Metabolic Engineering
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Lecture-35
Metabolic Engineering for Biofuel Production-Part B

In this lecture on metabolic engineering and its application in biofuel production the following concepts will be covered.

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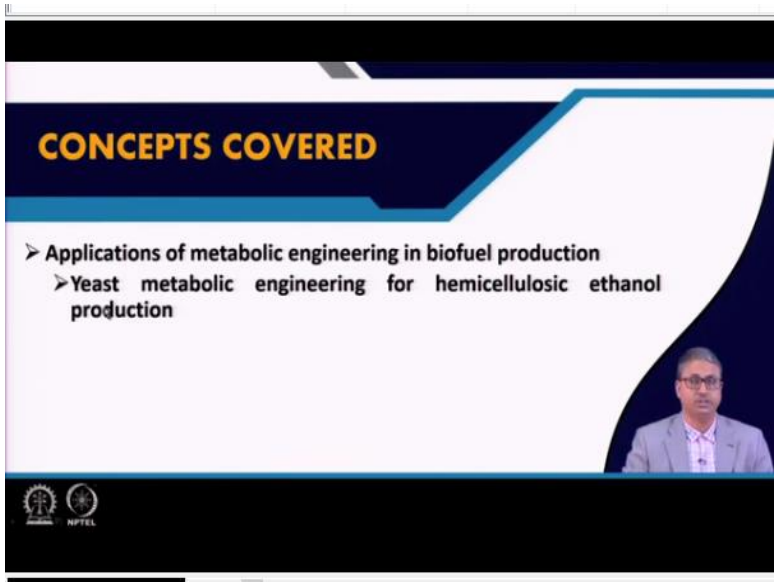

We will be discussing the application of metabolic engineering in biofuel production and we would emphasize on the metabolic engineering strategies adapted for improving the hemicellulose fermentation towards ethanol production in yeast.

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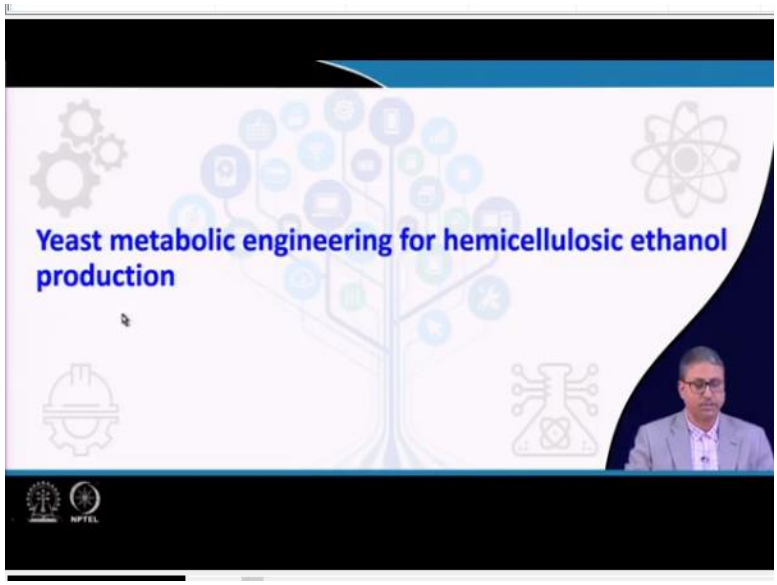

So, yeast metabolic engineering for hemicellulosic ethanol production will be discussed.

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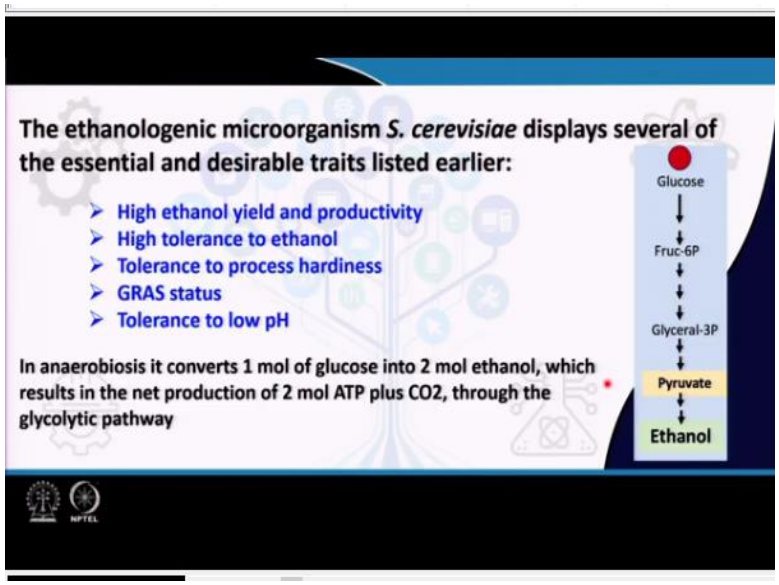

Now the ethanolgenic microorganisms which basically include the *Saccharomyces cerevisiae* displays several of the essential and desirable traits listed earlier including the high ethanol yield and productivity, high tolerance to ethanol, tolerance to process hardliness, GRAS status and tolerance to low pH. So, a number of desirable characters or essential characters are highly qualified by *Saccharomyces cerevisiae*.

And it is also found that following this scheme of metabolic reactions which is the Embden-Meyerhof-Parnas pathway mainly which allows the yeast cells to convert the glucose to pyruvic acid and then 2 step fermentation process often leads to the production of ethanol. Now in anaerobiosis these particular metabolic reactions can convert 1 mole of glucose into 2

moles of ethanol which with resulting net production of 2 moles of ATP plus carbon dioxide through the glycolytic pathway.

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Now bioconversion of lignocellulose to ethanol must occur at high rate in good yield and to concentrations that are economically recoverable. With hemicellulose these goals are much more difficult to achieve why? Because hemicellulose is composed of the pentose sugars and when we talk about high yield high concentration of ethanol etcetera the overall the process is impaired or affected adversely by the presence of the pentose sugar in the hemicellulose and also by the inability of the *Saccharomyces cerevisiae* in utilizing or fermenting pentose sugars like xylose to alcohol.

But otherwise with cellulose or with sucrose these kind of organisms are very efficient to convert the glucose or hexose residues to ethanol.

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Now for hemicellulose it is the use of glucose, xylose, mannose, galactose, arabinose and rhamnose and because these are the constituents of the hemicellulosic biomass, in the presence of acidic and ferulic acids which are produced during these processing of the pretreatment and hydrolysis process along with various degradation products from thermochemical pretreatments.

So, the challenges are multiple the organism *Saccharomyces cerevisiae* is very well equipped to produce ethanol utilizing glucose, but when the glucose is present along with the many other pentose sugars and further with the inhibitors like acetic acid and ferulic acid and different other thermochemical pretreatment generated ingredients. The overall fermentation of the carbon substrates including the hexose and pentose sugars out of the lignocellulosic biomass becoming very challenging for the *Saccharomyces cerevisiae*.

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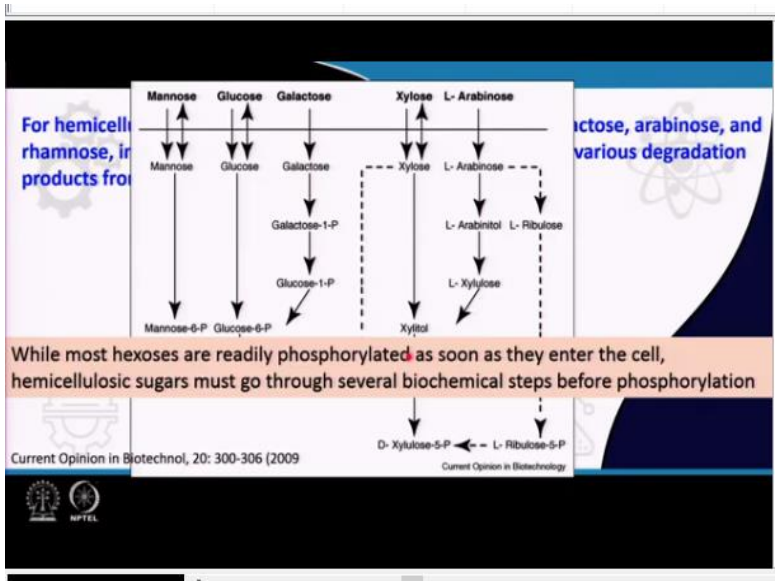

So, here is the schematic diagram by which we can clearly see that the different hexose sugars can very well or readily can be incorporated into the Embden-Meyerhof-Parnas pathway to produce the pyruvic acid and ethanol because mannose, glucose, galactose, everything can be converted essentially to fructose 6 phosphate and fructose 6 phosphate will be converted to fructose 1-6 bisphosphate and then subsequently phosphoglyceraldehyde and phosphoenolpyruvate pyruvic acid.

And then from pyruvic acid ethanol can be produced. But with respect to the xylose and arabinose and other pentose sugar though there are steps but the steps are not always present or even they are present they are not properly regulated and some of the intermediate like xylitol or arabinitol are often found to have different kind of toxic effects on the cellular systems.

Now the most of the hexose sugars are readily phosphorylated as soon as they are entering into the yeast cell or the *Saccharomyces* cells. But the hemicellulosic sugars which are representing the pentose sugars like the xylose and arabinose must go through several biochemical steps before phosphorylation. So, the phosphorylation which is essential to enter or to allowing them to enter into the pentose phosphate pathway because then only these pentose sugars will be eventually converted to intermediate metabolites which will be easily utilized by the Embden-Meyerhof-Parnas pathway requires a number of prior steps.

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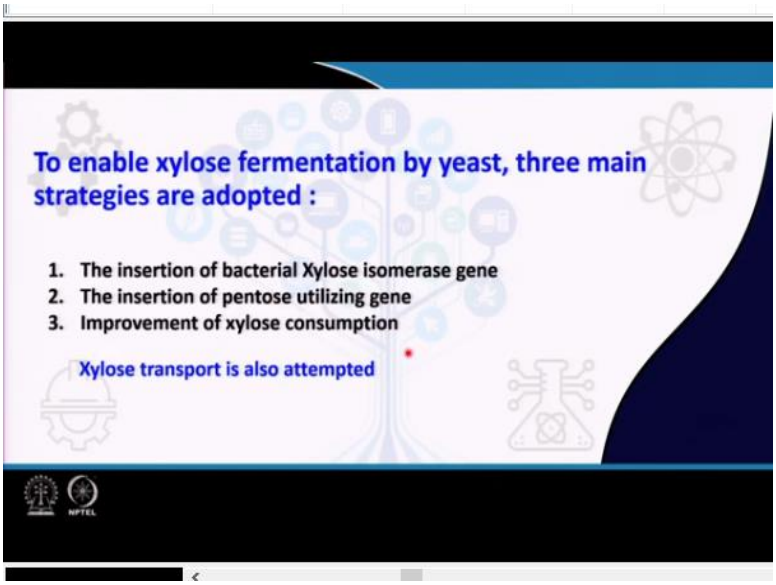

So, to enable the xylose fermentation by yeast 3 main strategies are adopted which are the insertion of bacterial xylose isomerase gene. So, that the xylose can be converted to xylulose and then xylulose-5-phosphate can be produced. The insertion of the pentose utilizing gene and improvement of the xylose consumption including the xylose transport also.

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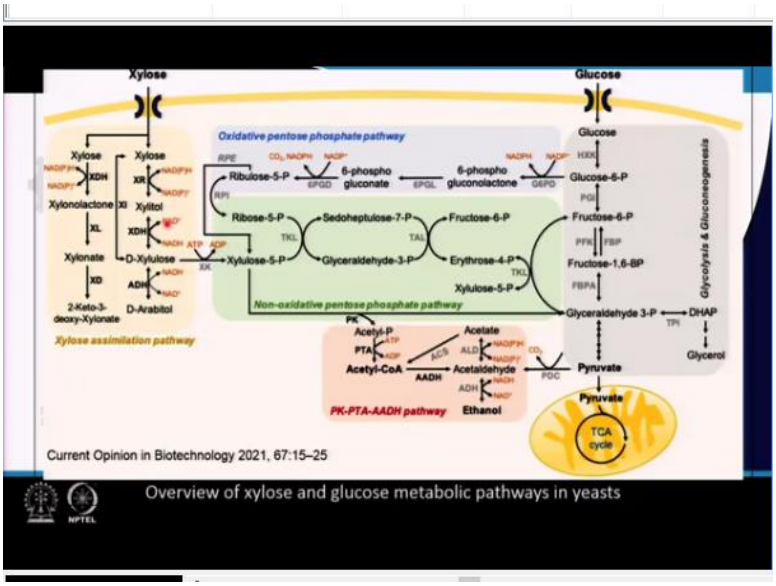

Now here is the broad overview of the xylose and glucose metabolic pathways in yeast or *Saccharomyces cerevisiae* in particular. As you can see over here we have the pentose phosphate pathway, entire pentose phosphate pathway the oxidative process is mainly highlighted over here and the glucose metabolism is present over here and once this pyruvate is produced the pyruvate is the essential metabolite in order to achieve the ethanol production.

Because once this pyruvic acid is produced the pyruvate can be metabolized through a number of steps either through acetate acetaldehyde acetyl-CoA and finally ethanol will be produced. So, when we have glucose as our substrate glucose can readily be converted through Embden-Meyerhof-Parnas pathway to pyruvic acid and pyruvic acid can be reduced to produce the ethanol.

But when we have the pentose sugars the first of all the pentose sugars has to be or the plantar sugars need to be converted to their phosphorylated form like the xylulose-5-phosphate which will then be processed through the pentose phosphate pathway enabling them to produce either the fructose-6-phosphate or the glyceraldehyde-3-phosphate which will take part in the main Embden-Meyerhof-Parnas pathway.

So, quite a few steps are involved in order to achieve metabolism of xylose by the organisms which are not native xylose utilizing organisms.

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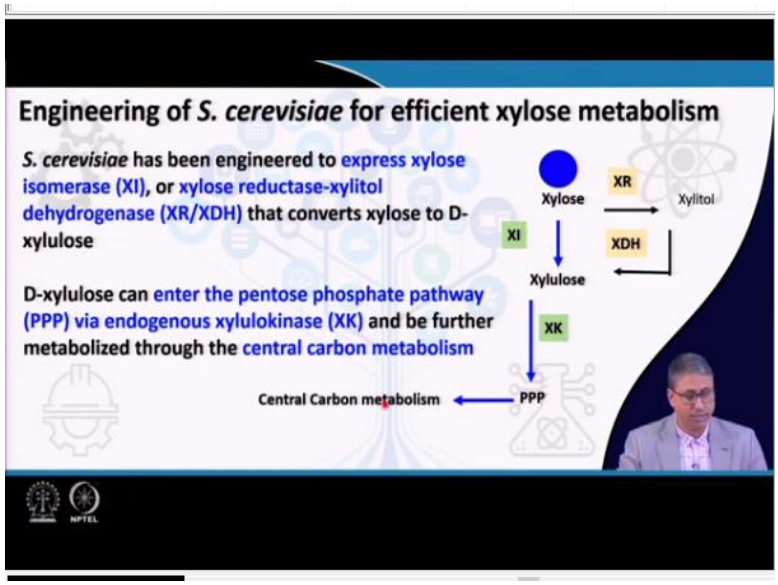

Now engineering of *Saccharomyces cerevisiae* for efficient xylose metabolism has been attempted for several decades and a great extent of success is achieved with some reservations or some limitations which the scientists and the researchers working on the metabolic engineering aspect of *Saccharomyces cerevisiae* towards utilization of the pentose sugar and the formation of ethanol and other biofuel.

They are called constantly working on that. So, fundamentally the Saccharomyces cerevisiae has been engineered to express the xylose isomerase which converts the xylose to xylulose or

the xylose reductase which can reduce the xylose to xylitol and then xylitol dehydrogenase which can convert the xylitol to xylulose and then these xylulose can be eventually converted to or processed to the xylulose-5-phosphate and xylulose-5-phosphate can enter into the pentose phosphate pathway leading to the production of the glyceraldehyde-3-phosphate and fructose-6-phosphate, thus allowing its entry into the central carbon metabolism.

So, the engineering could be done either by improving the xylose isomerase or xylose reductase and xylitol dehydrogenase or keeping in mind the entire system as well as the xylulokinase which is responsible for converting or phosphorylating xylulose to xylulose-5-phosphate and thereby allowing the entry of xylose into the central carbon metabolism.

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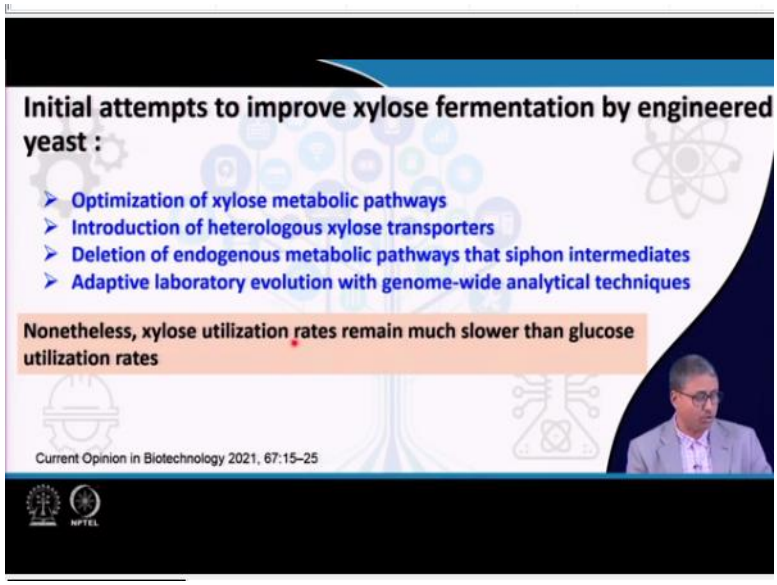

Now initial attempts to improve the xylose fermentation by engineered yeast particularly the *Saccharomyces cerevisiae* were targeted towards the optimization of xylose metabolic pathways, introduction of heterologous xylose transporters from different organisms including other yeast and bacteria, deletion of endogenous metabolic pathways that take out or siphon out the intermediates and adaptive laboratory evolution with genome wide analytical techniques.

After targeting or utilizing all these diverse type of approaches or strategies xylose utilization rates remain much slower than the glucose utilization rate, because these organisms are intrinsically glucose metabolizers, so, they use these *Saccharomyces cerevisiae* in particular. They are intrinsically capable of utilizing glucose very efficiently and not utilizing pentose sugar like xylose efficiently or sometimes not at all.

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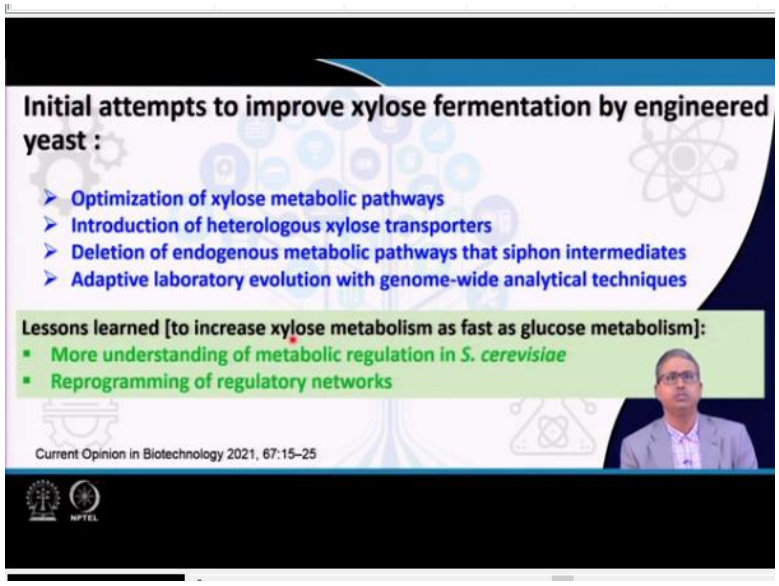

So, while trying to improve the xylose utilization abilities of these microorganisms we have learned or the scientist working on metabolic engineering of *Saccharomyces cerevisiae* have learned a number of things which are basically to increase the xylose metabolism as fast as glucose metabolism, because glucose metabolism to ethanol in *Saccharomyces cerevisiae* is considered to be very efficient, very high, but not xylose metabolism.

So, more understanding of the metabolic regulation of *Saccharomyces cerevisiae* and reprogramming of the regulatory networks found to be very important in order to enhance the glucose metabolism and make it a kind of comparable with that of the glucose metabolism.

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Recently adaptive evolution strategies were used to improve the xylose metabolism by the *Saccharomyces cerevisiae*. In this study the researcher used a combined genome sequencing proteome profiling and metabolomic analysis of this Saccharomyces cerevisiae and with a series of experiments and development of different mutants. They were able to achieve improved cell growth, xylose consumption and specific ethanol productivity by engineering the Saccharomyces cerevisiae, xylose isomerase pathway.

So, and this is the paper which actually elaborates the method for directed evolution for highlighting the epistatic interactions that alter the metabolic regulations and enable anaerobic xylose utilization by *Saccharomyces cerevisiae*.

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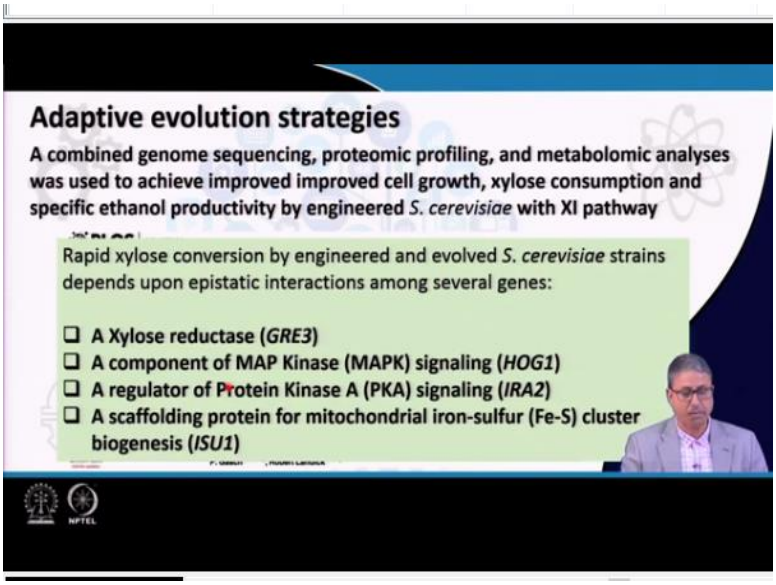

And this metabolic engineering attempt highlighted that the rapid xylose conversion by engineered and evolved *Saccharomyces cerevisiae* strains depend upon epistatic interaction among several genes that include xylose reductase GR3, a component of the MAP kinase MAPK signaling which is called HOG1, a regulator of the protein kinase A or PKA signaling that is called IRA2.

And a scaffolding protein for mitochondrial iron-sulfur cluster biogenesis that is ISU1. So, the interaction between all these different regulators and scaffolding protein enables the metabolically engineered *Saccharomyces cerevisiae* to produce high ethanol from xylose.

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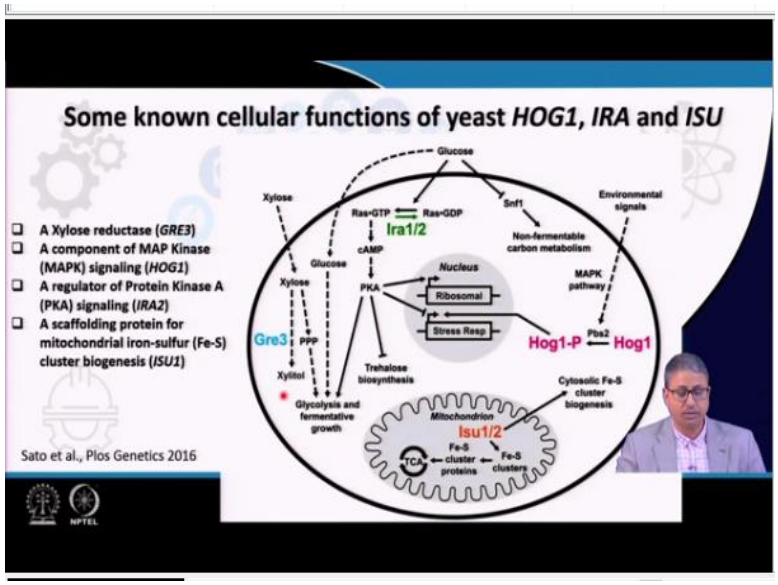

And here are these genes within the metabolic pathway or the broad general scheme of the metabolic pathway where the cellular functions of this xylose reductase, MAP kinase, regulatory protein IR and also the scaffolding a protein for mitochondrial iron-sulfur protein is indicated.

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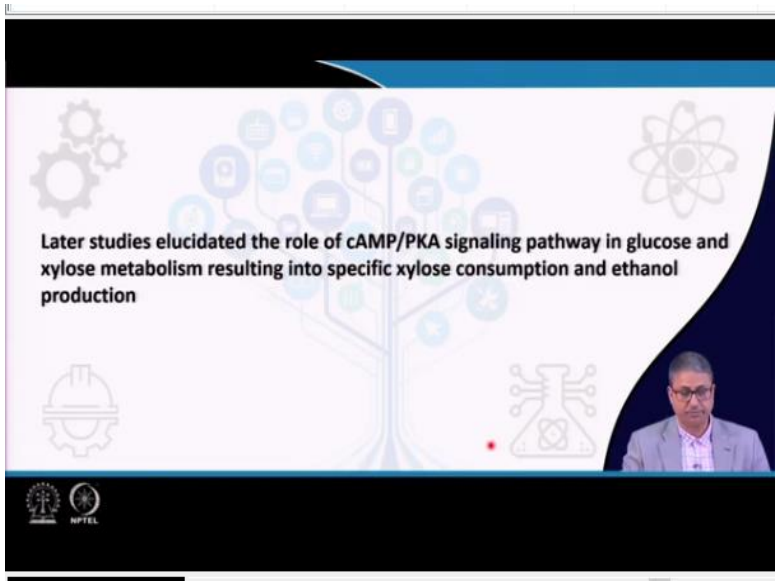

Subsequent studies elucidated the role of cyclic AMP and PKA signaling pathway in detail with respect to glucose and xylose metabolism because an intricate balance between the glucose and xylose metabolism is there and always the glucose is preferred. So, in order to achieve xylose metabolism independently or along with glucose it was found to be essential to understand these regulation between the glucose and xylose metabolism and that resulted into specific xylose consumption and ethanol production by the engineered organism.

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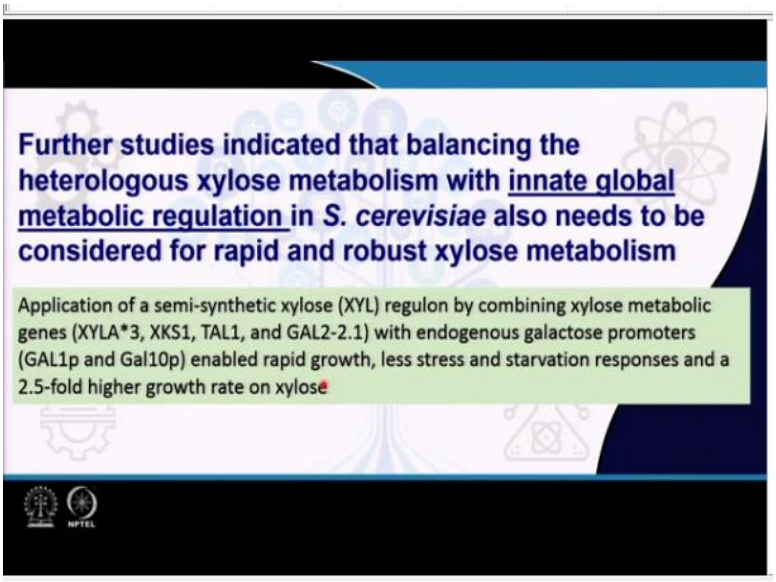

Further studies indicated that balancing the heterologous xylose metabolism with innate global metabolic network or metabolic regulation in the yeast also needs to be considered for rapid and robust xylose metabolism. So, it is not merely expressing 1 or 2 genes rather the xylose metabolism ability of *Saccharomyces cerevisiae* is connected to the inner global metabolic regulation.

And application of semi-synthetic xylose regulon by combining the xylose metabolic genes a number of xylose metabolism genes with endogenous galactose promoters, enabled rapid growth, less stress and starvation response and 2.5 fold higher growth rate on xylose.

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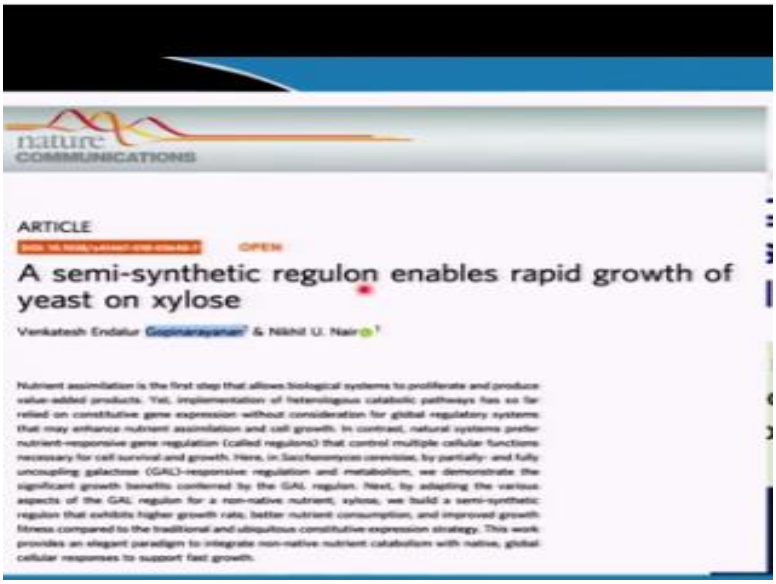

So and here is the kind of screenshot of the paper which is published and then it discussed about these particular semi-synthetic regulon which enables the rapid growth of yeast on growth.

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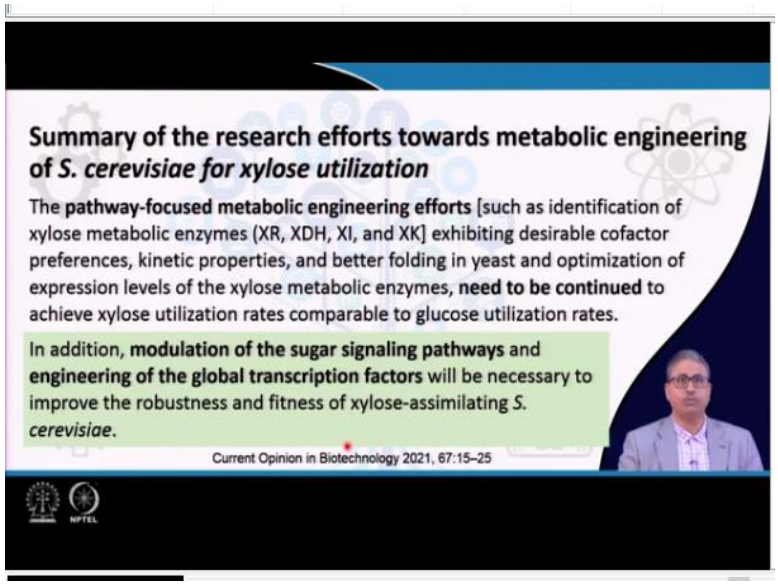

So, overall it was summarized that the research effort towards metabolic engineering of *Saccharomyces cerevisiae* for xylose utilization can be suggested that the pathway focused metabolic engineering efforts such as identification of xylose metabolic enzymes like the xylose reductase and dehydrogenase, xylose isomerase, etcetera exhibiting desirable cofactor preference kinetic properties and better folding in yeast and optimization of expression levels of xylose metabolic enzyme need to be continued to achieve xylose utilization rate comparable to glucose utilization rates.

And in addition modulation of sugar signaling pathways and engineering the global transcription factors will be necessary to improve the robustness and fitness of the xylose assimilating *Saccharomyces cerevisiae*.

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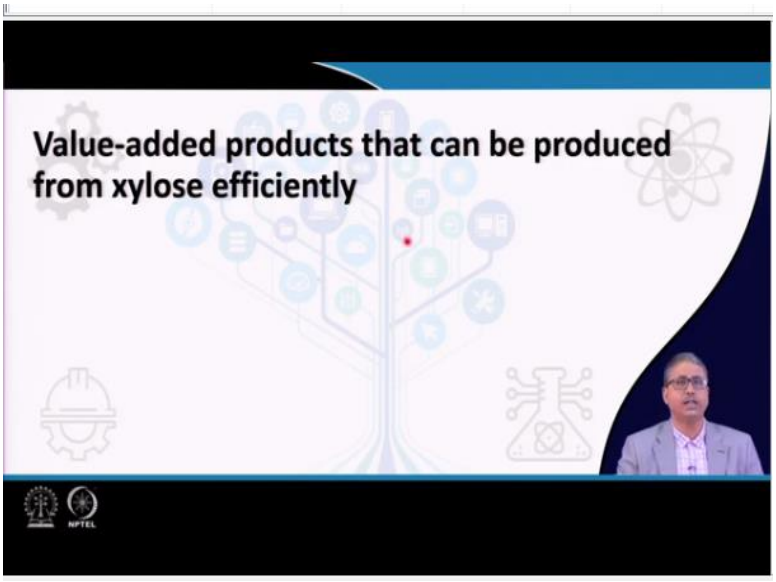

Now apart from the production of ethanol using these xylose as a part of the lignocellulosic feedstock several value added products were also targeted and found to be produced from the xylose efficiently.

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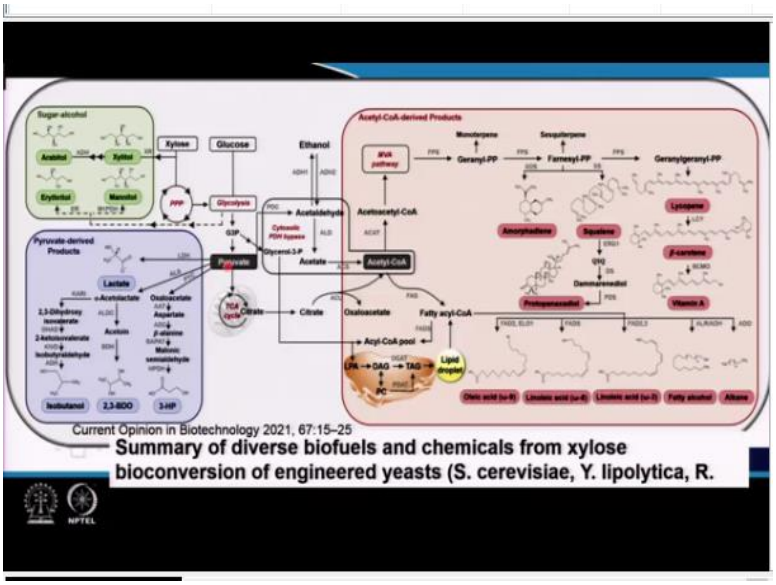

So, here is the broad outline of the diverse biofuels and other chemicals which can be produced from xylose through bioconversion of engineered yeast and as you can see that from the pyruvic acid the glucose through glycolysis the pyruvate is going to be produced.

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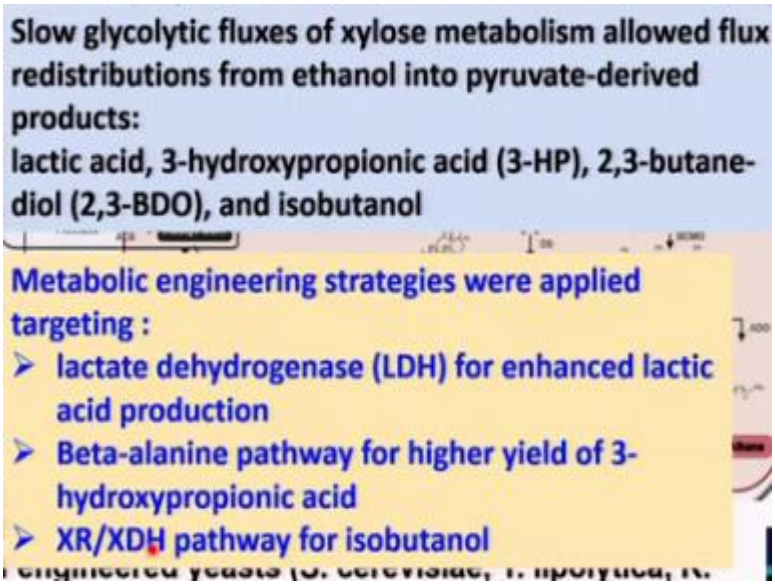

And this pyruvic acid can be utilized to produce a number of products. So, the slow glycolytic flux of the xylose metabolism allowed flux redistribution from the ethanol into pyruvate derived product. Since the native ethanol production might be reduced because of the xylose, because xylose is not readily metabolized. So, a number of pyruvate derived product including the lactic acid, isobutanol, 3-hydroxypropinic acid and 2,3 butanediol were found to be produced from the pyruvic acid.

And metabolic engineering strategies were applied targeting lactate dehydrogenase for enhanced lactic acid production, beta-alanine pathway for higher yield of 3-hydroxypropanic acid and also the xylose reductase or xylose dehydrogenase pathway for isobutanol production.

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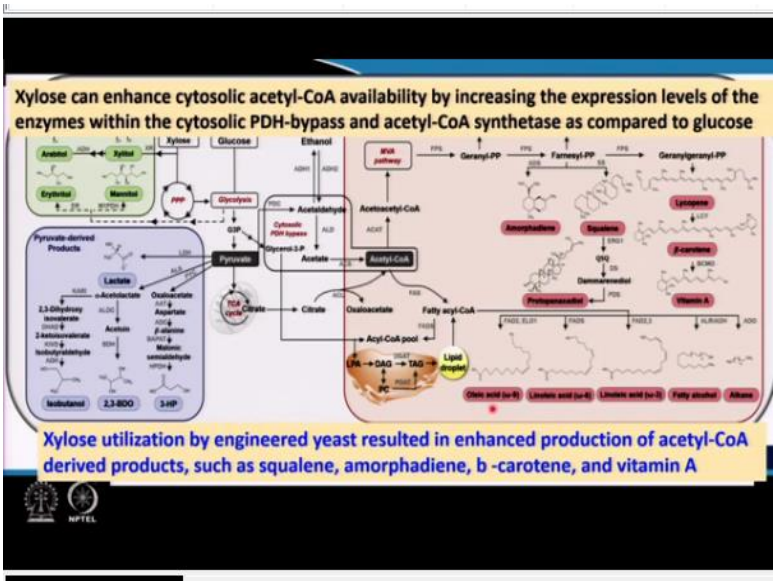

It is also observed that xylose can enhance cytosolic acetyl-CoA availability by increasing the expression level of the enzymes within the cytosolic PDH-bypass and acetyl-CoA synthetase as compared to glucose. And xylose utilization by the engineered yeast resulted into the enhanced production of a number of compounds including the acetyl-CoA derived products such as the squalene, amorphadiene, beta carotene and vitamin A etcetera.

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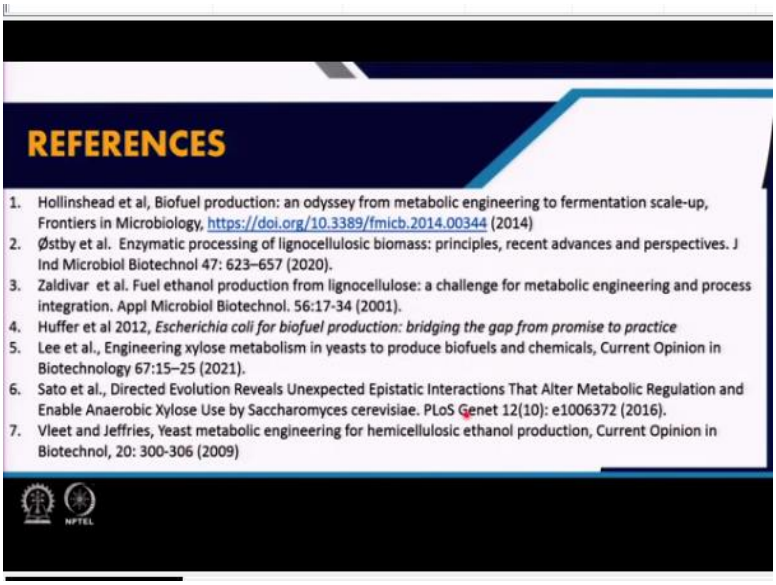

So, in this part of the lecture I have used the following references particularly the recent paper published by Lee et al in current opinion in biotechnology engineering xylose metabolism in yeast to produce biofuel and chemicals and yeast metabolic engineering for hemicellulosic ethanol production. Another paper review published in current opinion biotechnology in 2009 were found to be very useful.

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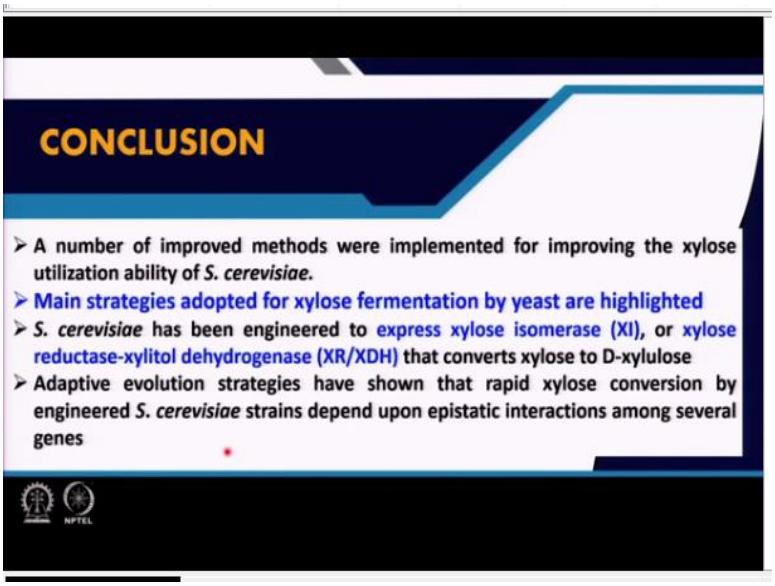

So, in conclusion a number of improved methods were implemented for improving the xylose utilization ability of *Saccharomyces cerevisiae*. Main strategies adopted for xylose fermentation by yeast are highlighted. *Saccharomyces cerevisiae* has been engineered to express xylose isomerase, xylose reductase, xylose dehydrogenase that convert xylose to D-xylulose (20:55).

And then subsequently metabolize through pentose phosphate pathway. Adaptive evolution strategies have shown that the rapid xylose conversion by engineered Saccharomyces cerevisiae strains depend upon epistatic interactions among several regulatory genes.

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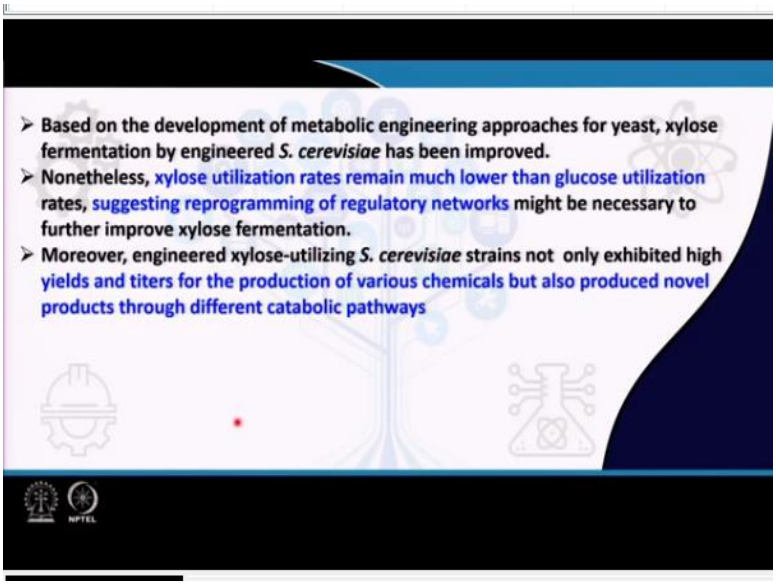

Based on the development of metabolic engineering approaches for yeast xylose fermentation by engineered Saccharomyces cerevisiae has been improved significantly. Nevertheless xylose utilization rates remain much lower compared to glucose utilization rate, suggesting that the reprogramming of regulatory networks might be necessary to further improve the xylose fermentation and to produce the biofuel including bioethanol successfully and cost effectively.

Moreover the engineered xylose utilizing Saccharomyces cerevisiae strains not only exhibited high yields and titers for the production of various chemicals, but also produced novel products through different catabolic pathways, thank you.