulated that is ACC and then that is the upstream pathway. So they generated the precursor metabolite that is Malonyl coenzyme A inside the cell which is far away from Tag Biosyntheses they ultimately want to improve the production of Tag. They want to improve the Tag by up regulating DGA1 that is diacylglycerol acetyltransferase, so this tag molecule that is the lipid molecules they want to increase in inside the cell triacylglyceraldehyde they want to improve.

And then for that they have targeted this 2 gene DGA1 and ACC. So in one hand is actually pushing the metabolite 2 are the malonyl coenzyme A, 2 are the fatty acid pathways. This malonyl coenzyme A is one of the precursor molecule in for lipid biosynthesis and then up regulating DGA1 try to pull the lipid to a tag molecule. In this way they have improved the production of lipid biosyntheses in Yarrowai lipolytica combining both these modifications.

You can see when they have not up regulated ACC1 that is minus minus then the production is just one, it is relative to the wild type. This is wild type production and then in the next step they up regulated the ACC1 which is basically the upstream pathway. The upstream pathway has been up regulated so the moment you up regulated the gene using a TEF promoter, which is a constitutive promoter, you have to replace the native promoter with a TEF promoter.

So this way you can up regulate the gene inside cell. So the promoter replacement is the technique that is followed by many people in metabolic engineering. So when you heard about that, the gene is up regulated that mean promoter is replaced with a some stronger promoter or a synthetic promoter. For example the yarrowia lipolytica has a native promoter that is step promoter which is very strong.

So that if you replace the promoter of ACC genetic promoter of BCC with a promoter then automatically the expression will increase and that is reflected in lipid content also. They become double, from one relative lipid counter is one in the wild type and then it become 2. So you see the production has doubled when you just increased ACC. The expression of ACC has been increased and then they have individually increase the DGA1 that is downstream pathway.

So they have up regulated the downstream pathway and see that the production has becomes four times compare to the wild type. When you up regulate the ACC1 then it is double and then when you up regulate the DGA1 only then it become 4 fold increases in lipid content. And then when they combine both of them then you can see it become 5 fold. So, this is mechanism where you can combine both ACC up regulation and DGA 1 up regulation where you can see the push pull mechanism is applied here.

And for this is you do not need any metabolic flux analysis data or anything it is just intuitive knowledge about the metabolic pathway which genes need to be targeted. You can decide on your own and try to get the maximum production. And this is very well followed in metabolic engineering. So, many of the metabolic engineering paper actually follow this technique. You might read many papers and this is a classical example of push pull mechanism where upstream and downstream pathways are actually up regulated where you see the maximum production of lipid in the form of a TEF.

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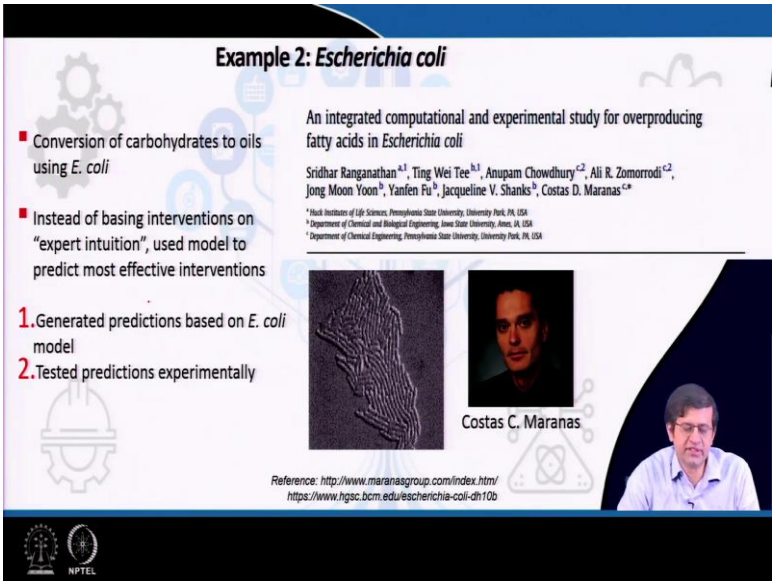

So, next example, the second example discuss about the E.coli metabolic engineering. In this case the model has been used, the metabolic model specially Optforce technique has been used. Optforce is very similar to Apnox which we have already discussed in previous lecture, the optknock will give you gene targets, multiple gene targets; where the biomass is maximized and at the same time the product is also maximized.

There are 2 objective function in this kind of algorithm. Optknock and Optforce is very similar. Just that in Optknock you have only gene knockouts that is you have to delete the gene but Optforce you have up regulation down regulation along with the gene knockout. So we have all kinds of genetic intervention were the product can be improved not just by knockout but also by up regulation and down regulation of the gene.

So, in this technique Costas C. Maranas group from Pennsylvania, he is one of the computational guy; who worked on designing new gene target and also when you apply those modification in the cell then they found that there is improvement in production of fatty acid. Fatty acid production has been improved in E.coli just by targeting some of the gene which has been predicted by a model. So they have generated the prediction based on E.coli model and tested experimentally.

So first I think you have to predict the gene target and then you have to perform the experiment to test that the predictions are right or wrong. So this way they have improved the production, even though E.coli is very well known organism. But the fatty acid production has been improved recently only, I think in the year 2013 that the production has been improved. E.coli is one of the organism where most of the things are known.

If you want to say that any organism in the world which is known more detailed everything is known it is E.coli. Most of the things are know most of the genes known and most of the regulations are known we have very well characterised metabolic network regulatory Network and also signalling network. So that is why Metabolic engineering is quite easy in E.coli. So, most of the metabolic engineering have been done on E.coli, tested in E.coli, and then they have extended in other organisms because E.coli molecular biology tools are very well known and is easy to engineer.

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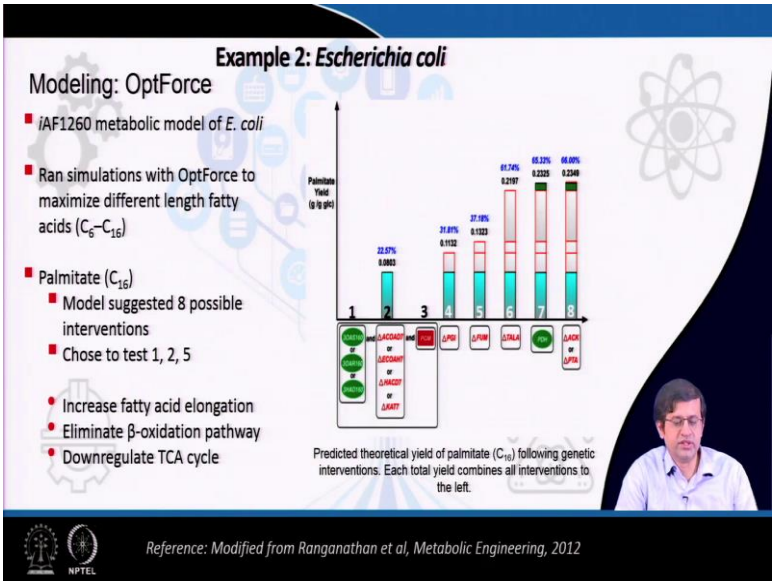

So using the OptForce they have improved the production of fatty acids though fatty acid molecules are actually at different lengths. Here I have given the example of palmitate where it has a 16 carbon, 16 carbon fatty acids can vary from C6 to C16 or C18. So, in this metabolic modelling where they have predicted the gene and model they have used is iAF1260 metabolic model of E.coli. And using the OptForce they have predicted 8 intervention.

Eight different predictions they have done the model suggested 8 possible interventions. So 8 intervention they have tested experimentally out of that I will just explain the test 1 which is given in 1 to 5, 1, 2 and 5. So, just by applying 1, 2, 5 they found an improvement is around 37% that is the palmitate yield. So, yield that I have already told you that in the previous lecture that yields is basically gram of fatty acid produced divided by the gram of glucose consumed.

So here we neglect the biomass. The biomass component is neglected and yield of palmitate is basically the amount of palmitate produced in gram divided by the gram of glucose the cell is consuming. And this way yield has been calculated earlier also in the isotope steady-state isotope labelling also we have calculate the yield of isotope level Glucose or isotope level compound that is synthesised inside the cell.

This way we can see that they have tested 1, 2, 5 interventions. In the 1, you can see they have targeted 3 genes, either of the three genes and then in target 2 they have targeted either of the 4 genes. They want to knockoff any of these 4 genes and on the 5th you can see they have down regulated or deleted fumarate. They have increased the fatty acid elongation pathway. So, these 3 interventions are present; the number one is present in fatty acid elongation pathway, number 1 which is here.

And then number 2 is basically elimination of beta oxidation pathway. So, beta oxidation pathway, as you know that the elongation pathway where the fatty acid chains are elongated or fatty acid molecules are formed. where the beta oxidation pathway is a degradation pathway. So, the fatty acid molecules which are formed they get degraded in beta oxidation pathway. So, those genes are eliminated in intervention 2 where any of these 4 gene can be knocked off to actually eliminate the beta oxidation pathway.

They want to eliminate the beta oxidation pathway so that the fatty acid produced should not be degrade than your production level will go down that is why they have actually eliminated the beta oxidation pathway and they have down regulated the TCA pathway. The TCA cycle they have down regulated because TCA cycle carries lot of flux from acetyl coenzyme A, that is why

if less amount of acetyl coenzyme goes in TCA cycle, then they can improve the production of fatty acid.

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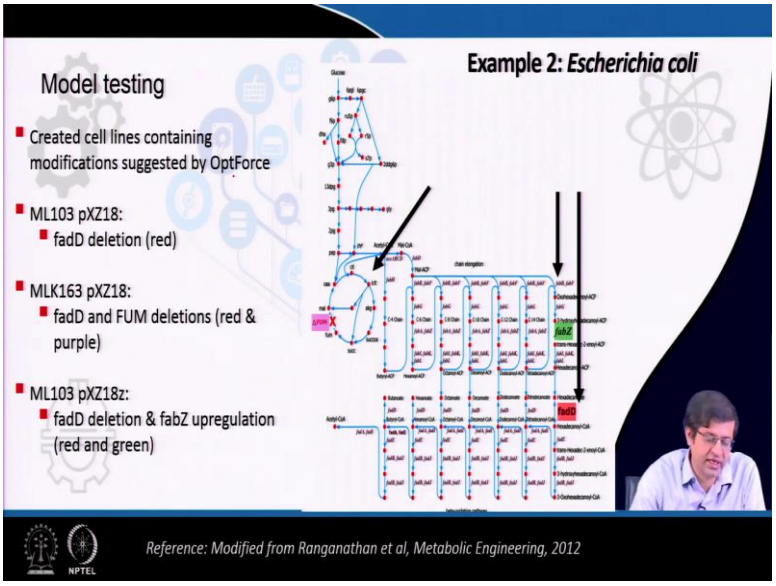

So, these are the 3 predictions that came from OptForce. Now they have tested this intervention using in difference strains. For that they have created 3 strains ML103 pXZ18 where they have deleted this gene but the beta oxidation pathway they have deleted the fadD. The fadD is deleted which is given in red colour and the strains is ML 103 pXZ18. So, they have created this 3 strains and they have created MLK163 strains where they have the plasmid pXZ18 that has fadD deletion and fumerate deletion. These 2 deletions they have red and purple.

And then in ML103, they have fadD deletion and then fadZ upregulation that is the green colour one they have upregulated one in this one actually deleted fumerate. So in this red and green that is ML103 pXZ18z they have a characterised these 3 strains to see how these actually improving the fatty acid production.

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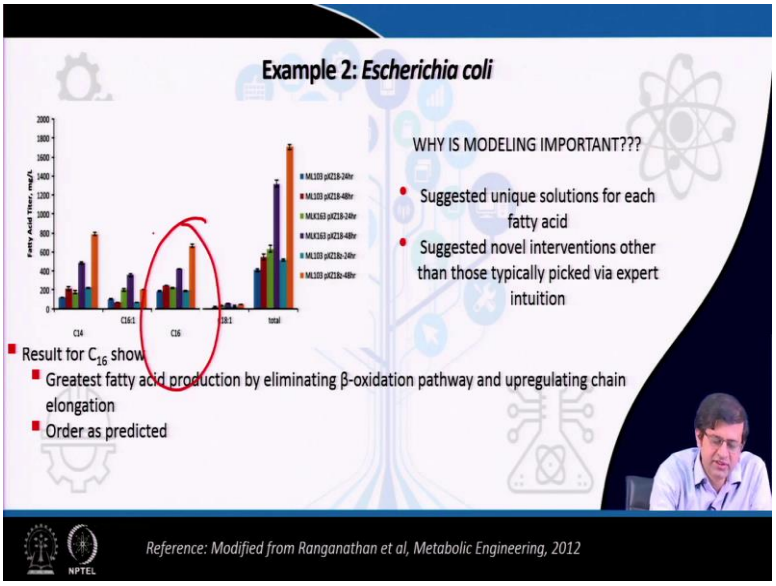

Here you can see the palmitate production C 16 the strains that is grown for 48 hours and 24 hours the is ML103 pXZ187 is actually producing the most. And they got around 6 to 700 milli gram of fatty that is palmitate. C16 has been produced the maximum in case of ML103 pXZ187. So, this strain, is actually producing the most and compared to the other strain. So they have suggested a unique solution for fatty acid production suggested novel intervention that can be used to improve the production.

And the greatest fatty acid productions are produced by eliminating beta oxidation pathway and the upregulation of the chain elongation. So this they got maximum when they actually deleted the gene in beta oxidation pathway and then upregulated and chain elongation pathway. These two interventions is actually very useful for production of fatty acid in E.coli that has been published in metabolic engineering in 2012.

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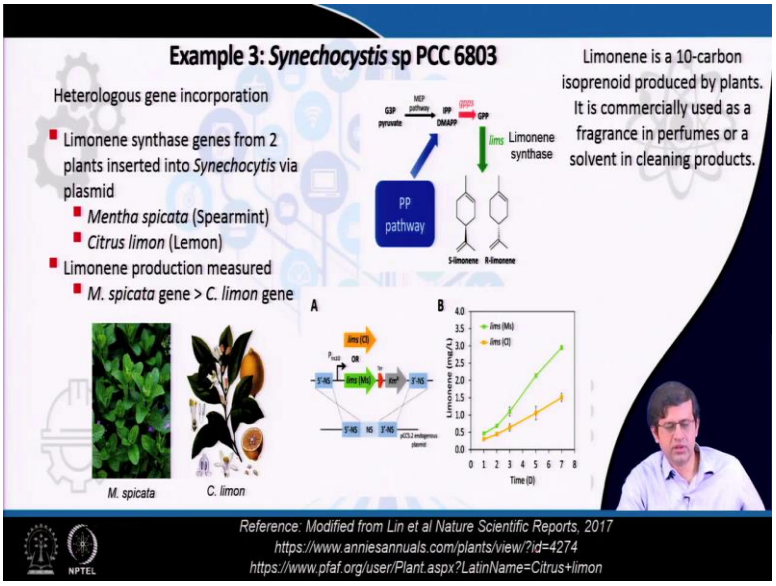

Now I will take another example, example 3, where they produce limonene compound. limonene compound is, very useful commercial compound which is a 10 carbon isoprenoid compound which is produced by a plant. So plant produces this compound. Now you want to synthesise this compound in microbial cell that is cyanobacteria. So this cyanobacteria species Synechocystis PCC 6803 has been used to produce isopreniod based compound limonene.

It is commercial used for fragrance in perfumes and as well as solvent in cleaning product and also it is used as a drop in biofuel for biodiesel and jet fuel. And this limonene synthase is not present in cyanobacteria. So it is actually a heterologous gene which is present in the lemon food or citrus food. Citrus foods have this gene that is limonene synthase which is meant for limonene. They have extracted the gene from these 2 species from C. Lemon and M. Spicata.

These 2 organisms they have plant organism, they are used to extract the gene and then they found that the limonene production is maximum for M psicata. M spicata produces less compared to C. Lemon. So the Lemon strain much more preferred. So limonene synthasis has been taken from citrus fruit and they found that the Lemon gene is much more active compared to M spicata. And this is only gene they have taken from other source.

Source from the plant have taken and then they introduce this gene in the cyanobacteria species PCC 6803.

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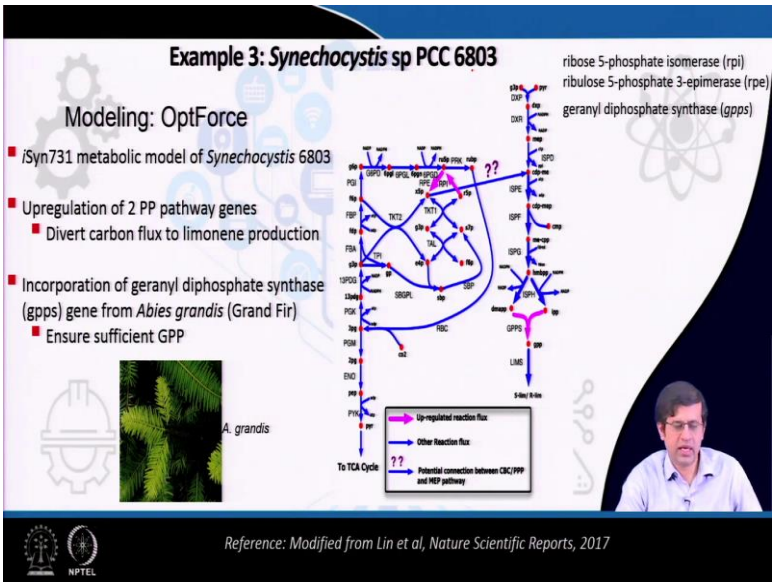

And using OptForce they actually predicted some of the gene which are actually important for metabolic engineering for important for production of limonene. So, the limonene synthase that they have taken from plant and some of the genes they have targeted based on OptForce prediction. So, the OptForce predicted 2 genes in PPP pathway that is phosphate pentose pathway and one gene from the downstream pathway that is geranyl diphosphate Synthase.

So GPPs has been need to be upregulated based on OptForce Modelling techniques they have predicted and also RPI that ribose 5 phosphate isomerase that is present in PPP pathway over here RPI. You can see RPI over here and then ribulose 5 phosphate 3 epimerase that is RPE which is present over here. So this 2 gene need to be upregulated based on OptForce prediction and also the downstream pathway that is GPPS you need be upregulated.

When they have upregulated this 3 gene and also added limonene synthase they found the production has improved.

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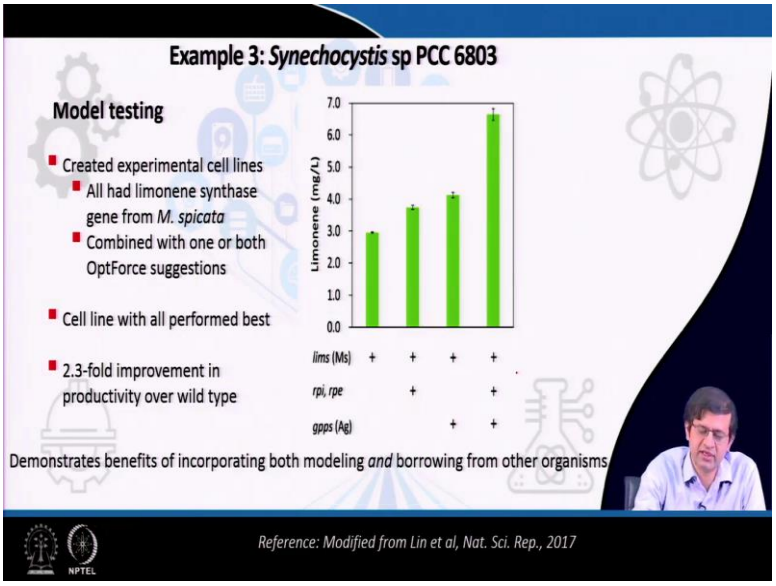

So this is their production profile they perform the experiment and they are measured the limonene production in cyanobacteria that is synechocystis sp. PCC 6803. They created the experimental cell line using the genetic intervention that is metabolically engineered strain and they have added the limonene synthase from M. Spicata and combine with one or both and then they have actually done the Optforce suggestion apply the OptForce suggestion and then they created the strain that have actually this limonene synthase integrated in the chromosome.

And then they have upregulated gene that is RPI and RPE when they have limonene synthase present they found limonene production is around 3 milligram per litre this one and then when they upregulated RPI and RPE that is from the PPP pathway this one and this one then they found the production has become very close to 4 milligram per litre. And then when they upregulated the downstream pathway that is GPPS which is just next to limonene formation and then they found there is a little improvement in limonene production is around 4 milligram per litre.

And then when they upregulated all the three things that is limonene synthase have been integrated and RPI and RPE upregulated and EPS is also upregulated then they found around very close to 7 milligram per litre. So this is how they have improved the production of that is 2.3 fold improvement, they have achieved. Where they upregulated some of the gene from based on the prediction of OptForce.

Using the OptForce they have predicted some of the gene which need to be upregulated and then they verified using the experiment.

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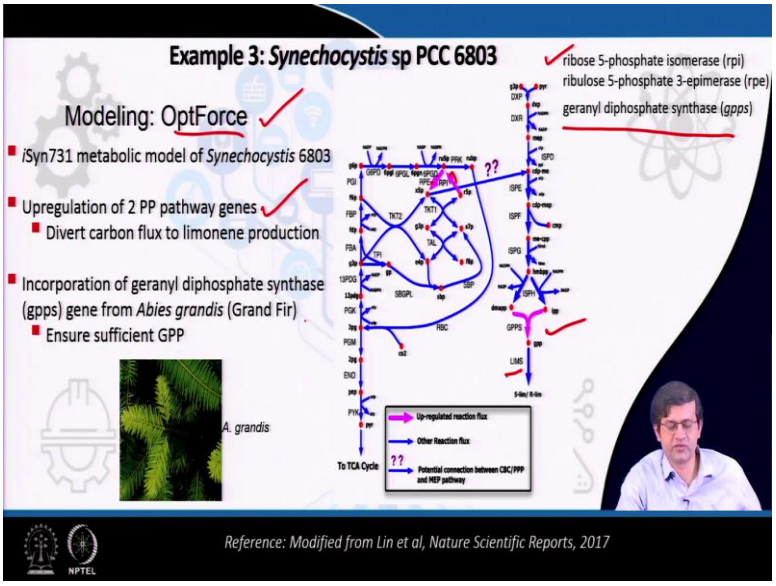

So these are the gene they need to upregulated based on OptForce prediction and using this prediction they have improved production where they found that there is a 2.3 fold improvement in limonene production compared wild type.

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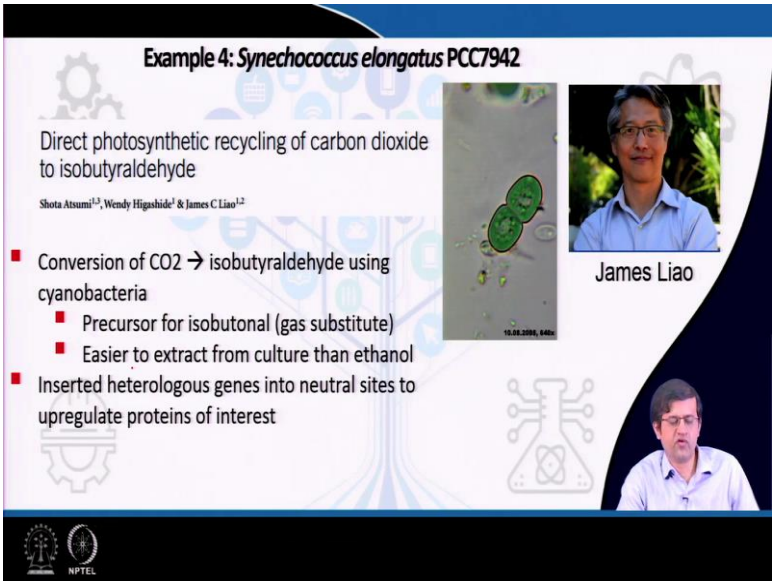

Now the 4th example, I will discuss about another cyanobacteria that is Synechococcus elongates that is species PCC7942 and this is another cyanobacterial species where have been engineered

for the production of isobutyraldehyde, Butyraldehyde has been produced isobutyraldehyde has been produced from CO 2. The carbondioxide is used for production of this compound which is very easy precursor for isobutanol production that is gas substitute.

So isobutanol can be produced from isobutyraldehyde where cyanobacteria consume this carbon dioxide and then produce this compound, precursor molecule using cyanobacteria. So, it is easier to extract from cultured ethanol and then inserted heterologous genes in into a neutral site to upregulate the protein of interest. Here they were engineered the cyanobacteria production of isobutyraldehyde and this work is done by James Liao from UCLA.

So you see he is from UCLA he work on this organism and they have shown that the production of this compound very useful for many compounds.

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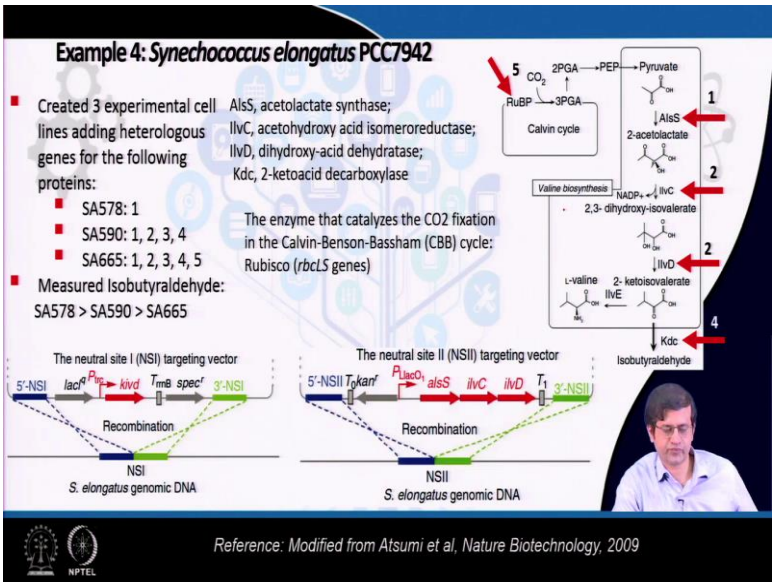

So these are metobolic engineering they have performed while they have engineered this trend where they have integrated some of the gene in the chromosome genetically engineered organism for the production of this industrially important compound. So they have created 5 genes they have actually integrated in the chromosome what are those 5 genes? They have located neutral site 1 and neutral site 2 where the genes are actually integrated.

The neutral sites are basically the site in the chromosome. If you actually integrate the new gene in that site it will not affect the other genes so that is why they are actually neutral site. So when you integrate a new gene on the heterologous gene in inside chromosome then make sure that you should not affect the other native genes. So these neutral sites are estimated based on several study. So that it does not affect the other native genes.

So this intervention they have done for basically 1, 2, 3, 4 and then 5 interventions. The 5 interventions is basically; the first one is basically the number one that is acetolactase synthase that they have actually taken from bacillus subtilis. There is another organism bacillus subtilis that is another organism bacillus subtilis. So this came from bacillus subtilis where they have taken this gene that is acetolactase synthase and then acetohydroxy and isomer reductase and dihydroxy acid dehydratase has been taken from E coli.

So, these two genes are actually taken from E.coli and ketoacid decarboxylase has been taken from L-lactase, so this one is taken from L-lactase these are taken from E.coli and this one taken from bacillus subtilis. These you can see there are 3 different sources which has been integrated in the cyanobacteria. So are the 4, 5 interventions. Fifth one is basically the enzyme that catalyzes the CO 2 fixation that is through Calvin Benson Bassham cycle that is CBB cycle.

The Calvin Benson cycle is actually required for CO 2 fixation the carbon dioxide fixation if you can upregulate that gene that is Rubisco which is present in the Calvin cycle. The rubisco genes can be upregulated by the carbon fixation can be improved, so, that you can have maximum isobutyraldehyde. So, isobutyraldehyde can be improved by adding these five genes. So these 5 genes have been taken from four different organisms.

And these are the neutral site they have integrated. Neutral site 1 where they have integrated the - 2 keto acid decarboxylase 2. Keto acid decarboxylase e has been integrated into neutral site 1 and this 1, 2, 3 these 3 has been integrated in neutral site 2. So this is synechococcus elongatus genomic DNA which is shown over here and on the position where only 1 is intergated integrated is also shown over here. And this strain they have number that is a SA578 where only

one is integrated and then in SA590 they have integrated 1, 2, 3, 4 and SA665 where they integrated 5 of them.

And an out of all these 3 strands they found that SA655 is producing maximum isobutyraldehyde. And isobutyraldehyde has been produced maximum in the SA665 strain.

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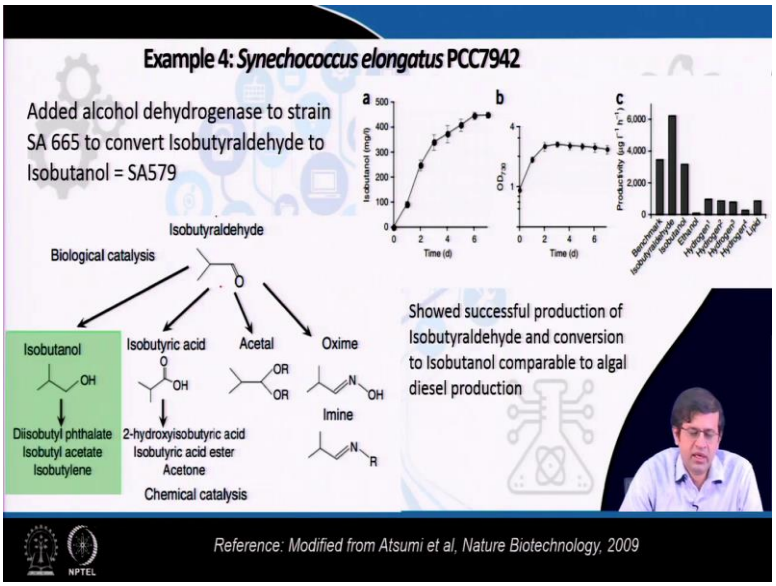

And they also added the alcohol dehydrogenase to the strain that is SA665 and they found that the isobutyraldehyde can be converted into isobutanol. Isobutalnol is again a very important industrial compound that can be produced from isobutyraldehyde that is isobutanol, which is shown over here. And then they have shown that the isobutanol production is around 4 to 500 milligram. And you can see the growth rate of the cell also it is producing isobutanol in the stationary phase.

So here you can see that the isobutanol is increasing with time and the cell is already in the stationary phase. It is very important result that you can see that the cell has stopped growing but still it is producing more isobutanol. And the compound that has been produced that isobutyraldehyde, isobutanol production has been compared with other bench mark production. Productivity levels that others strain have produced like hydrogen, Lipid, ethanol, isobutanol that is produced by other benchmark for organism.

And then in all cases you can see that isobutyraldehyde is actually produced more compared to other organisms. That is the productivity that is microgram per litre per hour.

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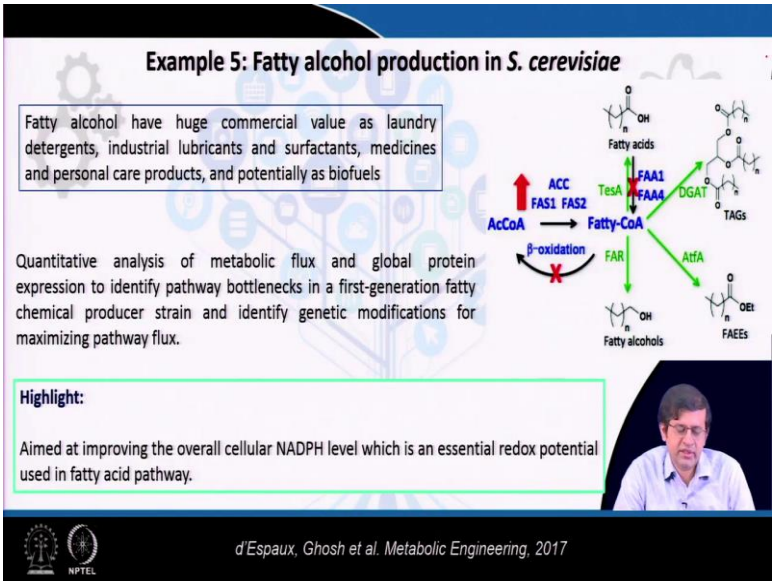

The last example that is a fatty alcohol production in saccharomyces cerevisiae this is another model which we will discuss now. Fatty alcohol is very important commercial compound which is used as laundry detergent industrial lubricants and surfactants and medicine, personal care product and potentially fatty alcohol can be used as bio-fuel. It has a wide application and fatty alcohol is used in many purposes.

And then the how we can improve the fatty alcohol production here fatty alcohol production has been improved through 13 c metabolic flux analysis. Quantitatively they measure the metabolic fluxes which are the bottleneck for fatty alcohol production. And they have improved the fatty alcohol production. First they have constructed the fatty alcohol pathway. For fatty alcohol production they have added the fatty acyl reductase that is the heterologous gene.

Otherwise all genes are present inside the cell. Only one gene they have taken from other source and otherwise most of the genes like ACC FAS1 FAS2 which is already there. They have just upregulated the gene by replacing the net promoter with a constitutive promoter like TAS1. So in this technique they aim to improve the production of fatty alcohol in saccharomyces cerevisiae

and then they have looked into the NADPH level which is essential for redox potential in fatty acid pathway.

They want to target the gene which are actually consuming NADPH because fatty acid production require a lot of NADPH molecule inside the cell but somehow if you can improve the level of NADPH level inside the cell you can improve the production of fatty acid in terms of fatty alcohol in saccharomyces cerevisiae.

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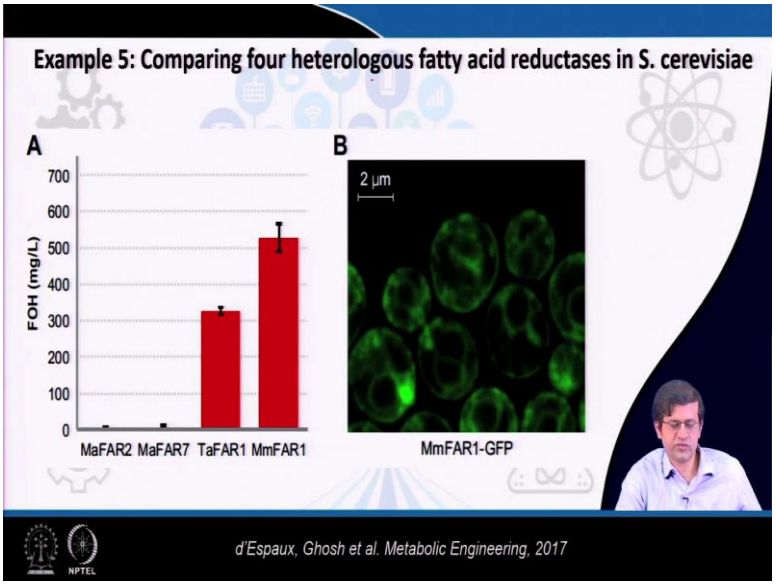

So as I told they have included only one gene from other organism that is FAR1 fatty acid reductase. Fatty acid reductase has been taken from other sources they have tried four different organisms out of that they found that mass muscular which is a mammalian source. While they found that the fatty alcohol production is more. So, that is why they have expressed this gene inside the cell and integrated the FAR gene using a GFP.

And using GFP they found that the gene is very well expressed inside the cell and this genes are actually codon optimised, this are not taken as it is from the mammalian cell, but it is actually codon optimised. So that, the expression level is more inside the cell in terms of Saccharomyces cerevisiae.

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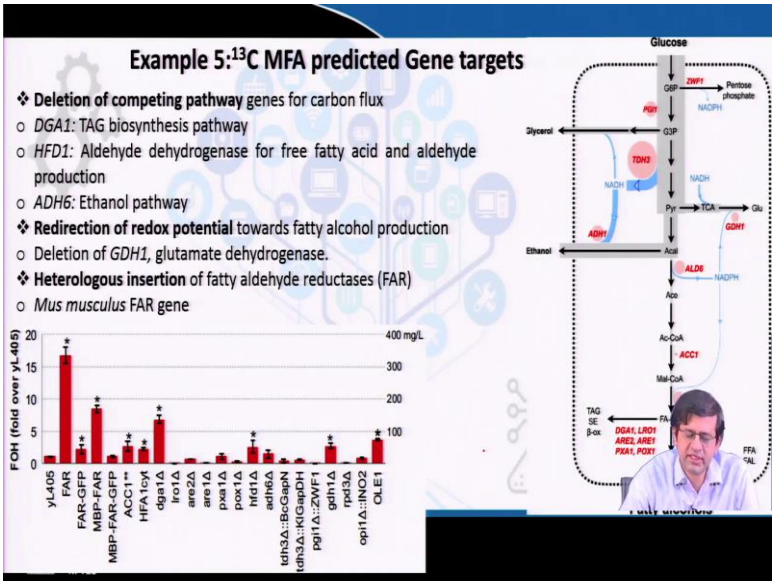

So they have targeted several pathways where they have integrated the hetrologous gene from other sources that is mass muscular. FAR gene has been integrated in the chromosome. But also they have look for competing pathway. The competing pathways are the pathway which actually consuming lot of carbon. So, competing pathway for example ethanol pathway that is consuming lot of carbon and also you it may consume NADH.

So this ethanol pathway they have targeted that is aldehyde dehydrogenase for free fatty acid and aldehyde production. So the aldehyde dehydrogenase, they have removed and also ADH6, they have removed. So this way they actually removed most of the carbon going into free fatty acid production and aldehyde production. So these are competing pathway they also deleted the TAG by biosynthesis pathway, DGA1. DGA1 is also deleted so that maximum carbon can go into fatty alcohol production. And also they have deleted the glutamate dehydrogenase.

Glutamate dehydrogenase GDH1 which actually consume a lot of NADH that GDH1 also they have removed. So there any interventions you can see that these are all join at genomic level.

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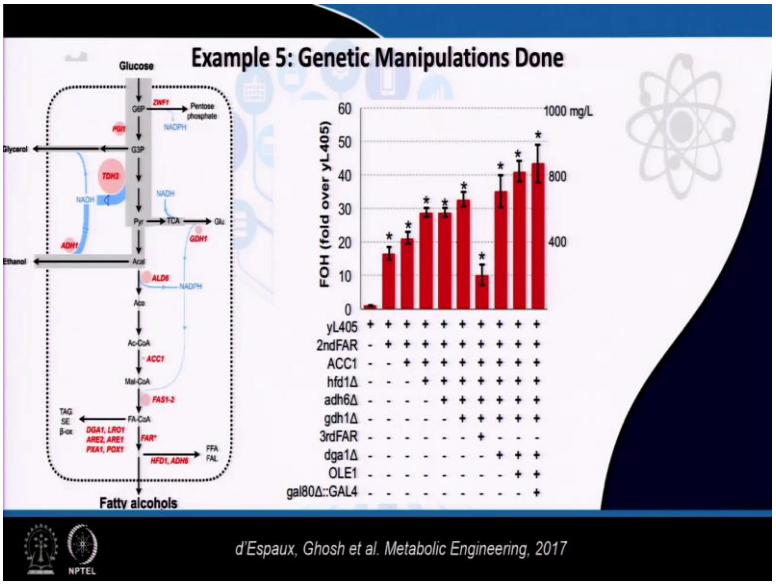

And they found that the production of fatty alcohol has significantly improved where they got around 800 milligram per litre. And the intervention they have used basically based on the flux prediction. This is this is the metabolic flux profile record from 13 C MFA and based on this prediction they actually targeted the gene. So they have integrated the FAR gene because they are integrating in the chromosome.

The plasmid best technology, you know that the high expression plasmid system can be used for production of protein but when you integrate in the chromosome, then integrate the gene at multiple locus. So they have integrated the first copy of FAR and third copy of FAR. And when the integrated third copy of FAR then they found that the production has not improved. So here you can see that the production has not improved.

So they have restricted in to the second copy of FAR that is fatty alcohol reductase. The fatty alcohol reductase they have been integrated upto second copy. And then they found that when they integrated upto second copy and then the production is more. And then they also deleted the competing pathway that is ADH6, HFD1 then they also deleted DGA1. So, once they have implemented all the genetic intervention then they found that the production is very close to 800 milligram per litre.

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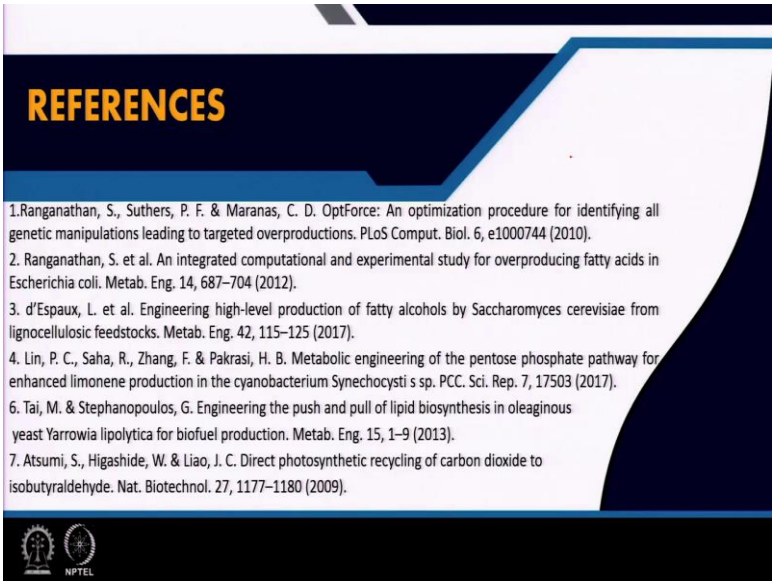

So if you want to read in more detail you can read this paper the fatty acid production in E.coli you can read the PLOS computational Biology paper and if want to read about the OptForce technique then you can read this paper. And also the fatty acid production in E.coli you can read the metabolic engineering paper published in 2012 for fatty alcohol, and you can read the metabolic engineering paper in publishing 2017.

And for production of limonene, production in cyanobacteria that you can read scientific reports paper published in 2017, and for the pull push mechanism which I told for Oleaginous for Lipid production in for that you can read the metabolic engineering paper published in 2013. And the production of butyraldehyde that you can read the nature biotechnology paper published in 2009. These are the reference you can go in detail and read more about this metabolic engineering in detail. Thank you. Thank you for listening.