Metabolic Engineering
Prof. Pinaki Sar
Department of Biotechnology
Indian Institute of Technology-Kharagpur

Lecture-33
Examples of Pathway Manipulations by Metabolic Engineering-Biofuels

In today's lecture on metabolic engineering, we are going to discuss about the examples of pathway manipulation, and we will start with biofuels.

(Refer Slide Time: 00:43)

So, the major concepts those are going to be covered will be the broad outline of the applications. Examples of few most significant developments on production of high volume chemicals to high value added compounds. And then application of metabolic engineering on biofuel production will be discussed. In this class, we are going to highlight the major roadblocks towards economical biofuel production, and lignocellulosic crops as raw materials for ethanol production.

(Refer Slide Time: 01:23)

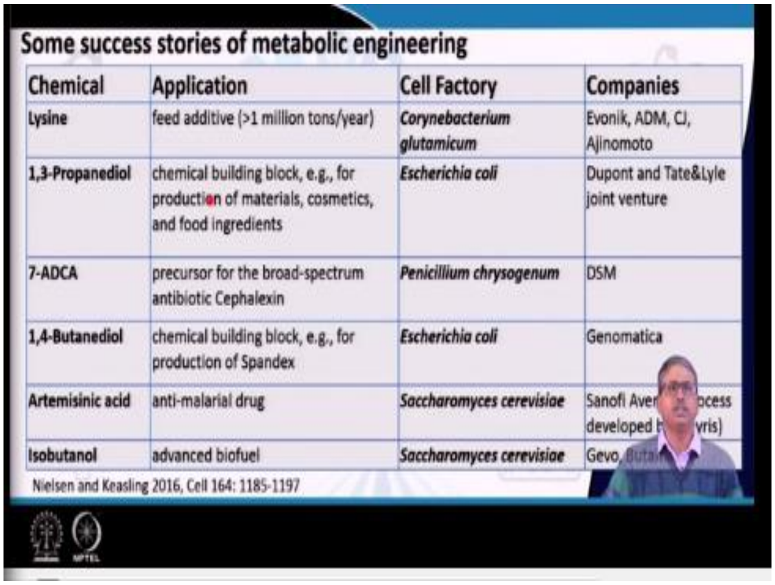
In last 2 decades, metabolic engineering has been exploited to improve traditional microbial fermentation processes, which were in place for over a century or maybe half a century and they have been improved. At the same time to produce chemicals that are currently used as fuel, materials and pharmaceutical ingredients were also targeted and successfully achieved.

(Refer Slide Time: 02:01)

So, a wide range of strategies have been developed. These strategies are the metabolic engineering strategies and they have been applied to efficient chemical production both in order to achieve the improved production of existing products. As well as the production of novel molecules or molecules which are naturally produced but with very low concentration in different microbial cell factorie.

That includes *Cornynebacterium glutamicum, Escherichia coli, Bacillus, Zymomonas mobilis* as well as the eukaryotic organisms like *Saccharomyces cerevisiae.*

(Refer Slide Time: 02:43)



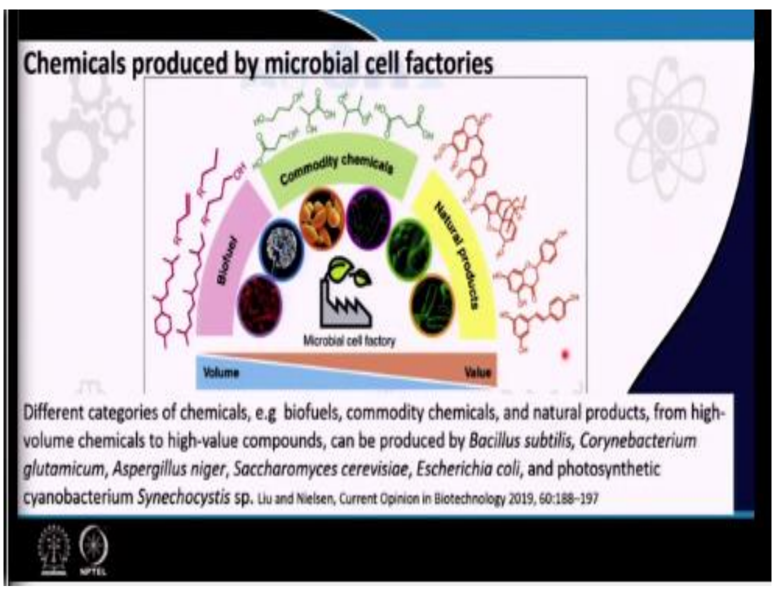
So, here are the some of the highlights of the success stories of metabolic engineering. And only few of the very important successful industrially deployed, implemented metabolic engineering products are only listed here. There are more such information available in the public domain and in the literature. So, for example, lysine production, 1, 3-propanediol production, butanediol production, Artemisinic acid, isobutanol.

All these compounds are successfully produced in a industrial scale and are being produced and adopted by a number of industries.

(Refer Slide Time: 03:32)

Now during these metabolic engineering strategies, a number of processes or approaches were used, including the genome editing and evolution part of that we have already discussed in our CRISPR-Cas based system. Tolerance engineering, rewiring of metabolic fluxes, growth based selection and adaptive laboratory evolution as well as subcellular compartmentalization and trafficking particularly when using the eukaryotic organisms.

(Refer Slide Time: 04:01)



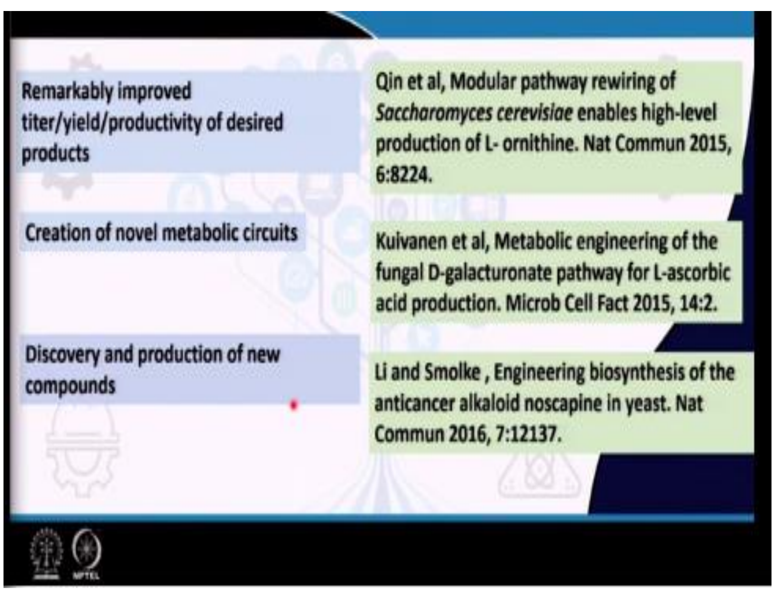
Now, the chemicals which are produced by microbial cell factories can be broadly categorized into biofuel, commodity chemicals and natural products. While for the biofuel like compounds the volume of the product is playing an important role, while the different natural products and other commodity chemicals it is the high value of the compound, which is a prime importance.

And these different categories of chemicals which include the biofuels, commodity chemicals and natural products from high volume chemical to high value compounds can be produced or have been produced successfully using *Bacillus subtilis*, *Cornynebacterium glutamicum*, *Aspergillus niger*, *Saccharomyces cerevisiae*, *E.coli* and photosynthetic cyanobacterium *Synechocystis* species.

(Refer Slide Time: 04:56)

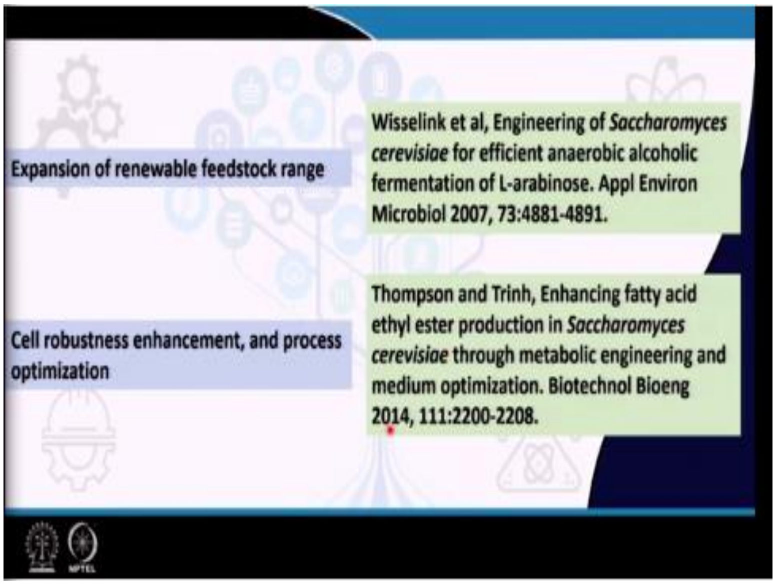
Now, there are some more examples on most significant developments on the production of high volume chemicals to high value added compounds.

(Refer Slide Time: 05:05)



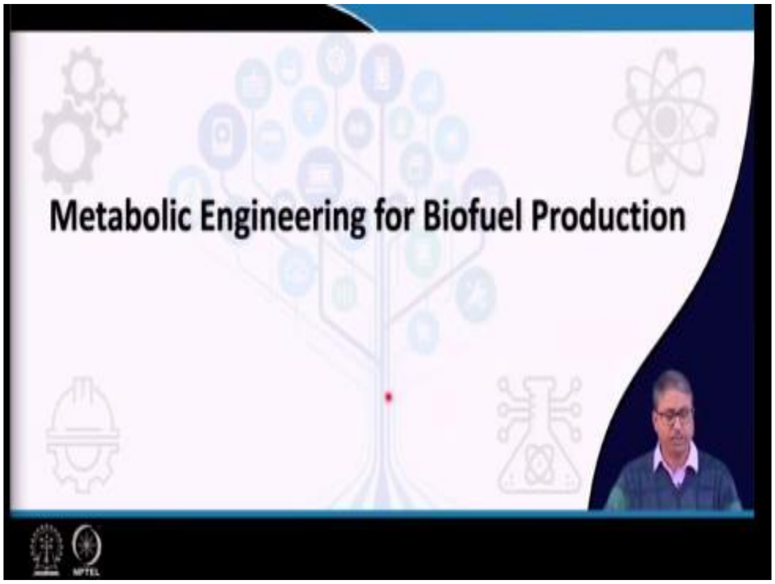
Particularly, remarkably improved titer or yield productivity of desired products have been achieved in *Saccharomyces cerevisiae* in particular. Creation of novel metabolic circuits is achieved in fungal strains to produce L-ascorbic acid. Discovery and production of new compounds is achieved through engineering device synthesis of anti cancer alkaloid in yeast.

(Refer Slide Time: 05:41)



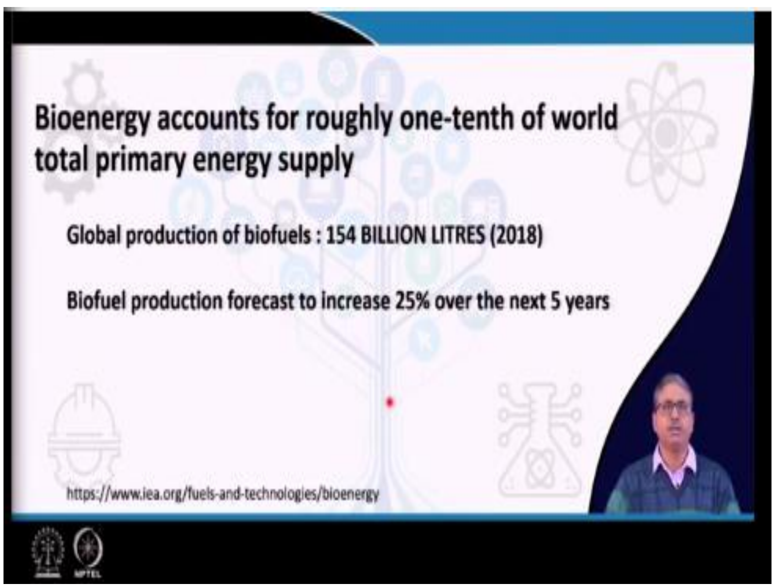
And expansion of renewable feedstock stock range is also achieved through metabolic engineering in yeast *Saccharomyces cerevisiae*. For efficient anaerobic alcoholic fermentation, and cell robustness, enhancement and process optimization is successfully done again in Saccharomyces cerevisiae through metabolic engineering and medium optimization process. So, there are a large number of such examples and only few are highlighted here.

(Refer Slide Time: 06:13)



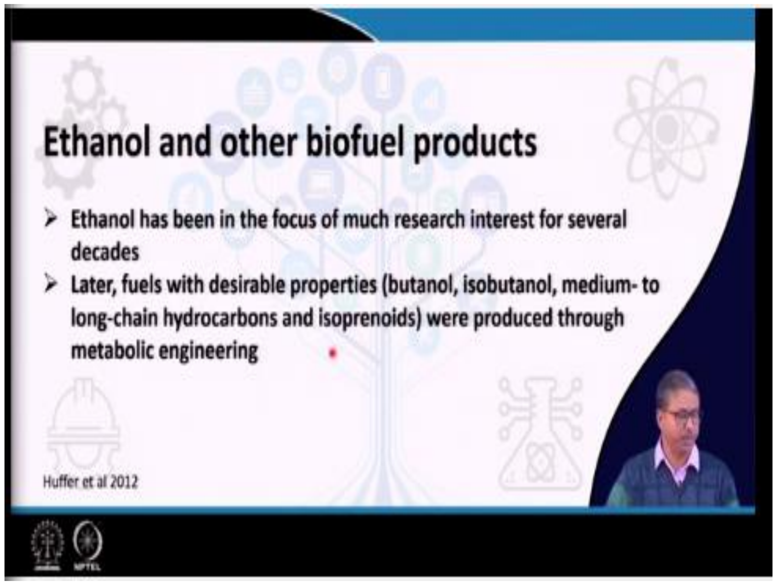
Now, we will move on to the application of metabolic engineering for biofuel production.

(Refer Slide Time: 06:20)



Now, bioenergy accounts for roughly 1/10th of world total primary energy supply, and it is increasing steadily. And according to 2018 report, the global production of biofuel is over 154 billion liters. And biofuel production forecast is there that it will increase to at least 25% more over the next 5 years that is up to 2024.

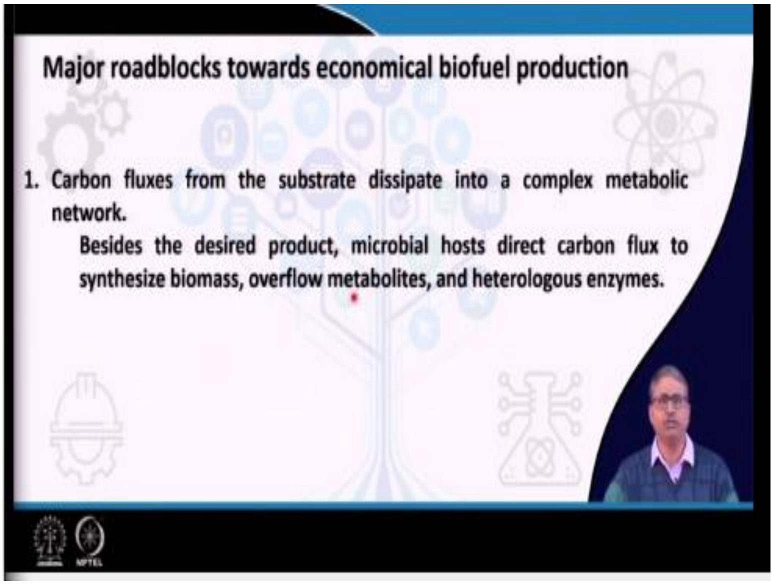
(Refer Slide Time: 06:55)



Now among these various chemical compounds, which are produced as biofuel using a large number of cell factories and cellular systems, including the prokaryotic organisms and eukaryotic organisms? Ethanol is found to have a very, very important place, ethanol has been in the focus of much research interest for several decades, and it is metabolic engineering for the improvement of the ethanol concentration, the expansion of the substrate range, reduction of the toxicity of ethanol.

All these parameters have been targeted and achieved successfully. However, with respect to certain shortcomings of ethanol as biofuel, in the later years we found that fuels with better or more desirable properties such as butanol, isobutanol, medium to long range hydrocarbons and isoprenoids were produced through metabolic engineering.

(Refer Slide Time: 08:03)

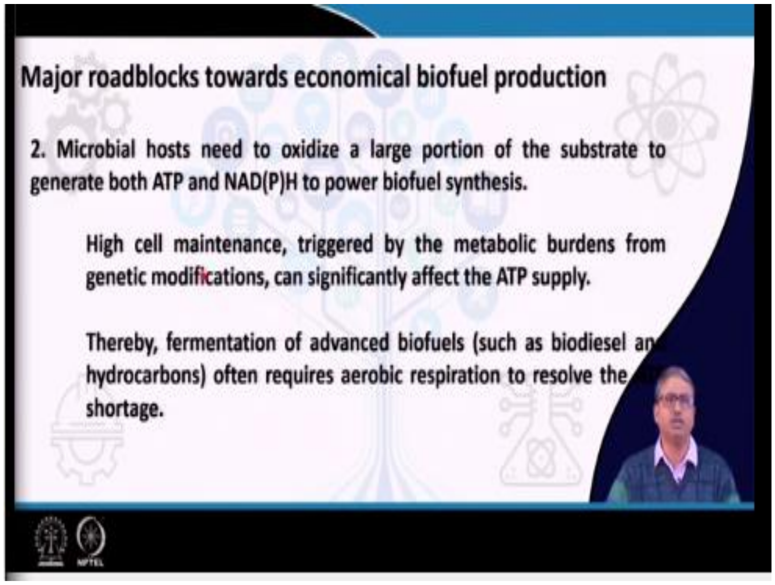


Now, major roadblocks towards economical biofuel production. First roadblock towards the successful or economical biofuel production is the issues related to carbon flux that is going through or that is being found in any prokaryotic or eukaryotic organism like either *Escherichia coli* or *Zymomonas mobilis* or *Saccharomyces cerevisiae.*

Now, carbon fluxes from the substrate whatever substrate the system like *E.coli* or *Zymomonas* or the *Saccharomyces* is utilizing the carbon flux from the substrate is found to dissipate into a complex metabolic network. As we have seen earlier also, that a large number of reactions and metabolic pathways are operating based on the carbon substrates which are being fed to the system.

And beside the desired product, like in case of ethanol production, that ethanol can be produced very successfully from pyruvic acid. However, beside the desired product, microbial host direct the carbon flux to synthesize biomass that is their own requirement, overflow metabolites, different other metabolites and heterologous enzymes.

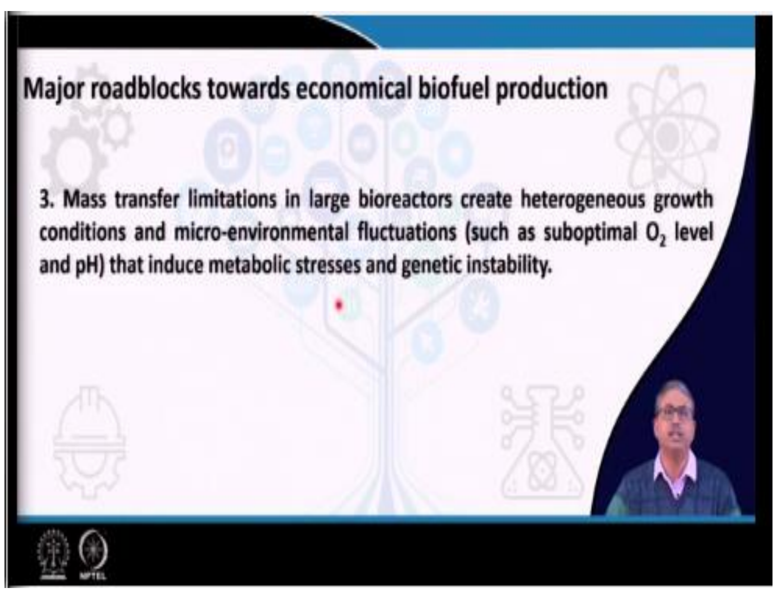
(Refer Slide Time: 09:22)



The second roadblock is that the microbial host need to oxidize a large portion of the substrate to generate both ATP and NADPH to power the biofuel synthesis. So, this is a kind of an obligatory requirement for the host system. Considering the high cell maintenance, which is triggered by the metabolic burden from genetic modifications including the presence of plasmid etcetera. It has been found that the requirement for these ATP and NADPH is significantly reducing the amount of biofuel which can be produced from a given amount of substrate.

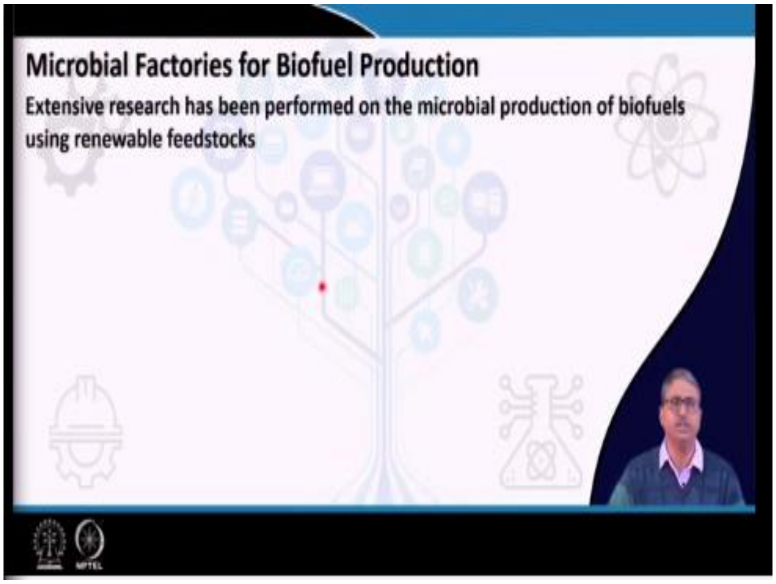
And thereby, the fermentation of advanced biofuels such as biodiesel and other hydrocarbons often require aerobic respiration to resolve the energy shortage.

(Refer Slide Time: 10:18)



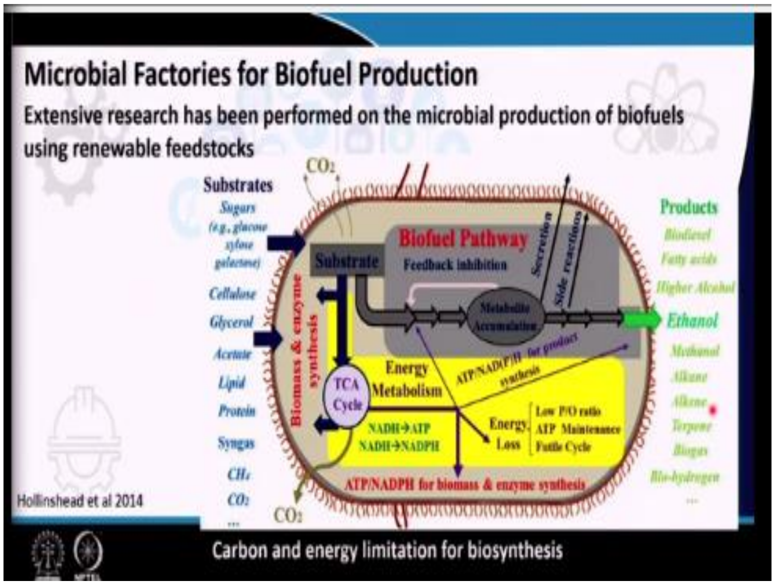
The third roadblock is the mass transfer limitation. In large bioreactor, which creates a kind of heterogeneous growth condition and micro environmental fluctuations, such as sub optimal oxygen level and pH that induce metabolic stresses and genetic instability. So, in very large bioreactors, often we found that these mass transfer limitations; they play a very significant role in reducing the concentration of the titer of the fuel molecule.

(Refer Slide Time: 10:50)



Nevertheless, there are a large number of cases and successful cases where metabolic engineering has been done, and they are all implemented successfully. Now, microbial factories for biofuel production these are extensive research which has successfully carried out and to produce the different kinds of biofuels using renewable feedstocks.

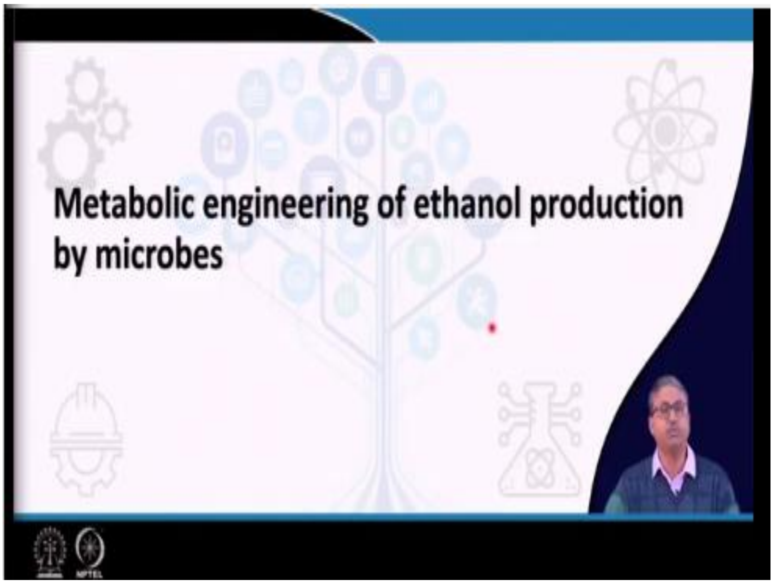
(Refer Slide Time: 11:17)



Feedstocks means, the resource which is provided to the microbial cells in order to gain the carbon and energy. Now, as you can see in this diagram, that a large number of substrates including the lignocellulosic biomass, the residues from the agriculture or the forestry or different products, organic products which might be produced from different other industries, including the food waste, or agricultural waste or food processing industries.

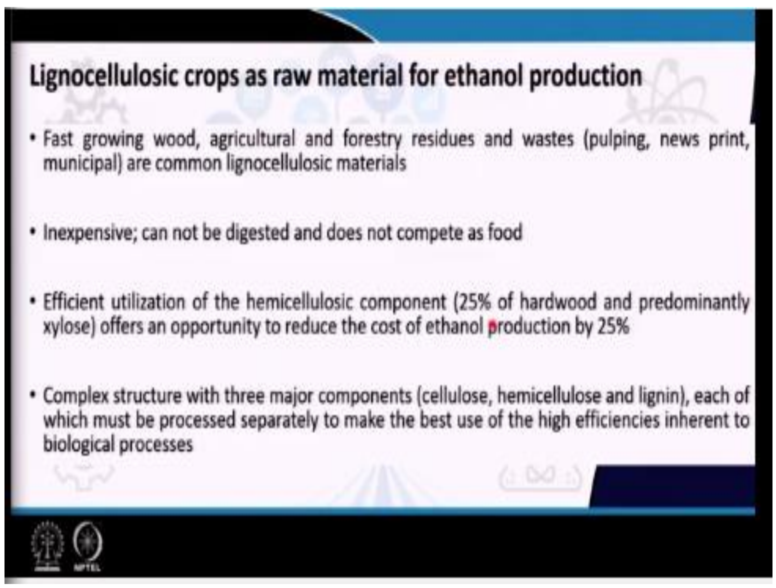
And different gases, including the syngas, methane, carbon dioxide, flue gas, these all can be utilized as substrate or raw materials for the production of the biofuels. And the products, which can be obtained from the microbial in metabolically engineered microbial cells would include the biodiesel, fatty acid, higher alcohol, ethanol, methanol, alkane, alkene, terpene, biogas and bio-hydrogen. A number of limitations or water lakes which are already highlighted in the previous slides are also indicated in this particular cartoon.

(Refer Slide Time: 12:27)



Now metabolic engineering of ethanol production by microbes.

(Refer Slide Time: 12:32)



Now lignocellulosic crops, which are available in most of the countries available in plenty, are found to be very important raw material for eternal production. So, because it is fast growing, including fast growing wood, agricultural and forest residue and different type of waste including the pulping, news print, municipal waste etcetera. These are the common lignocellulosic materials, which are often used for the production of the biofuels.

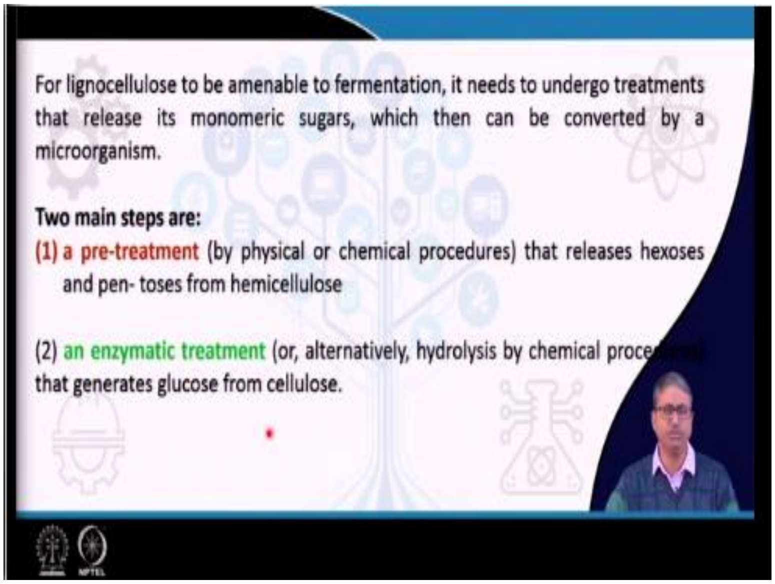
These are inexpensive and cannot be digested and does not compete as food. So, these are often not competing with food for either for human consumption or for the consumption by the cattles.

So, these are often selected over the other residues like over the corn and other which are specifically grown for biofuel production. Now efficient utilization of the hemicellulose component which is a very interesting and important part of the lignocellulosic biomass.

Because they contain both the cellulose as well as hemicellulose up to 25% of hardwood and predominantly composed of xylose offers an opportunity to reduce the cost of ethanol production by 25%. Because if metabolic engineering can address the issue of hemicellulose fermentation or hemicellulose utilization during the ethanol production, then this could possibly reduce the cost of eternal production to up to 25%. Because within any kind of lignocellulosic biomass, we see that on an average it is 25% or around 20% is the hemicellulose component.

Now complex structure with three major components cellulose, hemicellulose, and lignin, each of which must be processed separately to make the best use of the high efficiencies inherent to biological processes. So, any kind of biochemical or biological process, which is utilized for production of ethanol from the lignocellulosic biomass should be able to utilize all the components, all the constituents or sugar residues which are present in the lignocellulosic biomass.

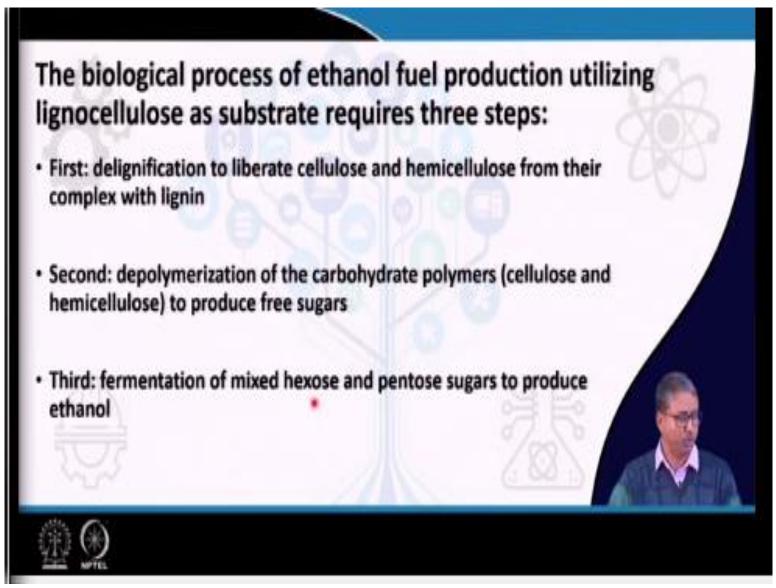
(Refer Slide Time: 14:58)



Now for lignocellulosic biomass to be amenable for fermentation, it needs to undergo treatment that release it is monomeric sugars, which then can be converted by the microorganism, because lignocellulosic materials are highly complex. And often recalcitrant towards or very slowly degradable by natural microorganisms. So, they need to be fast pretreated and there are very clear cut steps. And these steps are like a first a pretreatment by physical or chemical procedure that releases the hexose and pentose sugars from the hemicelluloses.

And followed by an enzymatic treatment or alternatively the hydrolysis by chemical process that generates the glucose from the cellulose. So, ideally, it is the cellulose and hemicelluloses, mostly which need to be processed or treated and the cellulose or the xylose or the ribose sugars. So, that means the hexose and pentose sugars, which are the constituents of the lignocellulose must be made available for microbial fermentation.

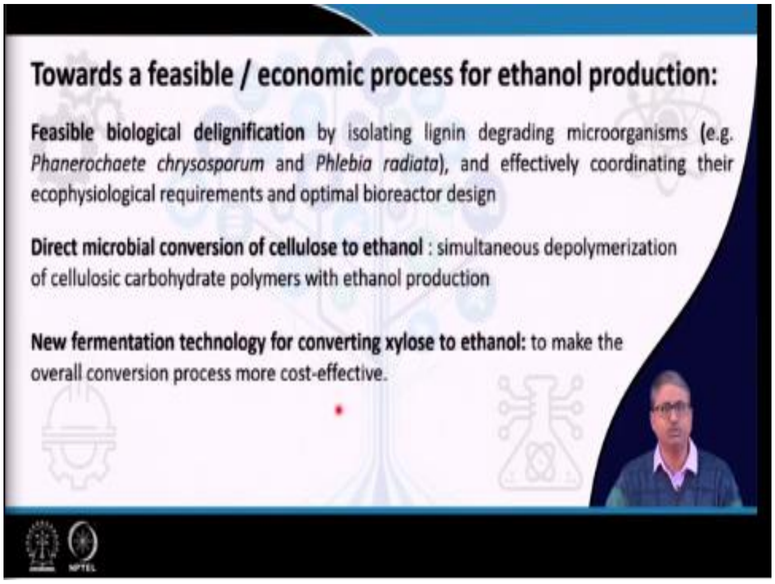
(Refer Slide Time: 16:02)



Now, the biological process of ethanol fuel production utilizing lignocellulose, as substrate requires three steps. The first step is the delignification, because it is a lignocellulosic biomass and we know it has very importantly the lignin as a important component. So, delignification is the first step to liberate the cellulose and hemicellulose from their complex with lignin. And the second is the depolymerization of the carbohydrate polymer that is the cellulose and hemicellulose.

So, out of the three components, cellulose, hemicellulose and lignin, lignin has to be removed first and then the cellulose and hemicellulose will be treated to produce the free sugars. And in the third step, the fermentation of the mixed hexose and pentose sugar that is the out of the hemicelluloses, the pentose sugar will come and the cellulose will give the most the hexose sugar. So, the hexose and pentose sugars which are coming out after the second treatment step are subjected to the microbial fermentation to produce the ethanol.

(Refer Slide Time: 17:10)



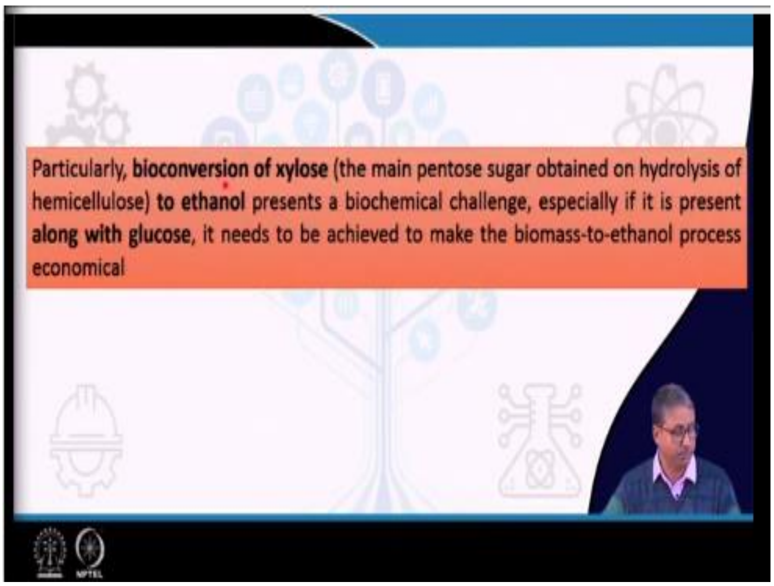
Now towards a feasible economic process for ethanol production requires certain kind of aspects. The first one is the feasible biological delignification. So, by isolating lignin degrading microorganism, for example different type of fungal strains are successfully isolated and microbial strains are isolated and effectively coordinating their eco physiological requirements and optimal bioreactor designing.

Direct microbial conversion to cellulose to ethanol, so in order to reduce the step 2 and then to step 3 direct microbial conversion of cellulose to ethanol is also targeted, where simultaneous depolymerization of cellulose carbohydrates, polymers with ethanol production. And new fermentation technology, which is basically targeted for converting these xylose to ethanol, because xylose is very important component which is a pentose sugar present within as a part of the hemicellulose.

And often we have found that most of the ethanol producing microorganisms, they are unable to utilize the pentose sugar like xylose. Or when they utilize xylose, they produce certain intermediates which are toxic, so they try to avoid the xylose utilization. Now since the hemicellulose component constitutes around 20, 25% of the total lignocellulosic biomass.

Successful utilization of both hexose like the glucose sugars, hexose sugars and the pentose sugars like xylose is found to be a very important and critical step to achieve the commercial deployment of the metabolically engineered organisms. And this has been particularly important to make the overall conversion process more cost effective, because as I mentioned, nearly 20% or 25% of the biomass lignocellulosic biomass is composed of hemicellulose which is made up of the pentose sugar.

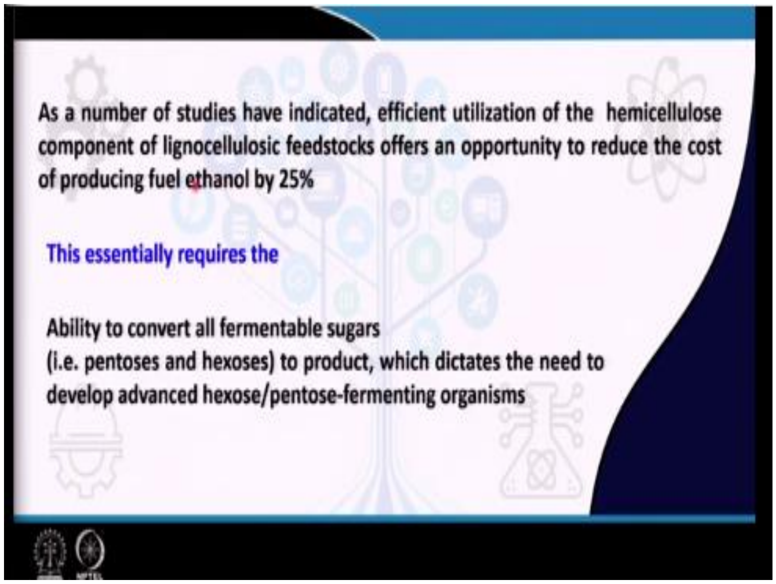
(Refer Slide Time: 19:15)



So, particularly, the bioconversion of xylose which is the main pentose sugar obtained on hydrolysis of the hemicellulose part to ethanol presents a biochemical challenge. So, especially if it is present along with the glucose, it needs to be achieved to make the biomass to ethanol process economical. Now the bioconversion of xylose like pentose sugar which is present along with the hexose sugar like glucose.

The complete metabolism of both the pentose sugar as well as the hexose sugar like glucose to ethanol that was one of the first challenges towards the metabolic engineer that how to achieve this.

(Refer Slide Time: 20:05)

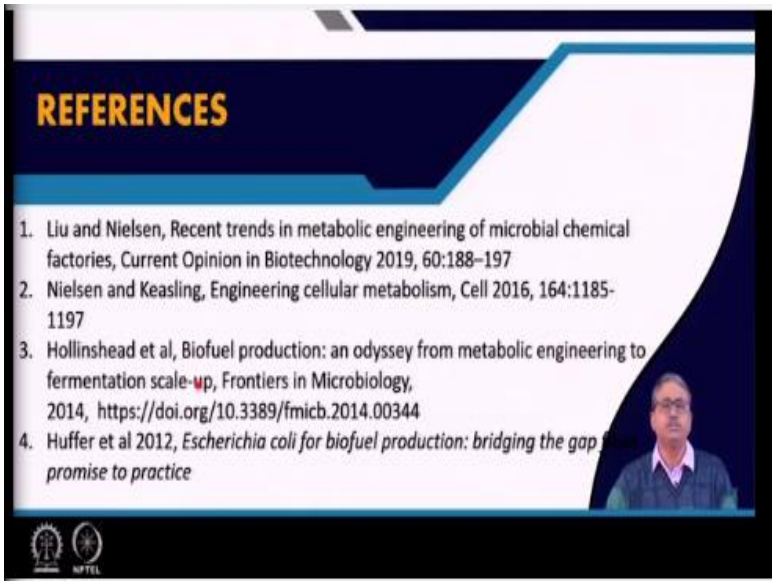


So, as a number of studies have indicated efficient utilization of the hemicellulose component of the lignocellulosic feedstock offers an opportunity to reduce the cost of production of fuel ethanol by 25%. So, for this reason, the cost effectiveness and the overall sustainability of the ethanol or bio ethanol production by microbial fermentation, initial attempts were made. So, that the organisms are capable of utilize the both the hexose and pentose sugar and then produce ethanol successfully.

However, this particular process that utilization of both hexose and pentose sugar and produce or convert them to ethanol essentially requires ability of this organism to convert all fermentable sugars that is the pentose and hexose both to the product that is the ethanol. Now, which dictates the need to develop the advanced hexose, pentose fermenting organism.

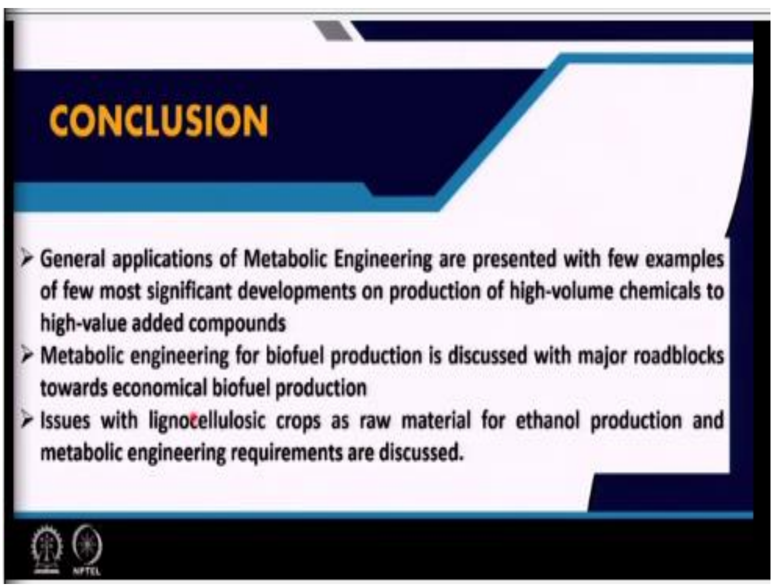
That means, any microorganisms which going to be selected for production of or cost effective production of the ethanol bio ethanol through fermentation either prokaryotic organism or eukaryotic organism, like Saccharomyces cerevisiae. It must be capable of utilizing both hexose and pentose together.

(Refer Slide Time: 21:29)



So, this part of the lecture is based on the following literature and we would be continuing this particular topic to it is further section.

(Refer Slide Time: 21:40)



Now, in conclusion, in this lecture the general applications of metabolic engineering are presented with few examples. More significant developments are highlighted; metabolic engineering of biofuel production is discussed with major roadblocks towards economical biofuel production. And issues with lignocellulosic crops as raw material for ethanol production and metabolic engineering requirements are also discussed, thank you.