Metabolic Engineering
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Lecture-34
Metabolic Engineering for Biofuel Production-Part A

In today's lecture we are going to discuss the application of metabolic engineering with reference to biofuel production.

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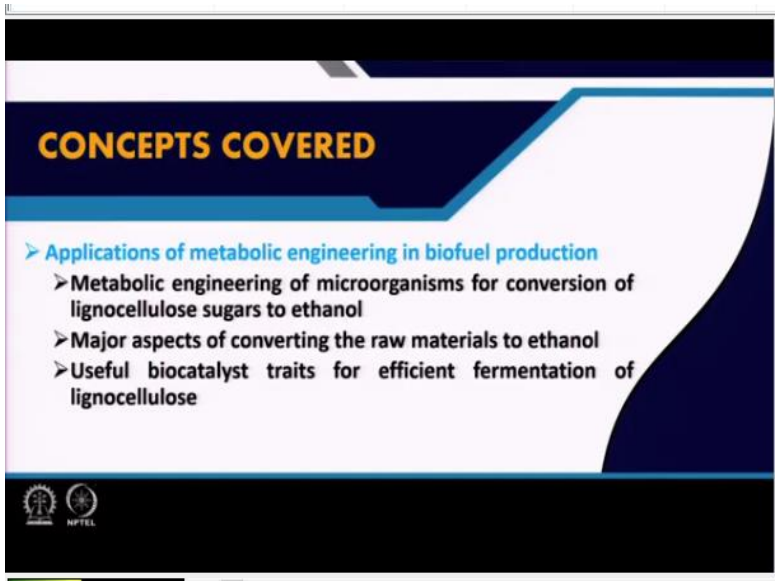

The major aspects those will be covered in this lecture would be the metabolic engineering strategies adopted for conversion of lignocellulosic sugars to ethanol. Major aspects of converting the raw materials present within the lignocellulosic biomass to ethanol and useful biocatalyst traits for efficient fermentation of lignocellulosic biomass.

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Now raw materials for ethanol production crops and lignocellulosic biomass and others. Now the raw materials which are used for biofuel or bioethanol production could be categorized into 2 types, one is the different type of crops; the other is the lignocellulosic biomass. There are other materials also like agro waste, forestry waste, even food waste and other industrial waste are being utilized and appropriate metabolic engineering strategies and process development processes are going on.

However, we are going to discuss mainly keeping in mind about these crops and lignocellulosic biomass, because most of the metabolic engineering processes are well developed based on these 2 raw materials. So, the crops are basically the sugar cane,beet, (02:18) corn and other cereals which are tested and utilized to varying extent for the production of biofuels particularly the bioethanol.

For example the use of corn and cereals particularly corn was very popularly used in USA and it is basically composed of starch which upon hydrolysis and subsequent depolymerization produces glucose residues. The sugarcane is mostly used as a resource for ethanol production in countries like Brazil and India. And the sugarcane is basically composed of sucrose which upon hydrolysis and depolymerization lead to the production of the fructose and glucose monomers.

On the other hand the lignocellulosic biomass which is obtained from a great extent of or range of forestry and other agricultural waste materials or agricultural products are composed of 3 important components, the cellulose, hemicellulose and lignin. Cellulose represents

around 45% dry weight of the lignocellulosic biomass. Hemicellulose represents around 30% dry weight of the lignocellulosic biomass.

And lignin corresponds to around 25% of the lignocellulosic biomass. Now both the cellulose and hemicellulose components which are basically constituted by different type of hexose and pentose sugars are subjected for the production of bioethanol or biofuel production.

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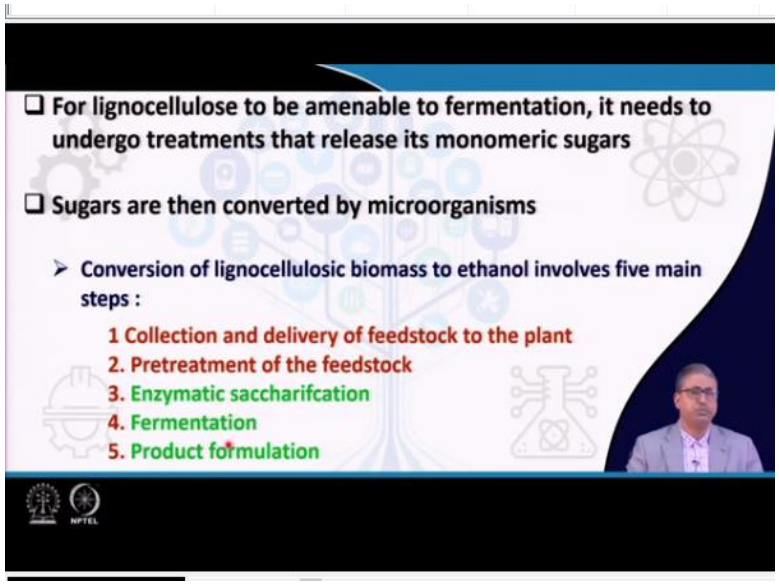

Now for lignocellulose to be amenable to fermentation ethanol production based fermentation or other biofuel based fermentation, it needs to undergo treatments that release its monomeric sugars because lignocellulose as we mentioned is composed of cellulose, hemicellulose and lignins. So, the delignification and then the subsequent treatments and hydrolysis are required to produce the monomeric sugars.

So, that the sugar monomers can be utilized by the microorganisms during the process of fermentation. Now these sugars which are released upon the depolymerization and hydrolysis are converted by microorganisms and conversion of the lignocellulosic biomass to ethanol basically involves 5 main steps. The steps are as follows, the collection and delivery of feedstock to the plant.

Feedstock in this case the lignocellulosic biomass, pretreatment of the feedstock, enzymatic saccharification, fermentation and then the product formation.

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Now in order to make the progress or the entire process viable all these steps need to be considered from the economic point of view with primary focus on feedstock handling, pretreatment and enzyme efficiency and enzyme cost.

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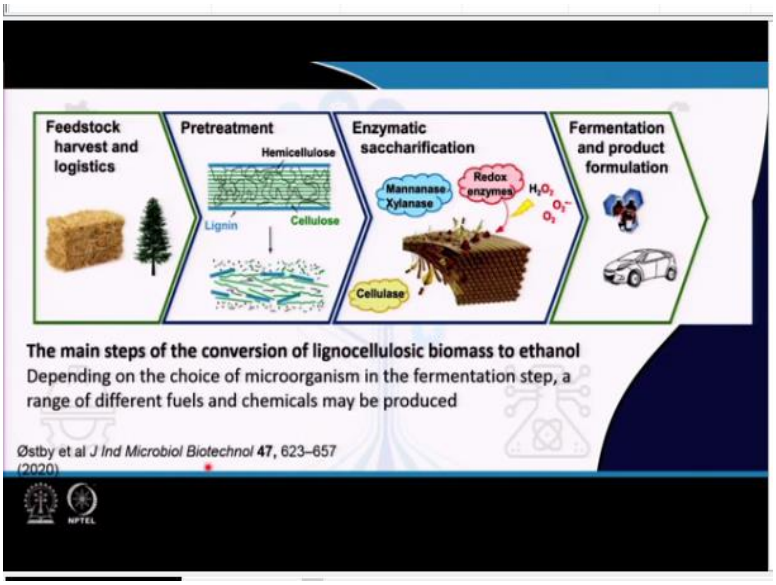

Now here is the diagrammatic representation of the main steps to be followed during the conversion of the lignocellulosic biomass to the ethanol and as we can see that the feedstock collection which includes the harvesting the biomass and its transportation to the facilities where it will be treated in order to release the constituting the polymer and then the monomers.

Like in this case you can see these hemicellulose and cellulose components or lignin components are depolymerized to produce the different type of a monomeric unit and then

enzymatic saccharification which leads to the final production of all the monomers and it is often used several chemical compounds as well and once these monomers are produced and monomers are available the syrup or this extract is subjected to the fermentation.

And then upon fermentation the fermented products including the ethanol and other targeted compounds are used to produce the desired formulation which can be used as a biofuel. Now depending on the choice of microorganisms particularly in the fermentation step which is the ultimate step of this entire process the fermentation ultimate and one of the key components towards the production of the ethanol.

A range of different fuels and chemical may be produced. Now the resource or the source material or the feedstock material is almost like constant because it is often the agricultural or forest residues or different type of glasses which are locally available or regionally available and their chemical constituents are almost like uniform except varying ratios between the hemicellulose, cellulose or lignin component.

But overall it is like the 45% cellulose and around 30% hemicellulose and 25% lignin and again the depolymerization to produce the hexose-pentose sugars after removal of the lignin and then these hexose-pentose sugars are available for microbial fermentation. Now it is the catabolic ability or catalytic ability of the microorganisms which facilitate the conversion of these sugars which are now available for fermentation towards the production of the desirable fuel molecules including ethanol.

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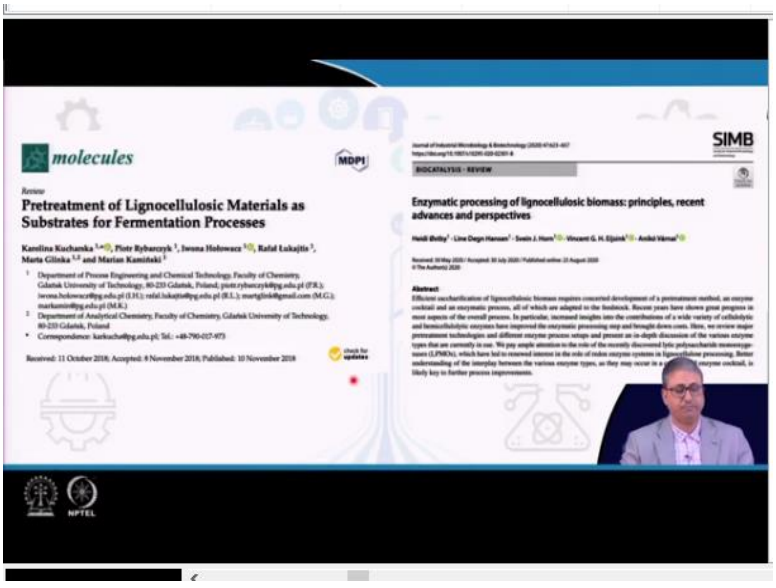

Now here are the 2 very relevant publications papers which might be useful for getting more information about the pre-treatment of lignocellulosic materials as substrate for fermentation process or specifically the enzymatic processes which are involved or used for lignocellulosic biomass and the principles recent advances and perspective. So, these 2 papers seem to be very relevant and quite useful to understand the things in detail.

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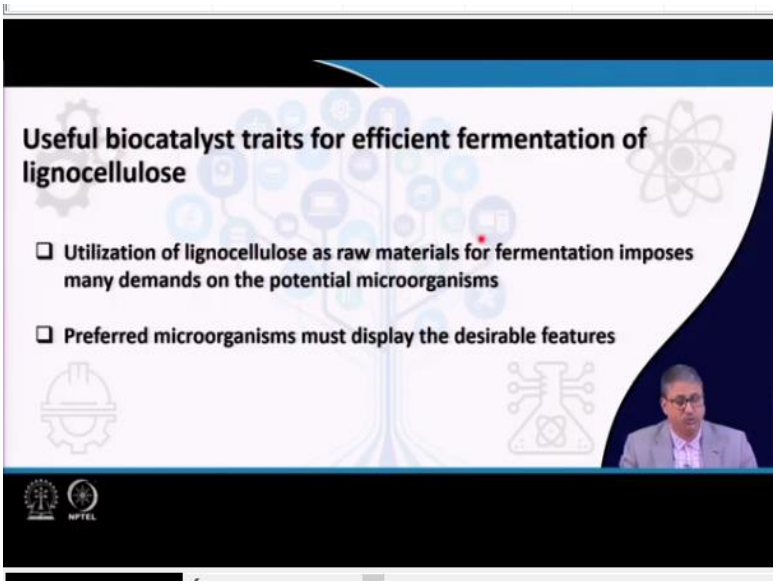

Now the useful biocatalyst traits for efficient fermentation of lignocellulose. Now utilization of lignocellulose as raw materials for fermentation imposes many demands on the potential microorganism. Preferred microorganisms must display the desirable features.

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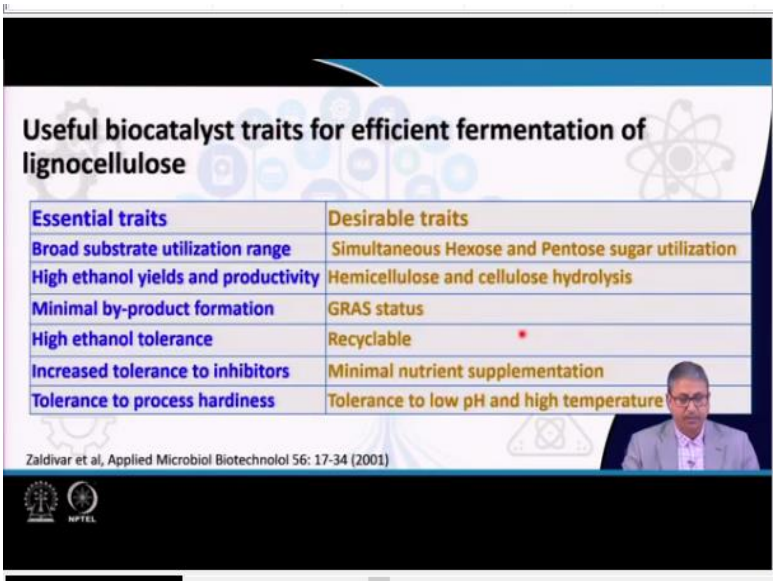

Now here are the lists of useful biocatalyst traits for the efficient fermentation of the lignocellulosic biomass. Now we must remember in this step that the traits which are being

tabulated here are based on the microbial traits that these are the traits or characters which are expected from the microorganisms which are to be selected as a candidate organism.

And one of the basic fundamental requirement from the microorganism site to be qualified for this kind of fermentative process for ethanol or other biofuel is the fact that the lignocellulosic biomass upon hydrolysis and depolymerization etcetera will produce both hexose and pentose sugars number 1 and number 2 it is going to have some other constituents which might have adverse or toxic effect on microbial activities.

And number 3 is the toxicity imposed by the ethanol or the biofuel molecule itself on the microorganisms. So, considering this general aspect that the lignocellulosic biomass which is hydrolyzed, pretreated and then fed into the fermenter to produce the ethanol or other biofuels by the microorganisms. The essential traits which must be there in the organism are the broad substrate utilization range.

It is directly connected to the microbial ability towards utilizing the hexose and pentose sugars which are produced or there in the lignocellulosic biomass which is subjected for fermentation. High ethanol yields and productivity, so in order to achieve the cost effectiveness and sustainability of the entire process the production of ethanol must be able to qualify the yield and productivity criteria.

The minimal byproduct formation, the microorganism while fermenting the lignocellulosic residues including the hexose and pentose sugar mainly, the byproduct formation should be minimum so that whatever product be it ethanol or butanol or other product it is less contaminated by other metabolites or other products for their further application. High ethanol tolerance, because ethanol itself is able to produce some kind of adverse effect on the cell in terms of its membrane and creates lot of stress on the cells.

So, the microorganisms which are involved in ethanol production after some time if they are producing higher concentration of ethanol in the reactor volume; these higher ethanol concentration might create or will create lot of stress on the organisms. So, the organisms must be capable of withstanding the ethanol toxicity. Increase tolerance to inhibitors, there might be inhibitors because of the hydrolysis, the chemical.

And there could be some physical inhibitors like other kind of compounds and certain range of temperature etcetera and tolerance to process hardness particularly when we operate the fermentation process within the different type of reactors. There are expected to be lot of process hardiness. So, the organisms must be capable of withstanding these adverse conditions.

So, these are the essential traits. Now apart from the essential traits there are some desirable traits and these traits are expected that these traits are present within the microorganisms which are being selected for the fermentation of the lignocellulosic biomass to ethanol and these traits include simultaneous hexose and pentose sugar utilized, both must be utilized together the repression which is often seen with respect to presence of glucose that glucose represses the utilization of other sugars present along with, that should not be there.

Hemicellulose and cellulose hydrolysis that if the organism is capable of hydrolyzing the hemicellulose and cellulose then one of the enzymatic pre-treatment of the biomass can be avoided or can be taken care of itself by the microorganisms. So, we can actually combine the enzymatic hydrolysis and the fermentation process together. Organisms must be or preferred to be safe for handling in the industrial or in the laboratory setup and in this case they must qualify the grass category status like the generally regarded as safe.

They must be recyclable, so the organisms are stable, they are genetic makeup is robust and stable, so that we can use these cultures continuously. Minimal nutrient supplementation, so maintaining the culture in active stage and overall cost effectiveness would be high if the nutritional requirements are minimal. And tolerance to pH and high temperature which are often expected in the process operating conditions are desirable.

So, these are all desirable characters and these are all essential characters. We need to remember that these essential traits are truly required for organisms which are to be selected or which are to be considered for ethanol or other biofuel production from the lignocellulosic biomass. And the desirable traits are like the traits which are if they are there it is preferred, it is better that if these traits are there.

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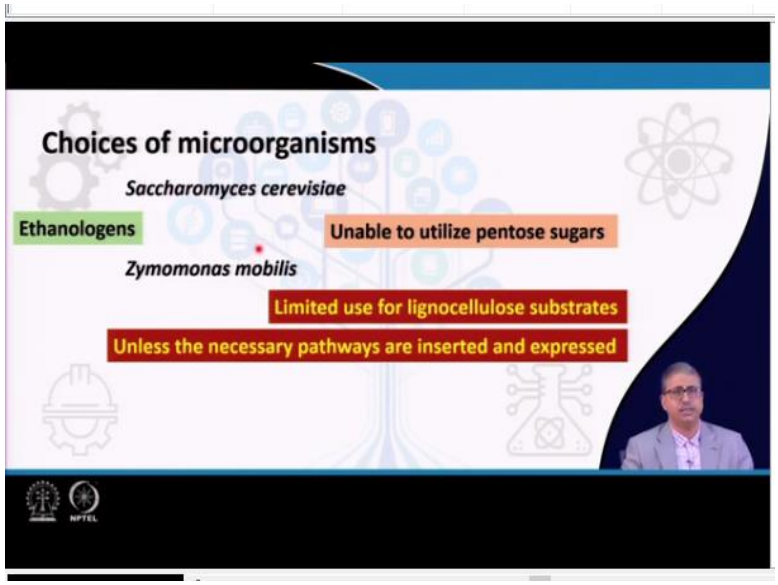

Now we will discuss about the choices of microorganisms. Now we have actually 2 types of organisms in front of us. One is called as ethanologens like they are known to produce high concentration of ethanol. These are the yeast *Saccharomyces cerevisiae* type organism and some other yeast are also identified and characterized, but it is basically the Saccharomyces cerevisiae and the bacterium Zymomonas mobilis.

These 2 organisms the *Saccharomyces cerevisiae* and *Zymomonas mobilis* are very well known, well reported for their high ethanol production. However, they both of these high ethanol producing strains or organisms are found to be incapable to utilize the pentose sugars. Now this pentose sugar is coming into picture or becoming relevant because any lignocellulosic biomass will have hemicellulose component.

And this hemicellulose component is composed of both hexose and pentose sugar. So, the pentose sugars are going to be an integral part of the hemicellulose and hemicellulose is an integral part of the lignocellulosic biomass and these pentose sugars particularly xylose or some arabinose they represent around 25% of the total sugar content. So, if we want to make our process the entire fermentation process cost effective, efficient and sustainable.

Then we must try to understand that utilization of pentose sugars along with the hexose sugars would be essentially required in order to achieve the metabolic engineering goal or the industrial success. Now with by defining this character that the most of the ethanol genes are known species of *Saccharomyces cerevisiae* and *Zymomonas mobilis* which are efficient in producing high ethanol but they are unable to produce, unable to utilize pentose sugar.

These are conventionally remain limitedly used for lignocellulosic substrates, because lignocellulosic substrates will have pentose sugars with them and if we plan to use *Saccharomyces cerevisiae* or *Zymomonas mobilis* they will not be able to utilize the pentose sugar and the overall efficacy of the process will be reduced. And in order to achieve the process efficiency and cost effectiveness that means we need to have necessary improvement or necessary pathway engineering through metabolic engineering by inserting and expressing the required genes.

Or required genes which will facilitate the utilization of pentose as well by this ethanol producing organisms like *Saccharomyces cerevisiae* and *Zymomonas mobilis*.

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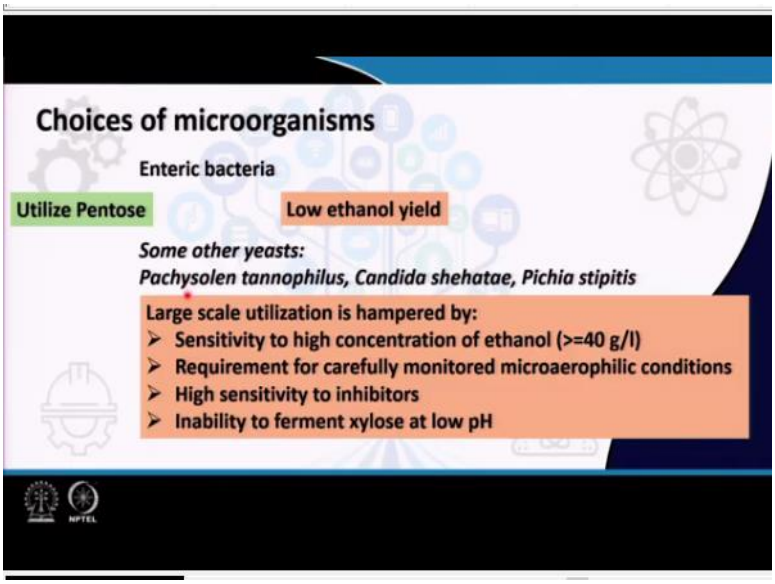

Now the second category of organisms are the enteric bacteria particularly is Escherichia coli and other members and some other yeast as well like the Pachysolen, Candida species and Pichia stipitis. Now these organisms are found to be capable of utilizing pentose, so unlike the previous category of organisms the yeast *Saccharomyces cerevisiae* and the bacterium *Zymomonas mobilis* who were restricted or limited with respect to utilization of pentose.

The Enteric bacteria including *E.coli* and some other yeast are capable of producing pentose or utilizing pentose as their carbon substrate. However, their ethanol production abilities are low. So, they have a low ethanol yield, particularly with respect to the yeast other yeast not the Saccharomyces cerevisiae the yeast which are listed over here. Large scale utilization in production of ethanol and other biofuels from the lignocellulosic biomass is hampered by

their sensitivity to high concentration of ethanol which is expected that the ethanol concentration following fermentation process will be high.

Requirement of carefully monitored microaerophilic conditions, so these organisms often require microaerophilic conditions so very strict monitoring is required within the reactors. High sensitivity to inhibitors, inhibitors which are expected from the pretreatment of the lignocellulosic biomass and inability to ferment xylose at low pH particularly if the medium pH is low the most of these yeast particularly, but not *Saccharomyces cerevisiae*. That *Saccharomyces cerevisiae* is different category; they are not able to use the pentose sugar, xylose very effectively.

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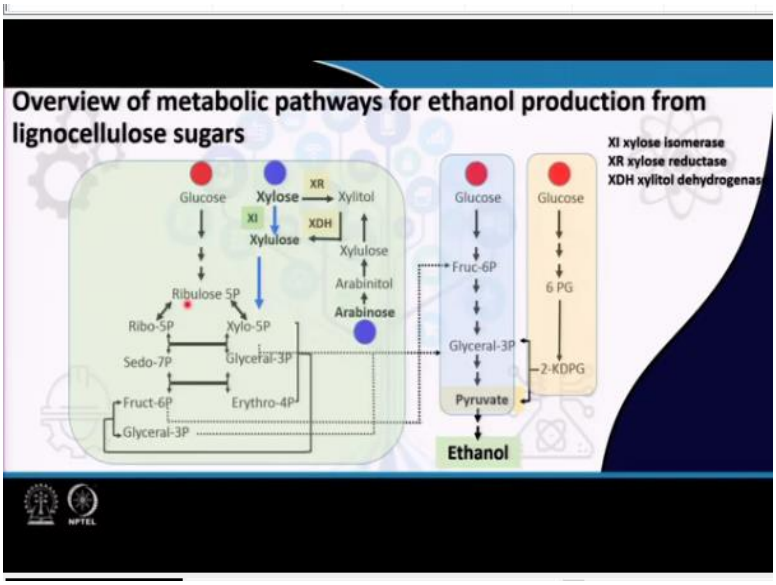

Now here is the overview of metabolic pathways for ethanol production from lignocellulosic sugars. So, by lignocellulosic sugar we refer to the glucose as representative of the hexose sugar and the xylose as representative of the pentose sugar. The other pentose sugar like arabinose might also be present and in subsequent time we will see that other hexose sugars can also be included in similar type of diagrammatic representation.

Now if we look into this particular part of the entire metabolic pathway. So, here is the pentose phosphate pathway from the glucose, the ribulose-5-phosphate is produced and then ribulose-5-phosphate is converted to ribose-5-phosphate and xylose-5-phosphate and these are then undergoing the transaldolase and transketolase reaction to produce couple of very interesting intermediates.

One is the glyceraldehyde-3-phosphate; the other is the fructose-6-phosphate and again the glyceraldehyde-3-phosphate. Now this glyceraldehyde-3-phosphate is connected or can feed into the Embden-Meyerhof-Parnas pathway the standard glycolytic reactions to produce the pyruvic acid and also the fructose-6-phosphate which is produced can also be fed into the Embden-Meyerhof-Parnas pathway now with respect to utilization of the pentose sugar like xylose or arabinose.

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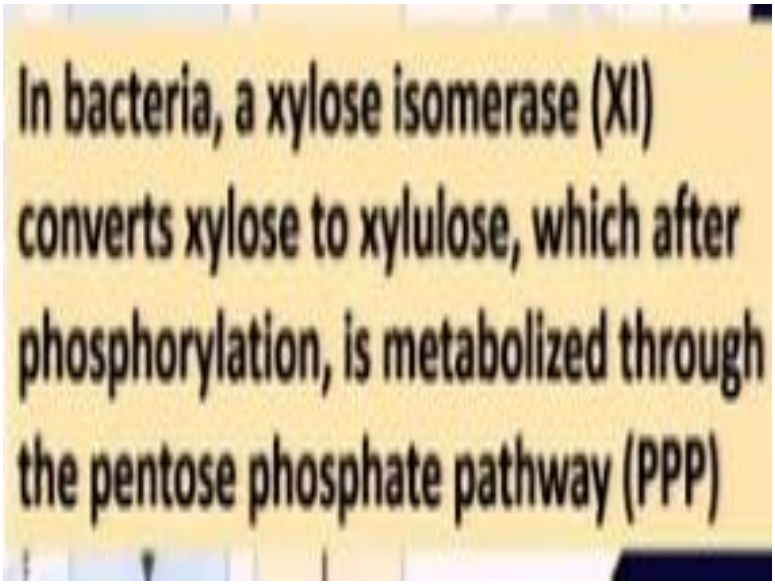

With respect to xylose it has been found that in bacteria the enzyme xylose isomerase is able to convert the xylose to xylulose and then this xylulose upon further phosphorylation is able to enter into the pentose phosphate pathway and then essentially metabolize through pentose phosphate pathway. That means the pentose phosphate pathway which is basically operated in bacteria using glucose as a standard substrate glucose to glucose-6-phosphate and then glucose-6-phosphate is decarboxylated to produce the ribulose-5-phosphate.

The pentose sugar like xylose can equally enter into the pentose phosphate pathway by kind of a 2 step reaction where xylose is first converted to xylulose and then xylulose is phosphorylated to form the xylose-5-phosphate and then xyluolse-5-phosphate is there in the metabolism of the pentose phosphate pathway.

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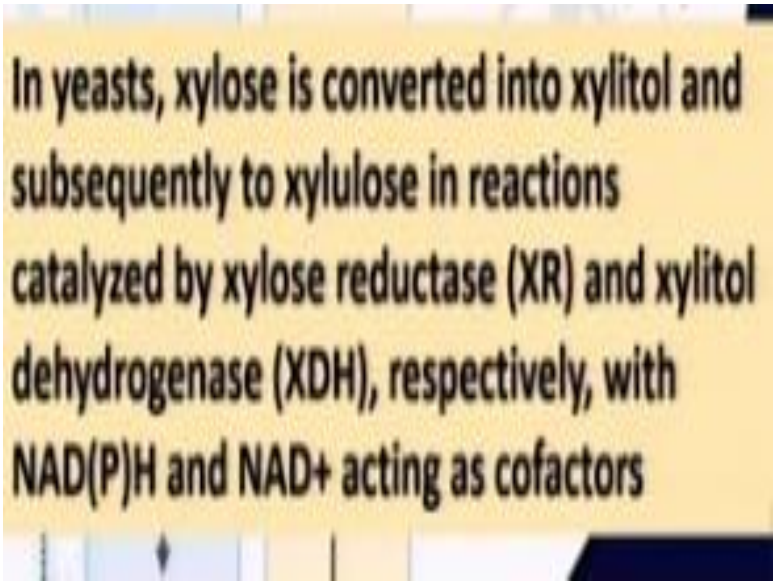

In case of yeast the xylose is converted **to it** through a different pathway. In case of yeast the xylose is converted to xylitol and then subsequently to xylulose by 2 reactions catalyzed by the xylose reductase and xylitol dehydrogenase and with any NADPH and NAD + acting as the cofactors. So, this is the kind of dehydrogenation reaction and ultimately the xylulose is produced.

Now the other main reaction which is capable of producing the ethanol is the Embden-Meyerhof-Parnas reaction where the glucose is converted to pyruvic acid and pyruvic acid is fermented to ethanol. So, all the products which are produced from the pentose phosphate pathway particularly the glyceraldehyde-3-phosphate and the fructose-6-phosphate. These 2 important metabolites or the products of pentose phosphate pathway can very well be fed into the Embden-Meyerhof-Parnas pathway to produce pyruvic acid.

And then the pyruvic acid can be converted ethanol using the fermentation reaction can be converted to ethanol. Similarly in some other microorganism the ED pathway operates and which produces the KDPG and 2 KDPG is then connected to the glyceraldehyde-3-phosphate and pyruvic acid of the Embden-Meyerhof-Parnas pathway.

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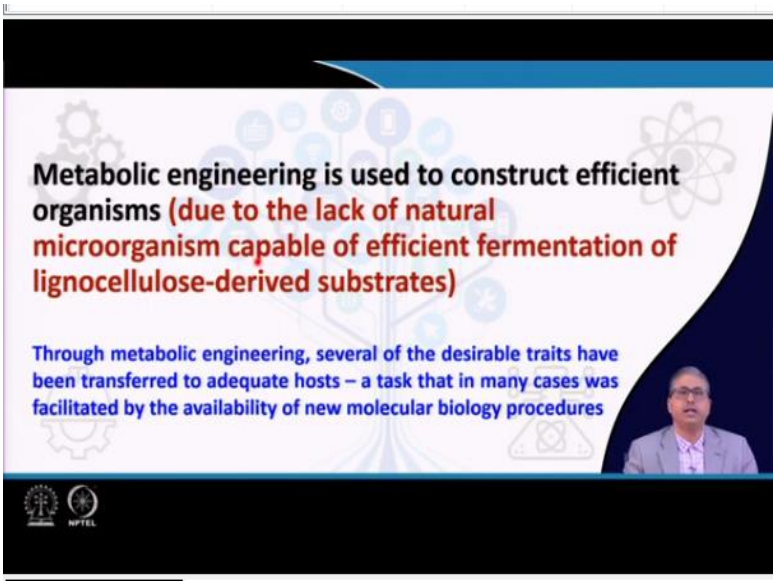

Now metabolic engineering is used to construct the efficient organism. Now why we need to construct the efficient organism is because there is a lack of natural microorganism capable of efficient fermentation of lignocellulose derived substrates. Two types of organisms we have discussed the yeast *Saccharomyces cerevisiae* and *Zymomonas mobilis* the bacterium who are capable of producing high ethanol.

But they are unable to utilize pentose sugar and on the other hand we talked about the Enteric bacteria including *E.coli* and other yeast not the *Saccharomyces cerevisiae* who are capable of utilizing pentose sugar, but are unable to utilize or utilize this pentose sugar and produce the ethanol in a cost effective way. Now through metabolic engineering several of the desirable traits which have been listed earlier have been transferred to adequate host like *Saccharomyces cerevisiae* or *E.coli.*

A task that in many cases was facilitated by the availability of new molecular biology procedures. So, we will see that largely these achievements were made because of the advancement in different molecular biology and genome engineering and other omics including CRISPR and other techniques.

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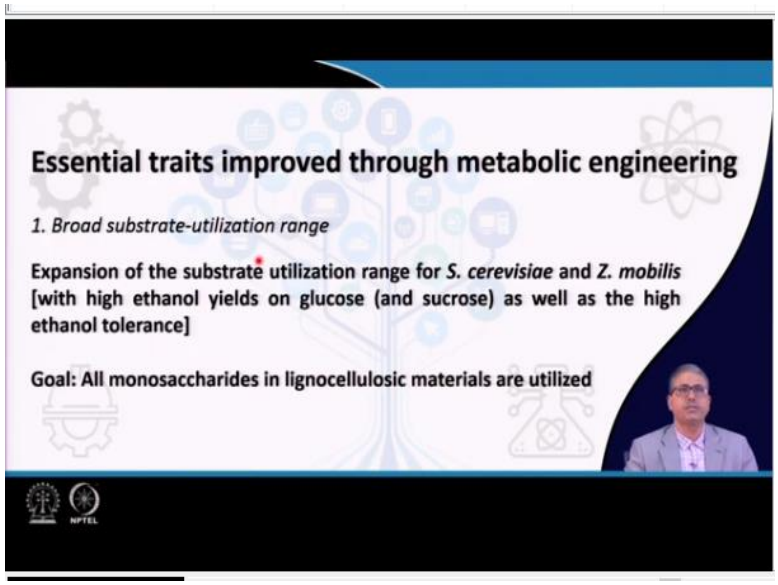

Now we will briefly discuss about the essential traits and their importance and in some cases we will highlight that why they are so relevant. Now essential traits improved through metabolic engineering are 6 or 7 characters are there or traits are there. The first is the broad substrate utilization range. Now expansion of the substrate utilization range for *Saccharomyces cerevisiae* and *Zymomonas mobilis*.

Because they are naturally capable of producing high ethanol from glucose and sucrose as well as they are highly resistant to the high concentration of ethanol. So, they are already ready with desirable traits including the high ethanol concentration ethanol yields from hexose sugars and high ethanol tolerance. However, they are limited or restricted with respect to their substrates.

They are unable to utilize pentose sugars. So, expansion of the substrate utilization range is very much required and metabolic engineering is able to serve this particular purpose and the goal for this particular category is that all monosaccharides including the hexose and pentose which are present in the lignocellulosic materials are should be utilized.

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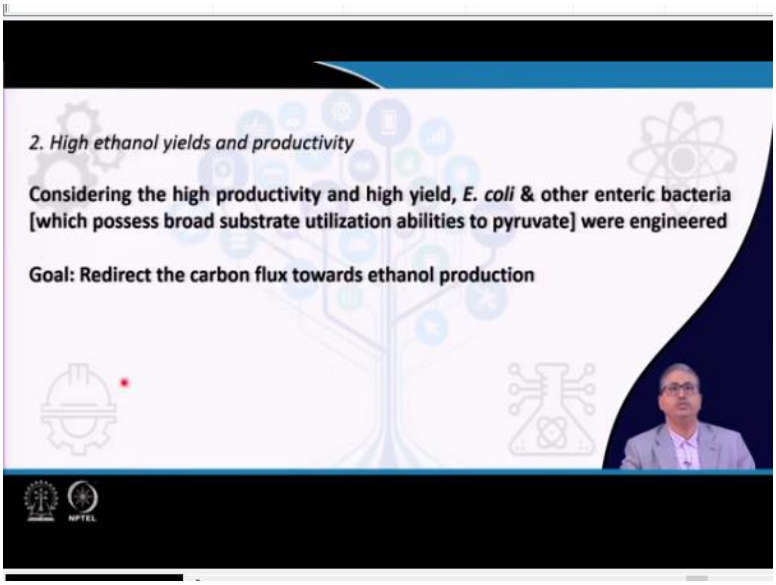

The second is the high ethanol yields and productivity. Now considering the high productivity and high yield *E.coli* and other enteric bacteria which process broad substrate utilization abilities to pyruvate where engineered. So, in this case *E.coli* and other enteric bacteria were selected because they are capable of utilizing pentose sugar and rather both hexose and pentose sugar.

But they are not capable of producing high ethanol. So, *E.coli* for example is selected and large number of experiments and metabolic engineering advancement has been made in order to achieve high production, high yield of ethanol in particular and as well as other biofuel as well within *E.coli* because they are capable of utilizing both the hexose and pentose sugar.

So, in this case the goal for the metabolic engineering was to redirect the carbon flux towards ethanol production, why this is so important this goal because in this *E.coli* or other enteric bacteria who are capable of using both hexose and pentose sugar the metabolic pathway for utilizing the carbon substrates like hexose and pentose to sugars are quite complex and they not only essentially produce ethanol but often they produce a number of products. That is how the redirection of carbon flux is essential in order to achieve high ethanol yield.

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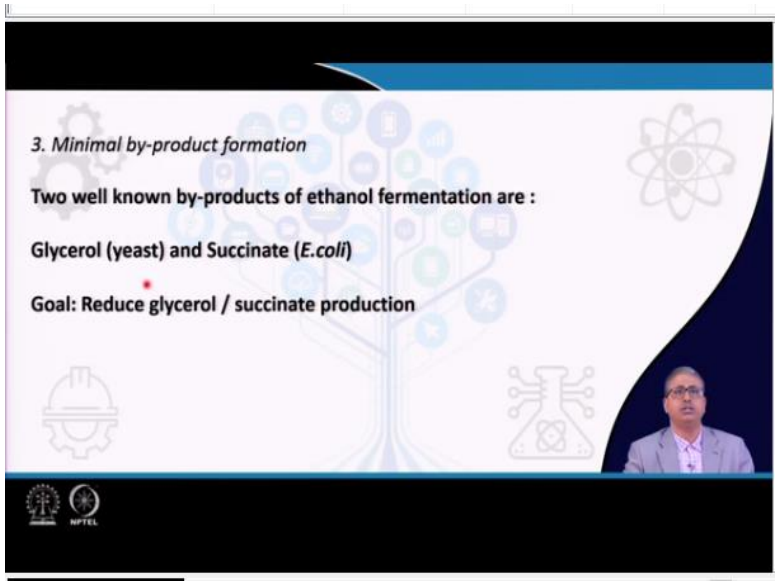

Minimal byproduct formation is the another or the next desirable trait. Two well known byproducts of ethanol fermentation are the glycerol in case of yeast and succinic acid in case of *E.coli*. Now these glycerol and succinic acid production either in case of Saccharomyces celibacy or in *E.coli* should be addressed because otherwise if we produce too much of glycerol or too much of succinic acid then there is a kind of a diversion of the carbon flux and overall the productivity and yield of the ethanol and biofuel production are going to be reduced. So, the reduced glycerol and succinate production is achieved through different metabolic engineering strategies in yeast as well as an *E.coli*.

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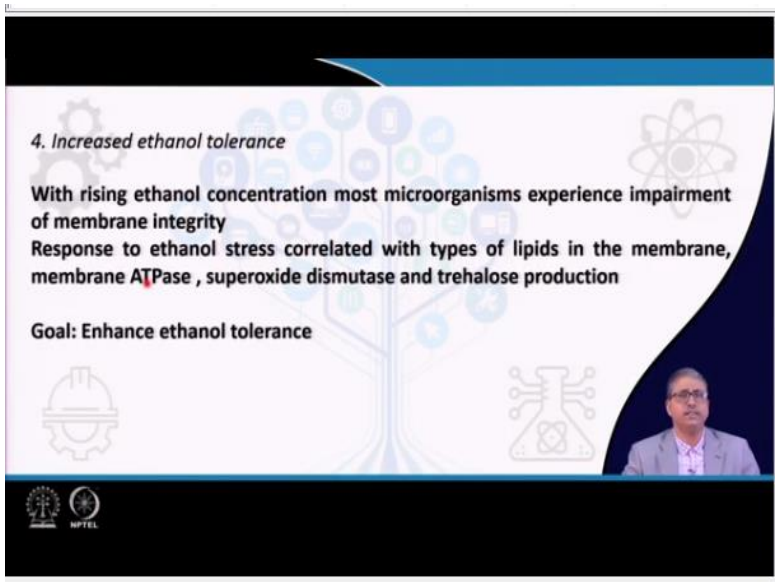

Increased ethanol tolerance; with rising ethanol concentration because in metabolic engineering for ethanol production one of the main targets is to achieve higher ethanol production or yield. Now as the high concentration of ethanol is produced and accumulated in

the fermenter the culture or the microorganisms are expected to experience lot of stress because of this high ethanol concentration, because ethanol often impair the membrane integrity and other cellular or physiological functions.

Now response to ethanol stress has been found to correlate well with the type of lipids that is present or they are present in the membrane. Membrane ATPase and superoxide dismutase and other trehalose production etcetera. So, enhanced ethanol tolerance remain one of the major goals in order to achieve increased ethanol tolerance.

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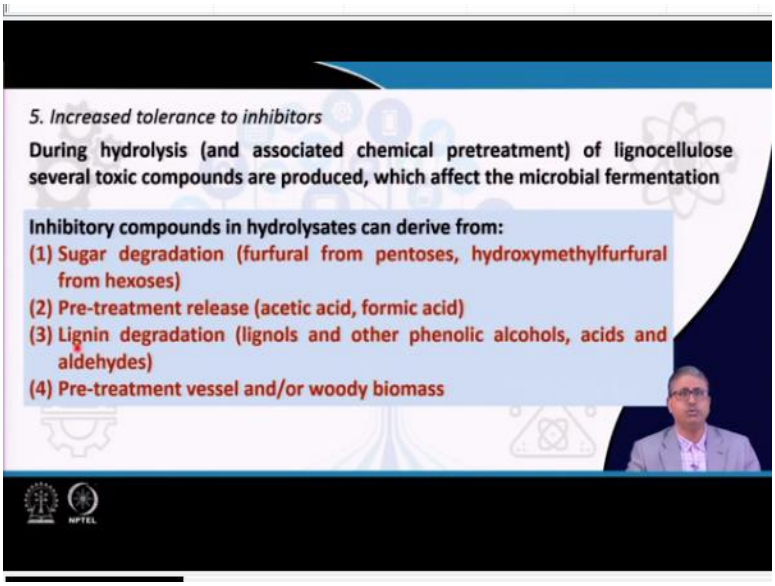

Next essential trait is the increased tolerance to inhibitors. Now during the hydrolysis and associated chemical pretreatment of lignocellulosic biomass several toxic compounds are produced which affect the microbial fermentation and these inhibitors affect the overall cell physiology result in decreased cell viability, ethanol yields and productivity. So, these inhibitors are often regularly they are present in the syrup which is produced from the hydrolysis and chemical pretreatment of the lignocellulosic biomass and these compounds are going to have and found to have very severe toxic impact or toxic effect on the fermentation abilities.

Now these inhibitory compounds in hydrolysates lignocellulosic biomass hydrolysates can derive from sugar degradation like furfural from pentose, hydroxymethylfurfural from hexose or pretreatment related release like acetic acid, formic acid, lignin degradation products like lignols and other phenolic alcohol acids and aldehydes and pretreatment vessel and or woody biomass. So, all these materials they put a kind of stress on the microorganism.

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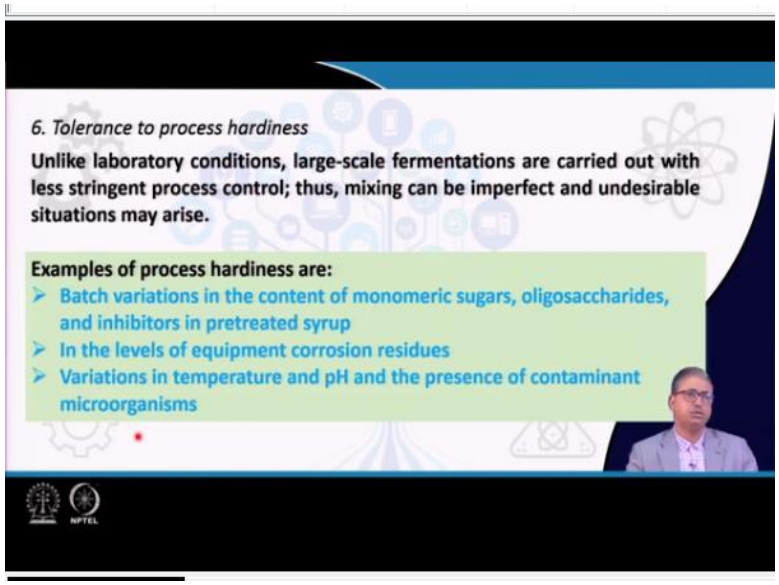

So, essentially we need organisms who are capable of withstanding these inhibitors the 6th essential trait is the tolerance to process hardliness. Now unlike the laboratory condition large scale fermentations are carried out with less stringent process control. Thus mixing can be imperfect and undesirable situation may arise.

It is therefore important that the microorganisms which are selected should be able to recover from the processing error, because there would be minor or some extent, some fluctuations in the process parameters because in the large scale reactors often that those may not be very strictly controlled, because we want to make the entire process simple and the cost effective. So, examples of process hardliness are batch variation in the content of the monomeric sugars, oligosaccharides and inhibitors in pre-treated syrups.

So, batch to batch the lignocellulosic biomass hydrolyzed might show different concentration of the monomeric sugars like the hexose-pentose sugars or the different oligosaccharides or the inhibitors which are often expected. So, these variations should be ok with the microorganisms. In the level of equipment corrosion residues, variation in temperature and pH and presence of contaminant microorganisms often in the syrup or in the other steps there might be some contaminant microorganisms which will be present there.

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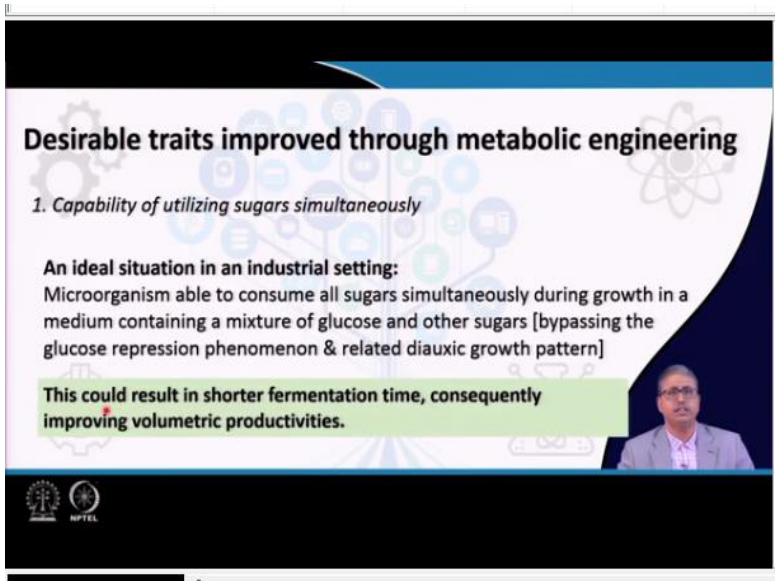

So, the ultimate process should not be hampered by these parameters which are often expected of any kind of lignocellulosic biomass based ethanol fermentation or other biofuel fermentation. Now the desirable traits which are improved through metabolic engineering are capability of utilizing sugar simultaneously. This is very important because this is considered to be an ideal situation for an industrial setting.

Because microorganisms able to consume all sugar simultaneously during growth in a medium containing a mixture of glucose and other pentose sugars particularly hexose-pentose together, bypassing the glucose repression phenomenon and related diauxic growth pattern is highly desirable. This could result in shorter fermentation time, consequently improving the volumetric productivities of the biofuel.

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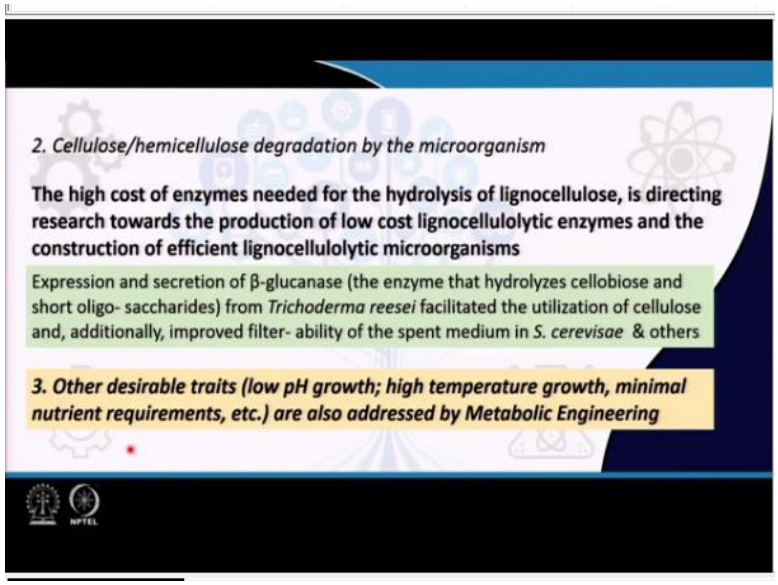

The cellulose, hemicellulose degradation by the microorganism. So, the microorganism should be able to produce the desirable enzymes which are capable of carrying out the hydrolysis of the cellulose and hemicellulose components released from the lignocellulosic biomass. The cost or the high cost of enzymes which are generally used or needed for the hydrolysis of the lignocellulose before the fermentation, directing research, further research on metabolic engineering towards the production of low cost lignocellulosic enzymes.

And the construction of efficient lignocellulolytic microorganisms. Now expression and secretion of beta-glucanase the enzyme that hydrolyzes cellobiose and short oligosaccharides from Trichoderma. For example facilitate the utilization of cellulose and additionally improve the filter ability of the spent medium in *Saccharomyces cerevisiae* and other organisms. There are other desirable characters like low pH growth, high temperature growth, minimal nutrient requirements etcetera which are also addressed by different metabolic engineering approaches.

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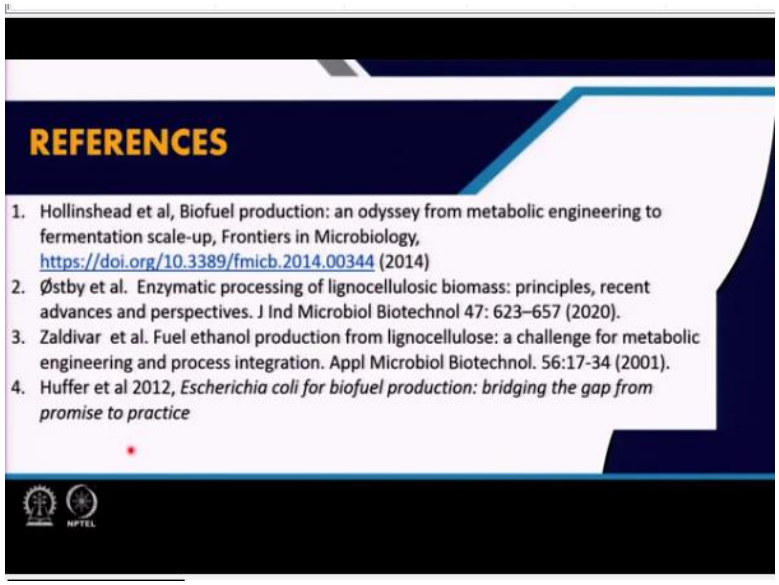

Overall in this section we have followed the following references particularly the reference used over here that is the review paper by Zaldivar on Fuel ethanol production from lignocellulose, a challenge for metabolic engineering and process integration and also the Ostby et al paper in 2020 Enzymatic processing of lignocellulosic biomass, principles recent advances and perspectives are found to be very useful.

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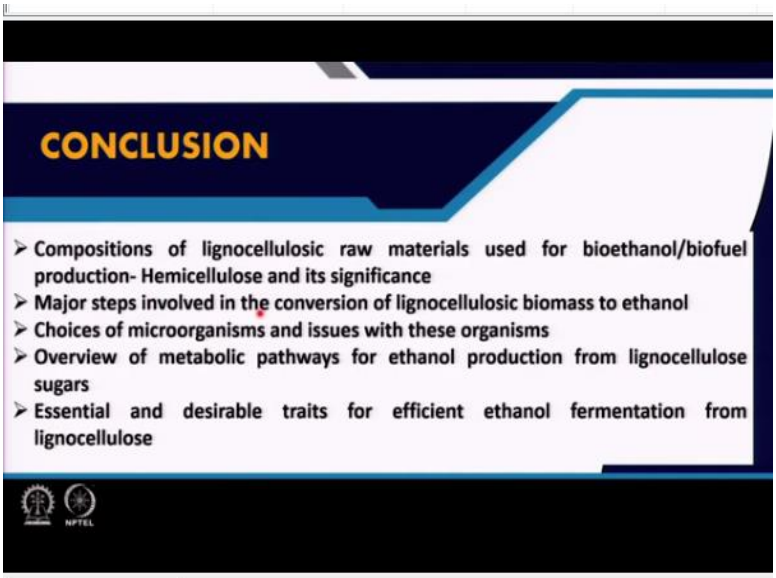

So, in conclusion composition of lignocellulosic raw materials used for bioethanol or biofuel production-hemicellulose and significance is highlighted. Major steps involved in the conversion of lignocellulosic biomass to ethanol is discussed. Choices of microorganisms and issues with these microorganisms are stated. Overview of metabolic pathways for ethanol production from lignocellulosic sugars. And essential and desirable traits for efficient ethanol fermentation from lignocellulose are also discussed, thank you.