Metabolic Engineering
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Lecture-36
Metabolic Engineering for Biofuel Production-Part C

In today's class we are going to discuss about the metabolic engineering of biofuel and this is going to be the last part of this particularly with respect to the biofuel production.

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And in this lecture the applications of metabolic engineering in biofuel will be discussed with specific emphasis on engineering is *Escherichia Coli* towards ethanol production. We will also briefly talk about the other aspects of the bioethanol production or biofuel production from *E.coli*.

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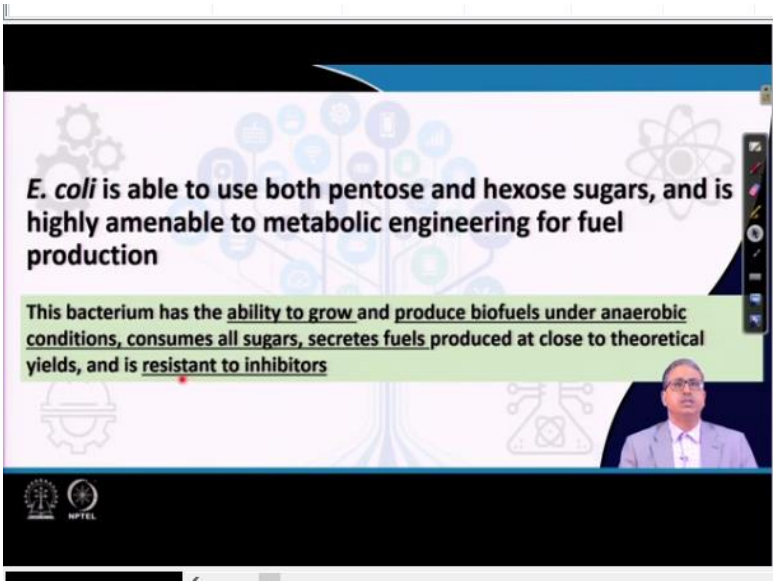

So, *E.coli* is able to use both pentose and hexose sugars and is highly amenable for metabolic engineering for fuel production. Now these pentose and hexose sugars are obtained from the lignocellulosic biomass and ability of *E.coli* to utilize both these pentose and hexose sugar simultaneously is one of the most notable advantages for selecting *E.coli* for bioethanol production.

E.coli apart from having the ability to use both the hexose and pentose sugars possess the ability to grow and produce biofuel under anaerobic condition as well as under aerobic condition, it consumes all the sugars, all the pentose sugars present in the lignocellulosic biomass, secretes the fuel produced at close to theoretical yields and is resistant to inhibitors.

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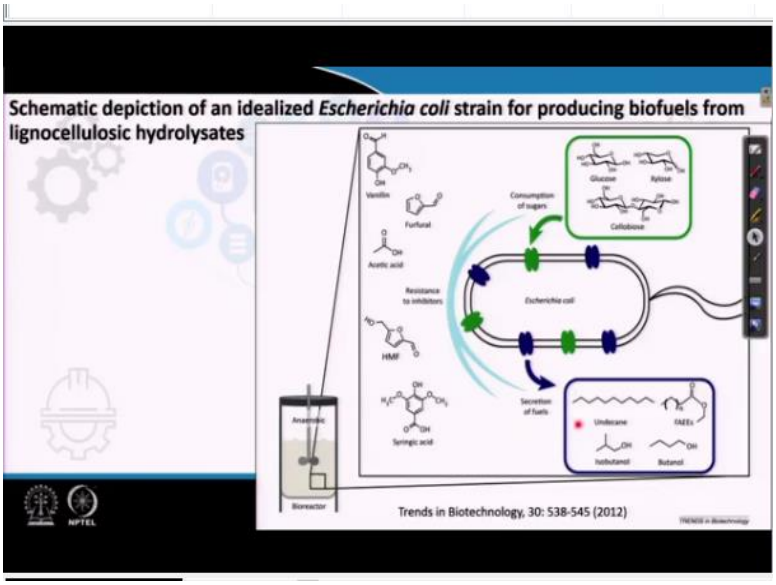

Now the schematic diagram of the idealized *E.coli* strain is presented here which is conceived as and have ideal system to produce the biofuel from lignocellulosic hydrolysates. So, as it can be seen from this diagram that there could be a number of toxic compounds which are often present in the lignocellulosic hydrolysate including the furfural compound, vanillin acetic acid, different other acids.

And the *E.coli* organism or *E.coli* strains which are to be used or developed for metabolic engineering through metabolic engineering for bioethanol production are often naturally resistant to these inhibitors or they are improved to withstand the higher concentration of these inhibitors. Along with this the ability to withstand the inhibitors ability to utilize different carbon substrates which are available in the lignocellulosic biomass and their transportation or secretion in the medium is also indicated here.

And the kind of biofuels or the fuel molecules which can be produced is enormous and it includes not only ethanol but also butanol isobutanol undecan and other compounds.

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Now *E.coli* is considered to be a preferred host for ethanol production from lignocellulosic biomass.

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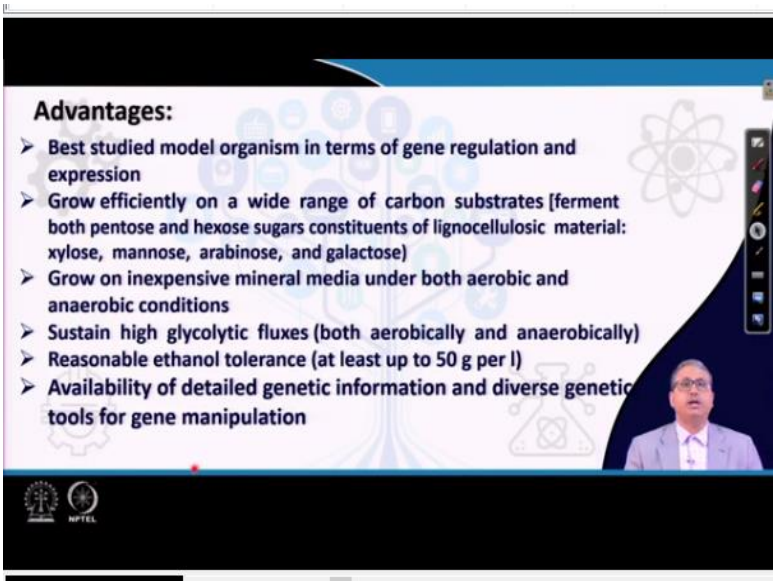

And the specific advantages of *E.coli* are it is one of the best studied model organism in terms of gene regulation and expression. So, a lot of information is available on the gene regulation mechanism, gene regulation processes and their expression profiles within *E.coli* system. *E.coli* grows very efficiently on a wide range of carbon substrates and they are often capable of fermenting both pentose and hexose sugars constituents of the lignocellulosic material that includes the xylose, mannose, arabinose and galactose while utilizing glucose as well.

E.coli grows on inexpensive mineral medium under both aerobic and anaerobic condition sustains high glycolytic flux both aerobic and anaerobic condition and also shows reasonably enhanced tolerance to ethanol at least up to 50 gram per liter and the availability of detailed genetic information and diverse genetic tools for gene manipulation is also considered to be one of the very advantageous aspect for selecting *E.coli* for bioethanol or biofuel production.

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However, there are a number of challenges which are enlisted here, these include need to improve the ethanol yield and productivity, because *E.coli* is not intrinsically ethanol producing strain, it can produce ethanol at higher concentration but the native *E.coli* strain so the wild strains are capable of producing ethanol at much lower concentration than what is expected for its industrial application.

So, the improvement of ethanol yield is one of the most important criteria. There is also a need to improve the tolerance to bioalcohols and other toxic compounds which are often present at high concentration as the cost of lignocellulosic biomass purification are to be considered. So, often the crude lignocellulosic hydrolysates are proposed to be used to minimize the cost of ethanol production.

And in order to do that lot of toxic ingredients are often present in the fermentation medium and the *E.coli* strains must be able to withstand those toxic compounds. Efficient utilization of low cost substrates not only including the lignocellulosic biomass but other waste materials which are more sustainable or often found to be more available in the local or regional scale, productivity towards advanced alcohol.

So, as we identify a number of shortcomings of ethanol and other alcohols like butanol or isobutanols are emerging or emerged as more potent or more suitable biofuel candidates. So, simultaneously the metabolic engineering must be targeted not only for ethanol production but also for advanced alcohol production by these organisms.

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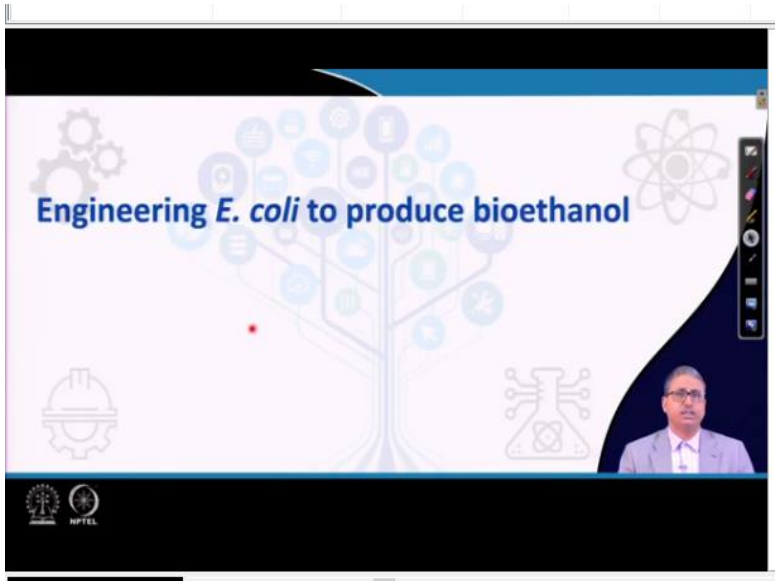

Now the engineering of *E.coli* to produce bioethanol will be discussed.

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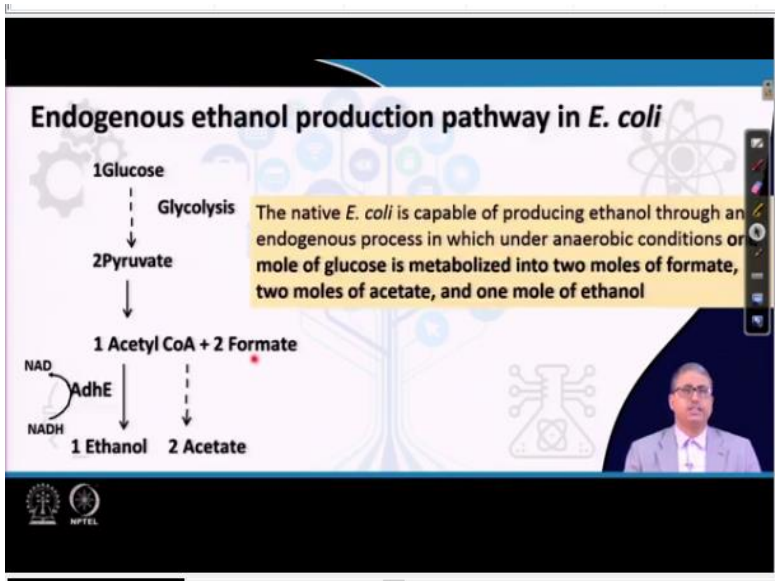

Now if we look at the endogenous ethanol production pathway in *E.coli* as I stated earlier that *E.coli* can produce ethanol but not at a very high concentration and the native *E.coli* is capable of producing ethanol through the endogenous process in which under anaerobic condition 1 mole of glucose is metabolized through the EMP pathway or the glycolytic pathway to 2 moles of pyruvic acid.

And these 2 moles of pyruvic acids are further reduced to produce 1 mole of ethanol, 1 mole of acetyl-CoA and 2 moles of formic acid.

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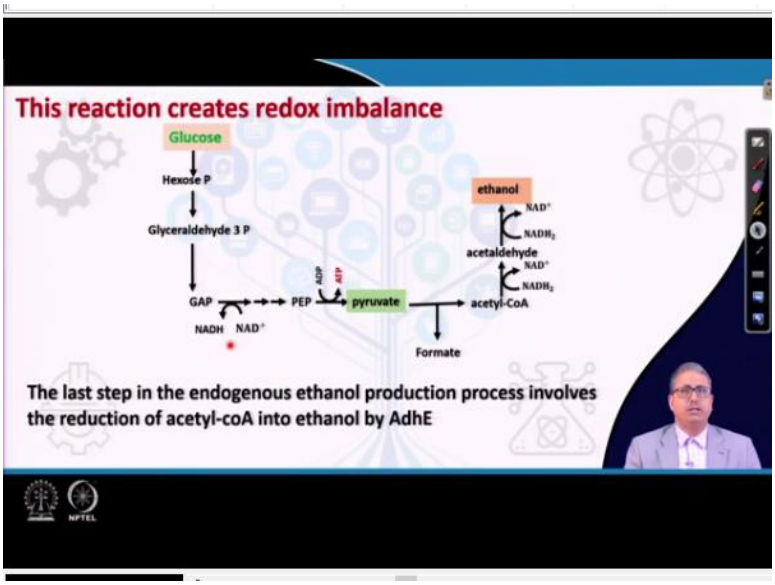

Now this particular reaction creates a redox imbalance. Now if we look at the metabolism of the pyruvic acid towards ethanol by native or endogenous *E.coli* system we will find that there are 2 steps where NADH H + or NADH2 are consumed. So, the reducing power is utilized in these 2 reactions. While 2 moles of NADH2 or NADH H + are consumed only 1 mole of NADH can be produced per moles of pyruvic acid from glucose.

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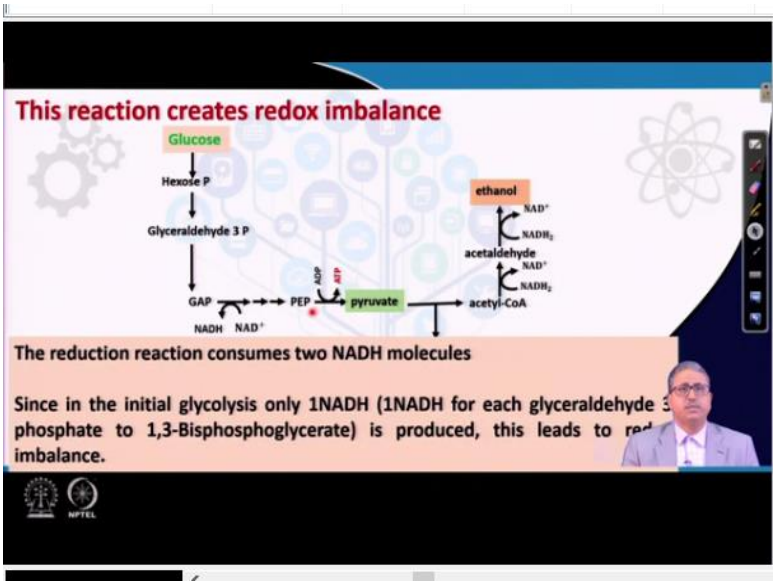

Now this creates a problem. So, the reduction reaction consumes 2 NADH molecules and only 1 mole of NADH H + can be produced per moles of pyruvic acid or pyruvate produced from alcohol. So, this leads to a kind of high demand or high consumption of NADH H + and thereby resulting into a redox imbalance.

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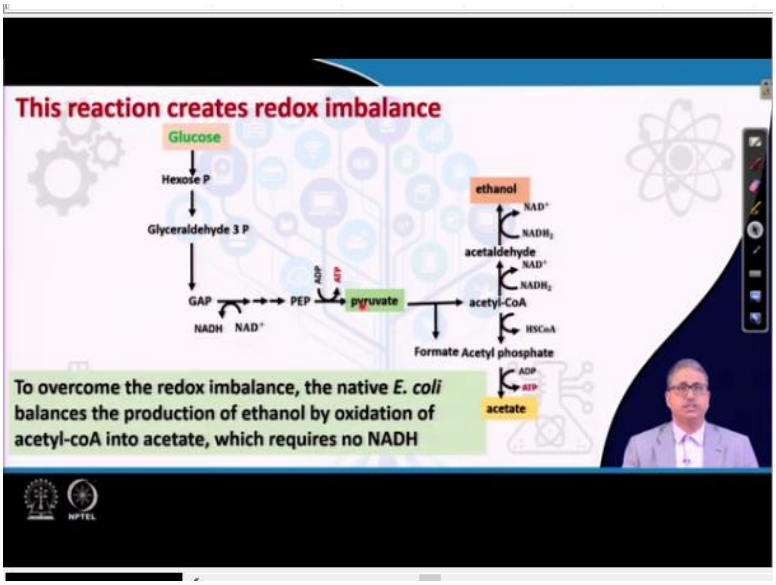

Now to overcome the redox imbalance the native *E.coli* balances the production of ethanol by oxidation of acetyl-CoA into acetate which requires no NADH. So, that means in native or wild type *E.coli* strains. The endogenous pathway for ethanol production is not very much functional, it functions but at much lower efficiency because of the consumption of the more consumption of the NADH H + would create the more imbalance in the redox conditions.

So, the endogenous pathway allows some moles of the acetyl-CoA to be processed towards ethanol while most of them will be processed through acetate. So, we will find that more of acetate and format are present in normal *E.coli* cell under the anaerobic condition growth or anaerobic growth and less concentration of ethanol are going to be produced.

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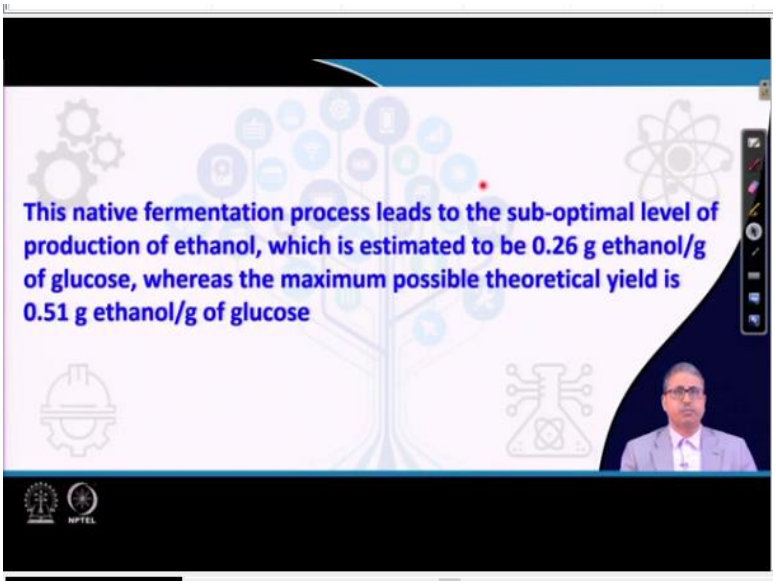

Now this native fermentation process leads to the sub-optimal level of production of ethanol which is estimated to be 0.26 gram ethanol per gram of glucose consumed, whereas the maximum possible theoretical yield is 0.51 gram ethanol per gram of glucose. That means theoretically it can be just doubled.

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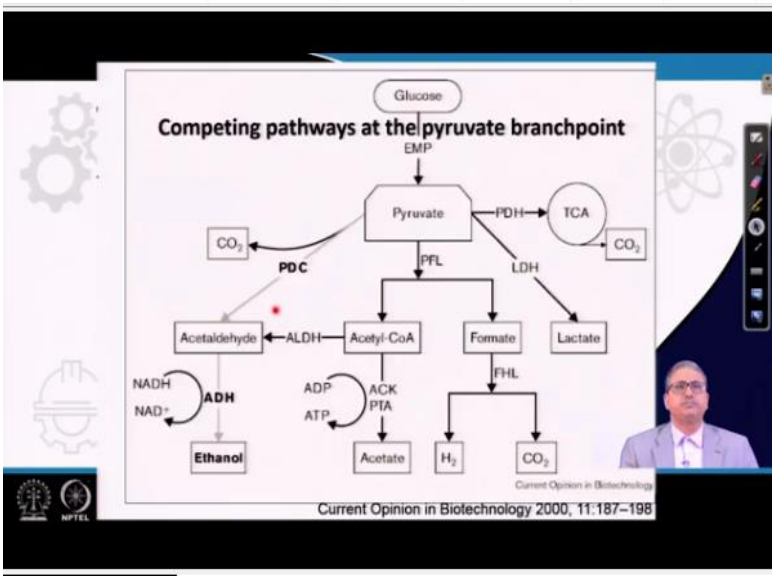

So, here are the competing pathways at the pyruvate branchpoint, which are most relevant for the *E.coli* system. So, as we can see that from the glucose to pyruvic acid or it is produced through this EMP pathway. Now once this pyruvic acid is produced there are number of reactions which can utilize or which utilize the pyruvic acid.

So, under aerobic condition mostly the pyruvate dehydrogenase complex is active it actually holds the TCA cycle leading to the complete oxidation of pyruvic acid to carbon dioxide. Pyruvate format lyase is another enzyme which also utilizes the pyruvic acid to produce the acetyl-CoA and formic acid and acetyl-CoA can be further converted to acetate molecule.

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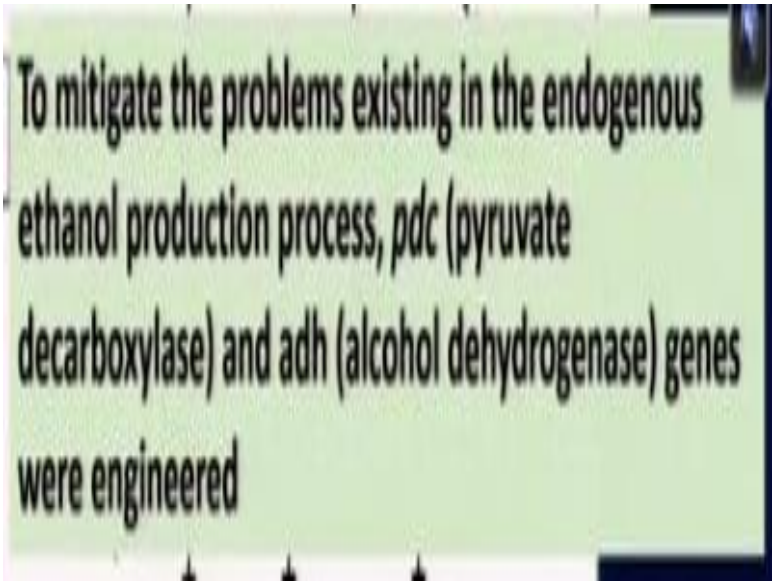

Now if we look at this pathway to mitigate the problem existing in the endogenous ethanol production process the pyruvate decarboxylase, this is the gene pyruvate carboxylase which is responsible for converting pyruvic acid to acetaldehyde and acetaldehyde can only be reduced further to produce the ethanol. So, both these genes are present in *E.coli* endogenous E.coli ethanol production system is there.

But they do not perform very well because of the redox imbalance which is often prevalent if the ADH and the PDC systems are very active. So, *E.coli* is not very efficient in terms of running this PDC and ADH although they are often found in the E.coli system. So, initially it was thought that to mitigate the problem of existing endogenous ethanol production process PDC that is the pyruvate decarboxylase enzyme which converts the pyruvic acid to acetaldehyde. And the ADH that is the alcohol dehydrogenase which reduces acetaldehyde to alcohol were engineered.

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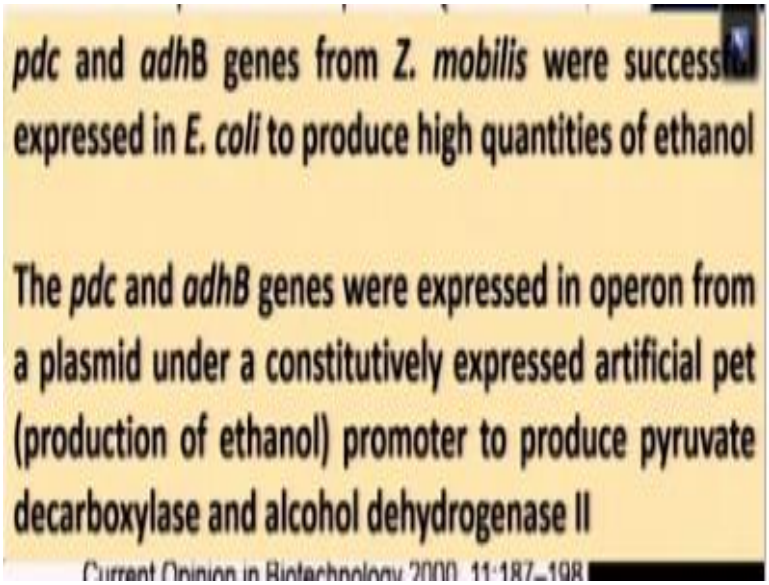

Now these 2 genes the PDC or the pyruvate carboxylase and ADH or alcohol dehydrogenase genes from *Zymomonas mobilis* were successfully expressed in *E.coli* strain to produce the high quantities of ethanol. Now why from *Zymomonas mobilis* because *Zymomonas mobilis* is the bacterium which is very efficient in ethanol production. *Zymomonas mobilis* cannot metabolize pentose sugar.

But it can metabolize hexose sugar, that is how it can produce very high concentration of pyruvic acid and from pyruvic acid the PDC and the ADH genes of *Zymomonas mobilis* can convert to the pyruvic acid to ethanol very effectively. So, these 2 genes were cloned into a plasmid vector and this plasmid vector was called as pet vector and these 2 are constitutively expressed within this pet vector and were engineered to E.coli strain.

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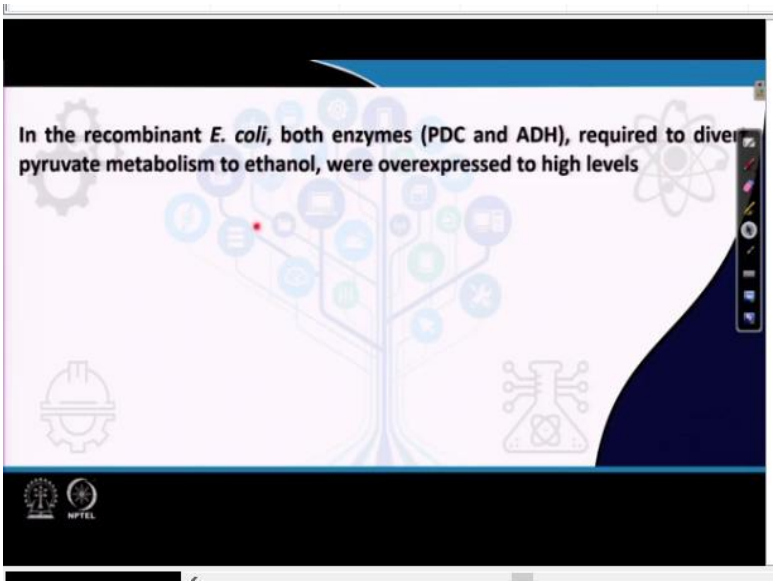

Now in the recombinant *E.coli* both the enzymes that the PDC and ADH from *Zymomonas mobilis* required to divert the pyruvate metabolism to ethanol were successfully overexpressed and high level of ethanol production was formed.

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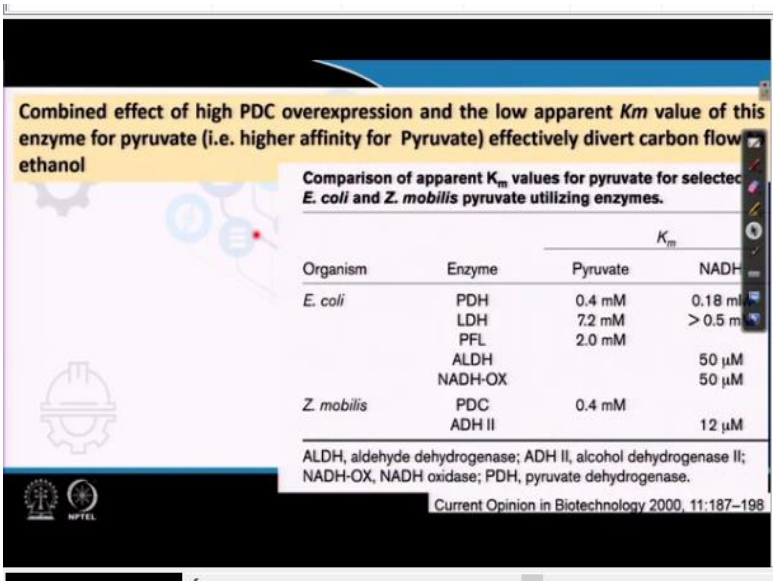

Now there are a number of advantages which are connected to the selection of these PDC and ADH genes of Zymomonas mobilis and their successful expression in *E.coli* which is more evident when we look at the Km values that is the enzyme affinity of these 2 particular enzymes the PDC and ADH towards their substrate are compared between the E.coli and *Zymomonas mobilis* strains.

So, the combine effect of high PDC over expression and the low apparent Km values of this enzyme for pyruvate effectively divert the carbon flow towards the ethanol.

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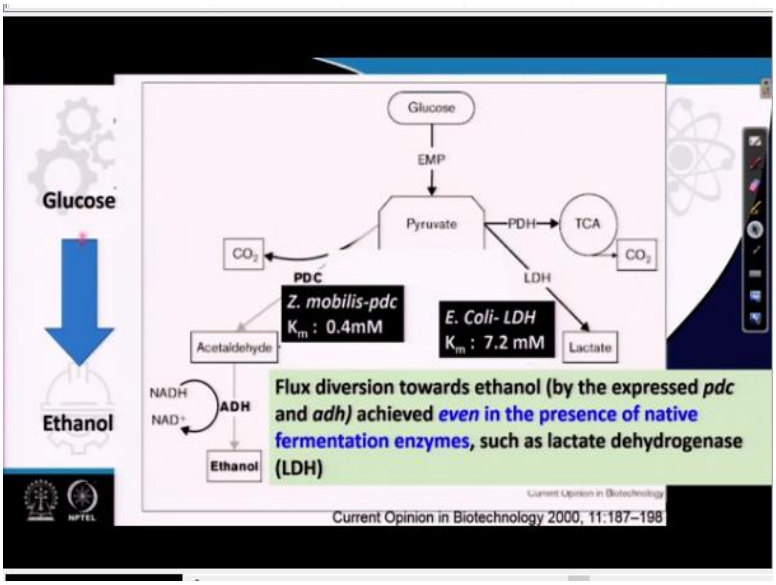

Now if we see this diagram again, the flux diversion towards ethanol by the express PDC and ADH is achieved even in the presence of native fermentation enzymes such as the lactate dehydrogenase, because the *E.coli* strains are often capable of expressing this lactate dehydrogenase very efficiently. So, there might be a competition between these over express PDC and ADH with the native lactate dehydrogenase which is *E.coli* endogenous enzyme.

So, it has been found that the E.coli LDH is having a Km of only 7.2 millimolar while the *Zymomonas mobilis* PDC is having a Km of only 0.4 millimolar. So, this significant lower Km value of Zymomonas mobilis PDC allows this PDC enzyme that is the pyruvate carboxylase to react to the pyruvic acid or pyruvate and effectively converts this pyruvic acid to acetaldehyde even if the LDH or the lactate dehydrogenase is operating in the engineered *E.coli* system.

And thereby even the LDH is operating in the *E.coli* system this engineered *E.coli* with PDC and ADH from Zymomonas mobilis can effectively convert glucose into ethanol.

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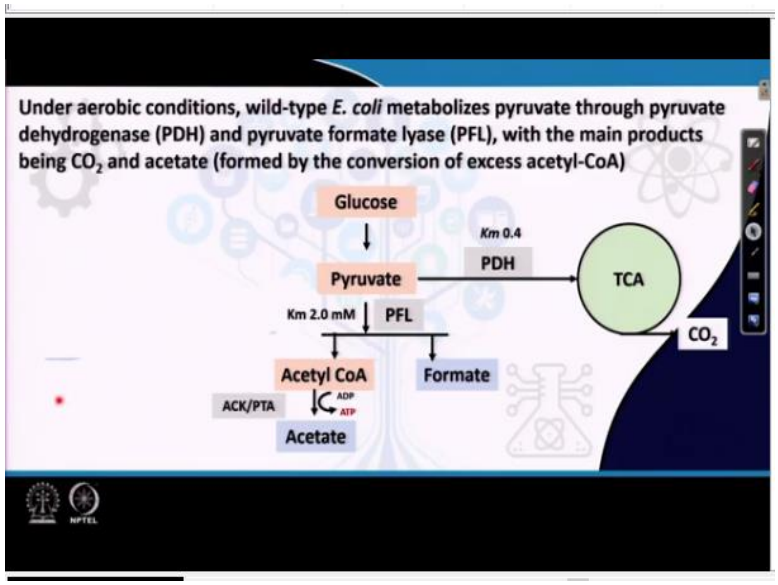

Now under aerobic condition the wild type *E.coli* metabolizes the pyruvic acid through pyruvate dehydrogenase as it is discussed earlier through this pyruvate dehydrogenase complex and pyruvate format lyase with the main products being carbon dioxide and acetate formed by the conversion of excess acetyl-CoA. So, under an aerobic condition both this PDH and the PFL pyruvate format lyase are quite active.

Now if we consider that the activity of this PDH and PFL along with the endogenous activity of LDH and the overexpressed activity of PDC and ADH, we will be able to understand the significance of this engineering process.

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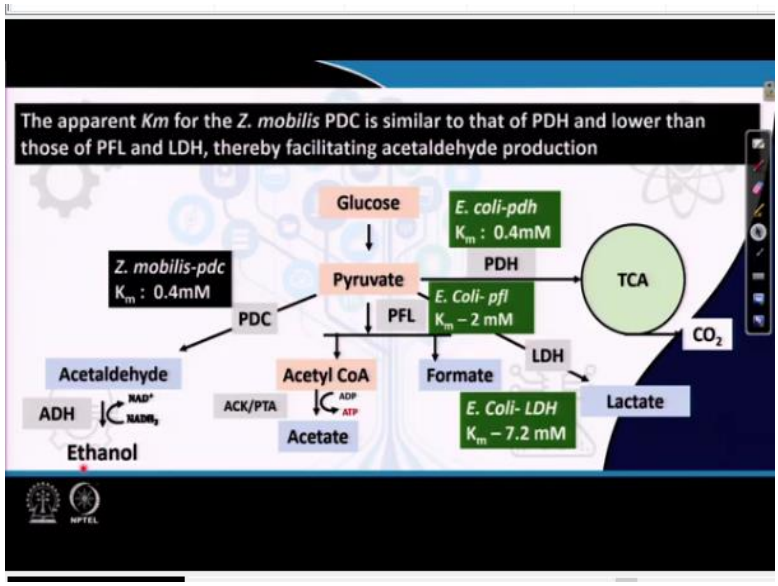

Now if we look at this the competing pathways for pyruvic acid utilization, there are pathways like the enzyme PDH which is redirecting the flux from pyruvic acid towards the

TCA cycle. There is a pathway for lactate production through LDH and also there is a pathway towards the formation of the formic as format and acetate through the enzyme PFL. Now if we look at this 3 enzymes Km value all these enzymes Km values are considerably higher than that of the *Zymomonas mobilis* PDC which is now in the engineered E.coli through the pet operon and it is constitutively expressed.

So, effectively under aerobic condition the engineered *E.coli* with the PDC and ADH from *Zymomonas mobilis* would utilize the pyruvic acid through pyruvate decarboxylase system and will convert it to acetaldehyde and acetaldehyde will be eventually converted to ethanol. Now the apparent came for this *Zymomonas mobilis* PDC is similar to that of PDH particularly if we compare the PDH to PDC.

So, these 2 are comparable so under aerobic condition a significant flux can still be allowed to flow through the PDC system, whereas the PFL and LDH will be significantly down because they are their Km values are much higher compared to the PDC.

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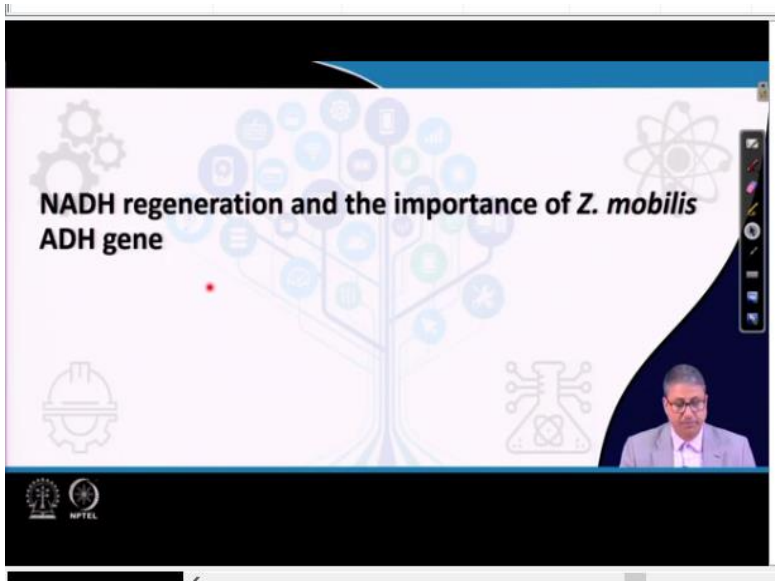

Now the another important aspect for these entire process is the NADH regeneration with the *Zymomonas mobilis* ADH gene.

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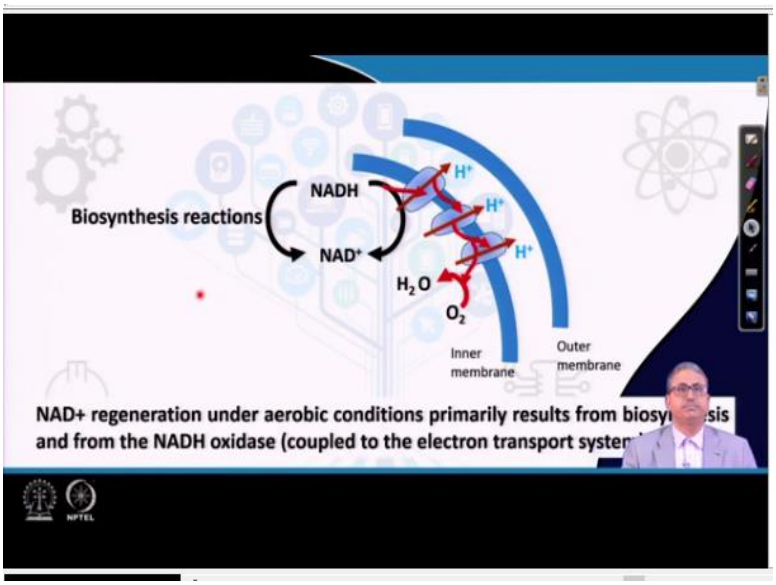

Now acetaldehyde is reduced with the help of NADH H + to produce the ethanol. Now if we look at the cellular fate of NADH H + which is produced during the different oxidative reactions. The NADH regeneration is a very important process and under aerobic condition it is primarily achieved through either the biosynthesis process so different electron consuming reactions often oxidize the NADH H + and converts it to NAD +.

Otherwise which is mostly allowed or preferred is the through electron transport system this NADH H + is oxidized.

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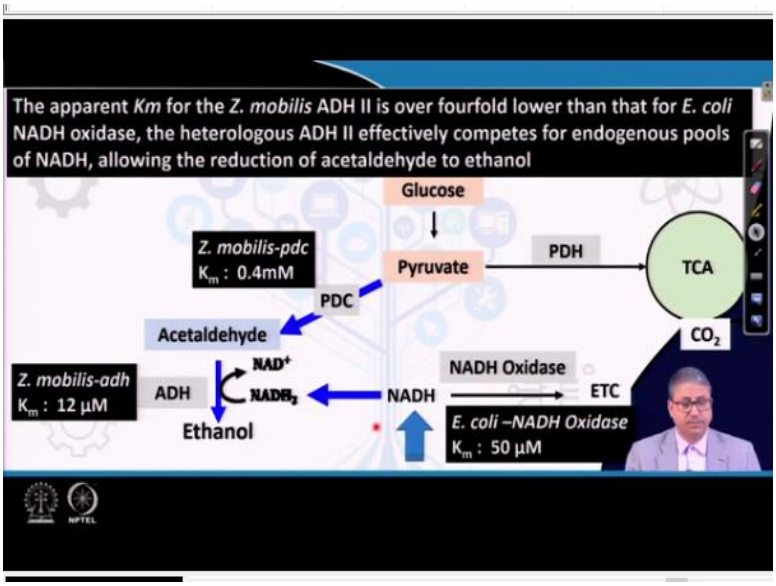

Now if we try to look into this ADH gene which is overexpressed and which is originally obtained from *Zymomonas mobilis*. So, we will be able to see that the NADH pool which is actually supplied from different oxidative reaction might go towards the electron transport

system where NADH oxidase will allow its oxidation or it might be flowing towards the acetaldehyde reduction process to ethanol, it depends on the enzyme activities.

Now the over express ADH activity will allow more efficient utilization of NADH because the *Zymomonas mobilis* ADH is having a Km value of 12 micromolar which is around 4 times less than that of the NADH oxidase. So, the apparent came for the *Zymomonas mobilis* ADH II which is a called ADH II is over fourfold lower than that of the *E.coli* NADH oxidase, the heterologous ADH II thereby effectively competes with the endogenous pool of NADH allowing the reduction of acetaldehyde to ethanol.

So, effectively the expression or over expression of these *Zymomonas mobilis* PDC and *Zymomonas mobilis* is ADH allows the engineered E.coli strain to redirect the flux from the pyruvic acid towards ethanol even in presence of the endogenous PDH or LDH or PFL or NADH oxidase.

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Now there are also advantages under the anaerobic condition.

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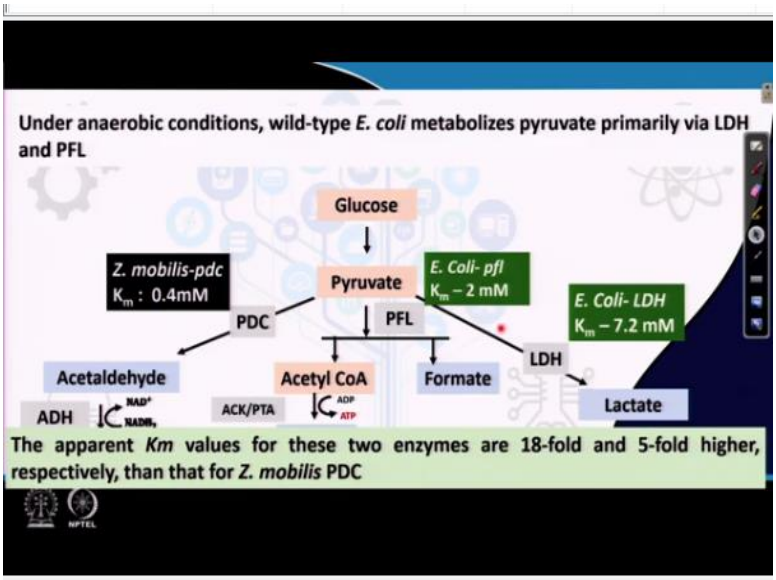

Now under the anaerobic condition it is mostly the wild type *E.coli* metabolizes pyruvate primarily by the lactate dehydrogenase and the pyruvate format lyase. So, these 2 enzymes LDH and PFL are more prominent during the anaerobic condition and allowing the conversion of pyruvic acid towards lactic acid and acetic acid or format. Now again if we try to compare the Km values of these 2 enzymes LDH and PFL along with the Zymomonas mobilis PDC.

We will identify or will find that Zymomonas mobilis PDC is significantly lower than that of the E.coli PFL and E.coli LDH.

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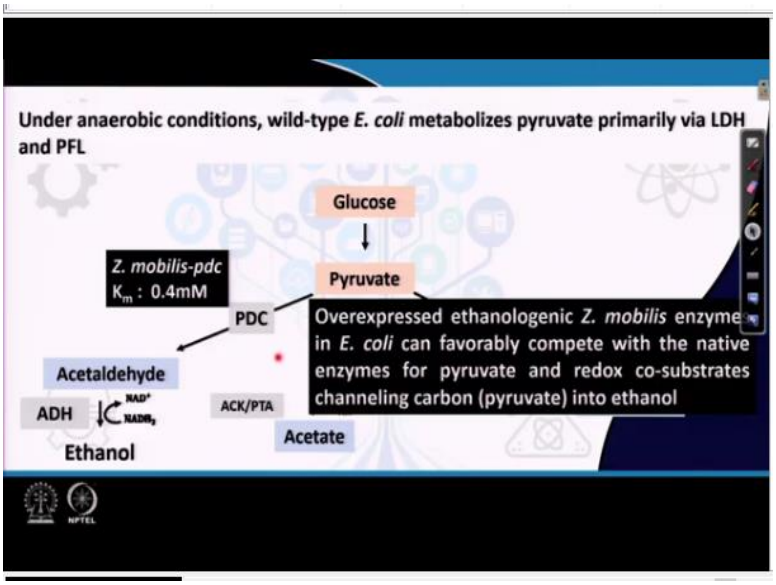

Thereby the apparent Km value comparison clearly indicates that the overexpressed ethanologenic Zymomonas mobilis enzymes in E.coli that is the engineered E.coli can

favourably compete with the native enzymes of pyruvate and redox co-substrates channeling carbon pyruvic acid into ethanol. So, under both aerobic as well as anaerobic condition this Zymomonas mobilis PDC and ADH which are engineered or used to engineer the E.coli cells are able to support the engineered E.coli strength to redirect the flux from pyruvic acid towards ethanol very effectively.

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Now this heterologous fermentation pathway produces 95% of the final products as ethanol without creating any redox imbalance and consumes only 1 NADH.

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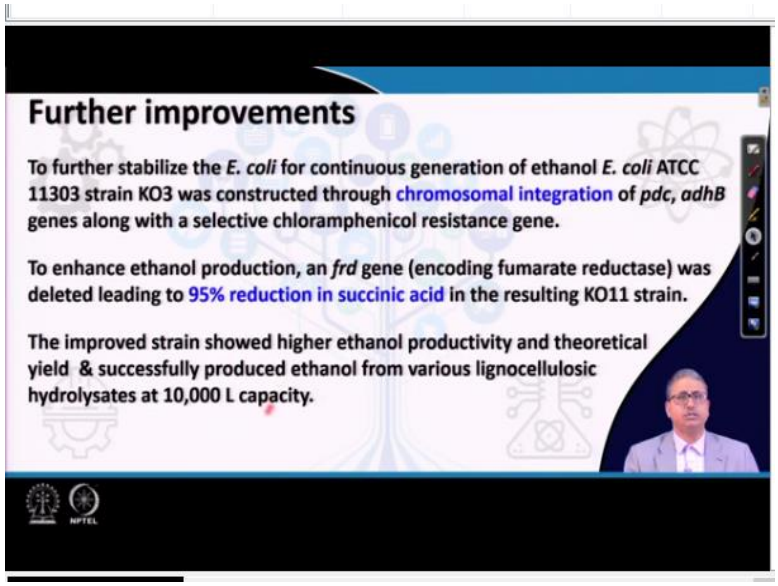

Now there are a number of improvements which are required or improvements which are achieved or done over this pet operon based system which included the Zymomonas mobilis PDC as well as ADH gene. So, these improvements include to further stabilize the E.coli for

continuous generation of ethanol. The E.coli ATCC 11303 strain K03 was constructed through chromosomal integration of pdc, adhB genes.

Because there might be some plasmid instability and maintenance of the plasmid copy numbers, the pet plasmid which is developed based on the Zymomonas mobilis PDC and ADH. So, there was initial attempt and a successful attempt to integrate these genes PDC and ADH into the *E.coli* chromosome. To enhance the ethanol production an important gene which is called fumarate reductase was deleted leading to 95% reduction in succinic acid.

So, succinic acid is one of the major issues with the production of the co-metabolites or which is actually the metabolite which is not required during the ethanol production. So, reduction of succinic acid is an important requirement for successful and cost effective strain development. So, this deletion of the frd gene was done and that allowed 95% reduction in succinic acid resulting into the KO11 strain.

Now the improved strain deriving from the KO11 strain showed higher ethanol production and theoretical yield and successfully produced ethanol from various lignocellulosic hydrolysates at 10000 liter capacity.

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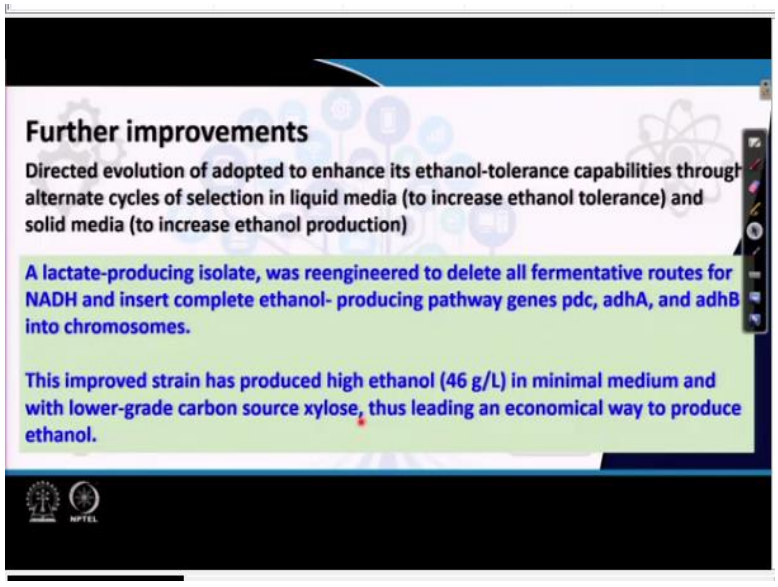

So, further improvements are also continued directed evolution of adopted to enhance the ethanol tolerance capacities through alternate cycle of selection in liquid media to increase the ethanol tolerance and solid media to increase the ethanol production. A lactate producing

isolate was re-engineered to delete all fermentative routes for NADH and insert complete ethanol producing pathway genes like pdc, adhA and adhB into the chromosome.

This improved strain has produced high ethanol 46 gram per liter in minimal medium and with lower grade carbon source xylose, thus leading to an economical way of producing ethanol.

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Now along with these developments through metabolic engineering to produce the bioethanol, other biofuel productions related advancements were also made. A number of techniques of metabolic engineering and synthetic biology are now available and used to enable the new fuel producing pathways to be constructed in E.coli.

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Particularly to increase the product concentration and productivities E.coli strains are developed to express heterologous enzymes with higher catalytic activities for key intermediates in the new pathway and gene knockouts constructed to channel greater carbon flux towards the desired product.

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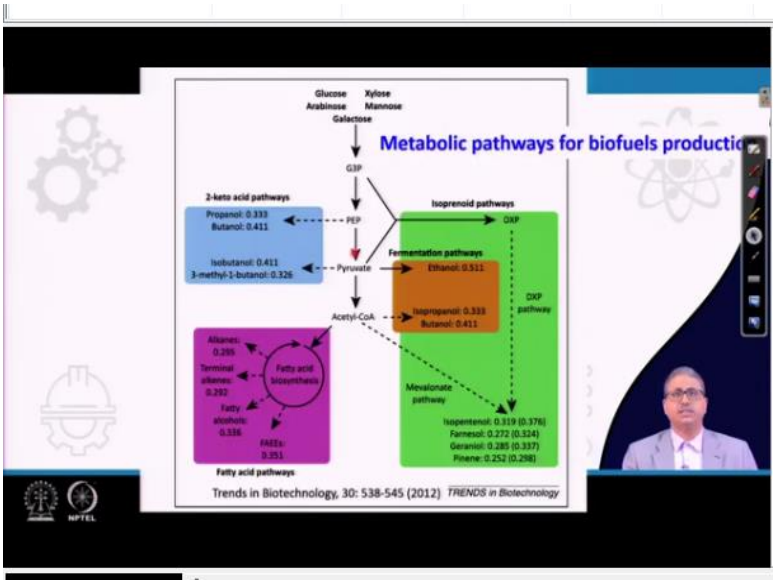

As presented in these schematics the metabolic pathway for biofuel production in E.coli is presented and we can see that the production of acetyl-CoA and pyruvic acid and phosphoenol pyruvic acid can be redirected to produce a number of advanced or other biofuel compounds other than ethanol and that includes propanol, butanol, isobutanol, different type of alkanes and through fatty acid biosynthesis and also other isoprenoid compounds along with the ethanol and isopropanol.

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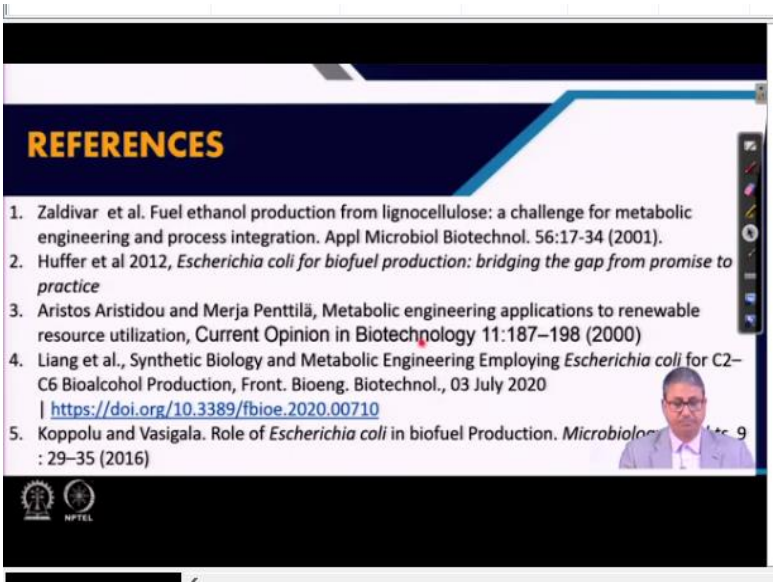

So, this part of the lecture has used the following references and particularly I would like to emphasize upon a few papers including the Zaldivar review on fuel ethanol production for lignocellulose. This Huffer 2012 paper on E.coli for biofuel production and the metabolic engineering applications to renewable resource utilization by Aristos and Merja.

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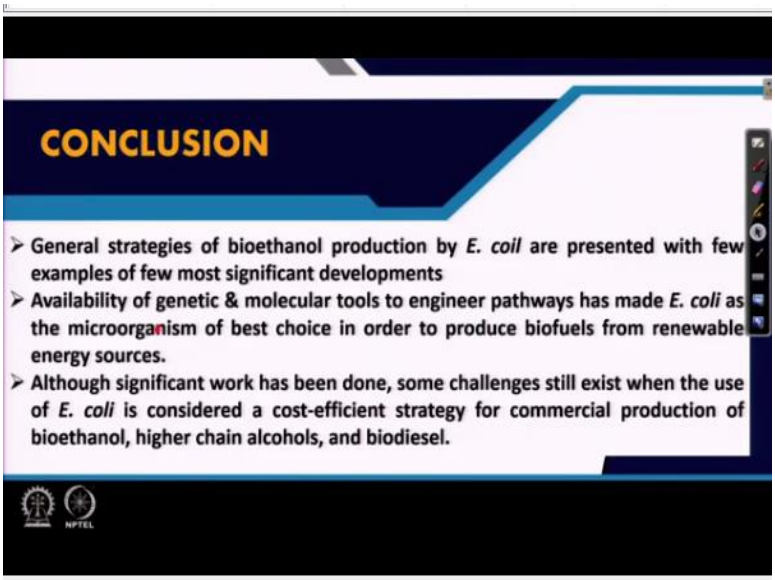

So, in conclusion the general strategies of bioethanol production by E.coli are presented with few examples of most significant developments. Availability of genetic and molecular tools to engineer pathways has made E.coli as the microorganism of best choice in order to produce biofuels from renewable energy sources. And although significant work has been done some challenges still exist when the use of E.coli is considered a cost efficient strategy for commercial production of bioethanol higher chain alcohol and biodiesel.

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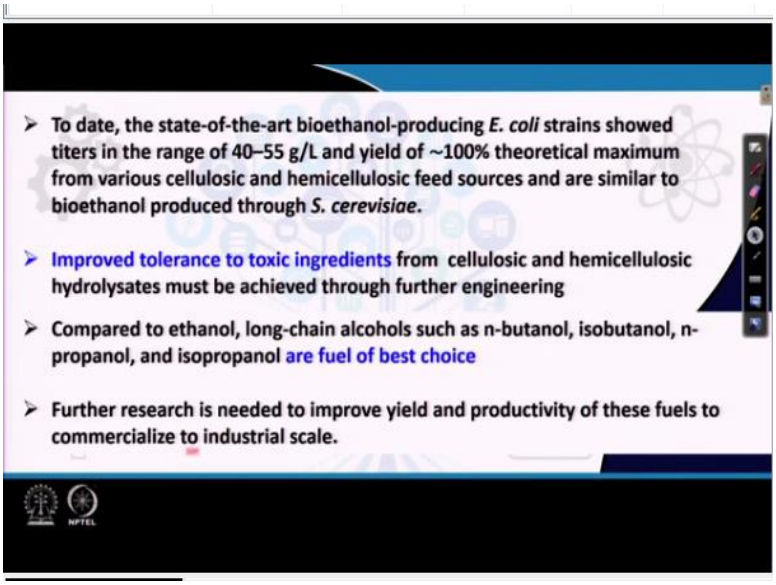

The state of the art bioethanol producing E.coli today showed the titers in the range of 40 to 55 gram per liter and yield of around 100% theoretical maximum from various cellulosic and hemicellulosic feed sources and are similar to bioethanol produced through Saccharomyces cerevisiae. However, considering the use of the hemicellulosic feed sources or cellulosic feed sources which are not purified and thereby likely to have many toxic ingredients.

So, improved tolerance to toxic ingredients from this cellulosic and hemicellulosic hydrolyzed must be achieved through further engineering. It is also emphasized that compared to ethanol production of long chain alcohol such as butanol, isobutanol, propanol, isopropanol are fuel of best choice and further research is needed to improve the yield and productivity of these fuels to commercialize the industrial scale application of E.coli, thank you.