MICROBIAL BIOTECHNOLOGY

Prof. Utpal Bora
Department of Biosciences and Bioengineering
Indian Institute of Technology Guwahati

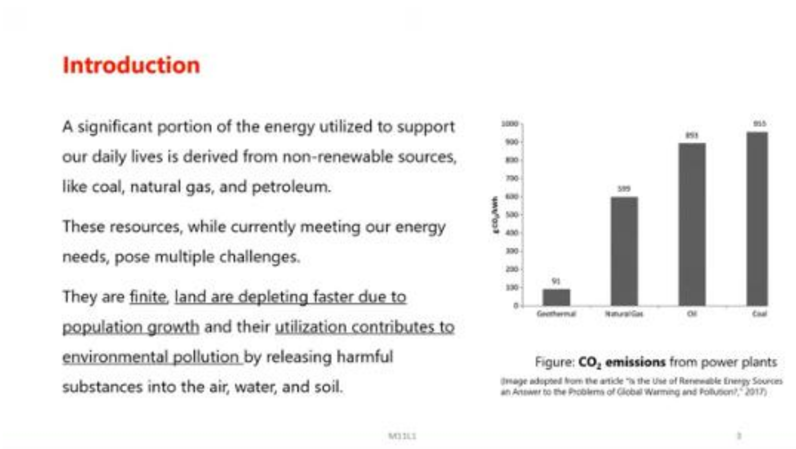
Lecture-36

Lec 36: Production of Biofuel using microbes

Hello friends, welcome to my course on microbial biotechnology. Today we are starting module number 11, which will deal with the role of microbes in producing alternative energy. So, in lecture 1, we are going to discuss the production of biofuels using microbes. This lecture is divided into three broad sections. In Section 1, we will start with an introduction to the topic.

We will discuss the need for alternative energy sources, how we utilize biomass, microbial biofuel production from various feedstocks, technologies available for converting biomass into energy, the definition of biofuels, types of biofuels, and microbes involved in biofuel production. We will also cover the role of microbes in biofuel production, pretreatment of solid waste, why microbial pretreatment is important, various fungal enzymes for lignocellulose degradation, lignocellulose degradation by lignolytic enzyme systems, hemicellulose degradation by hemicellulolytic enzyme systems, and cellulose degradation by cellulolytic enzyme systems. We will describe the remaining two sections as we come across them. A significant portion of the energy used to support our daily lives comes from non-renewable sources like coal and natural gas. These resources, while currently meeting our energy needs, pose multiple challenges because they are finite.

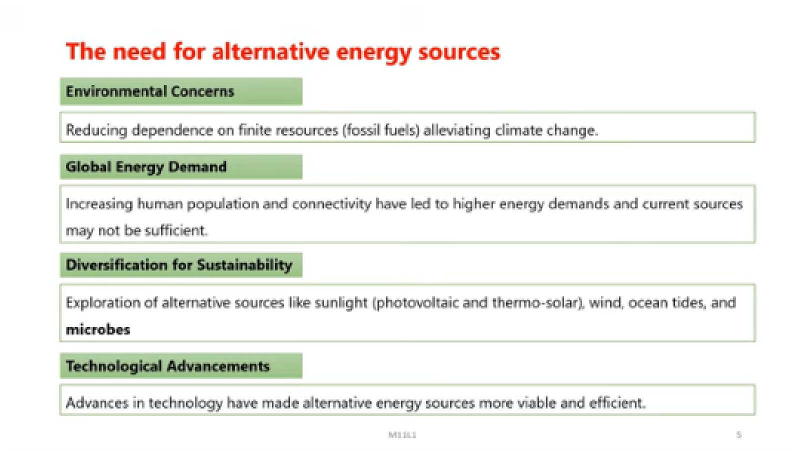
They are depleting faster due to population growth and infrastructure development. Their utilization contributes to environmental pollution by releasing harmful substances into the air, water, and soil. Here we can see the carbon dioxide emissions from various power plants compared to geothermal energy, natural gas, oil, and coal. Coal is the most polluting, followed by oil and natural gas. The entire life cycle of these fossil fuels—mining, transportation, refining, and consumption—leaves behind a substantial carbon footprint, thereby exacerbating global warming.



Addressing these issues is imperative for fostering a sustainable and healthy future. A key solution is replacing fossil fuels with clean, renewable energy sources. An alternative approach involves the utilization of biomass, enabling the production of biofuels by microbes. These biofuels can match or even surpass the performance of fossil fuels. Let us examine the need for alternative energy.

It is important because of environmental concerns, reducing dependence on finite resources like fossil fuels, and addressing climate change. There is a global energy demand. Increasing human population and connectivity have led to higher energy demands, and current sources may not be sufficient. Diversification for sustainability is important. Exploration of alternative sources like sunlight (photovoltaic and thermo-solar), wind, ocean tides, and microbes is important.

Then, from the technological advancements point of view, Advancements in technology have made alternative energy sources more viable and efficient. Let us now focus on the utilization of biomass. Biomass is the total cellular dry weight or organic material an organism generates, usually from carbon dioxide and sunlight. Mostly, these are plant-based or algae-based.

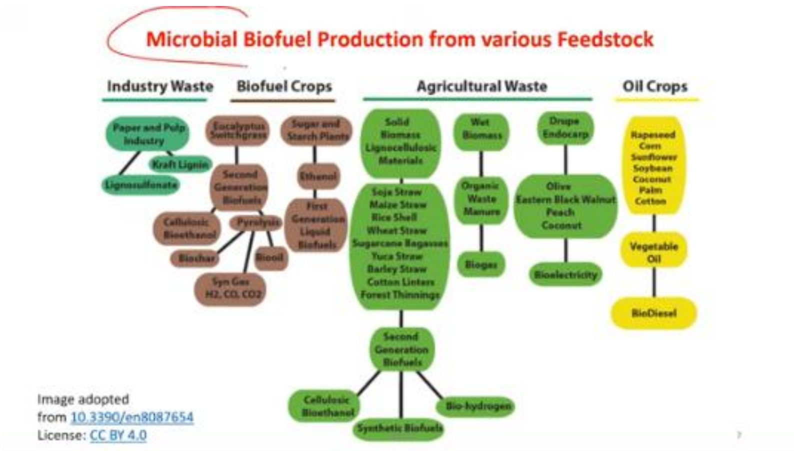


Waste biomass is categorized into starch, lignocellulosic, and oil-based biomass. Various microorganisms convert these types of organic biomass into biofuels like bioethanol, biodiesel, biogas, biohydrogen, butanol, acetone, and advanced biofuels. Starch and lignocellulosic biomass are pretreated with microbes or enzymes. To break down their structure, microbes directly convert water-soluble sugars into biofuels, while oils and fats are transesterified to produce biodiesel.

Let us look into microbial biofuel production from various feedstocks. It includes industrial waste, biofuel crops, agricultural waste, and also oil crops directly. Waste from industries like paper and pulp generates a lot of kraft lignin and lignosulfonate, which can be converted into biofuels. Then we may directly grow biofuel crops like eucalyptus or switchgrass, as well as sugar- and starch-producing plants, which can be converted into ethanol—the first-generation liquid biofuel. Biofuel crops like eucalyptus and switchgrass provide us with second-generation biofuels by supplying cellulose for biobutanol production or being used for pyrolysis to produce biochar and syngas, such as hydrogen, carbon monoxide, and carbon dioxide.

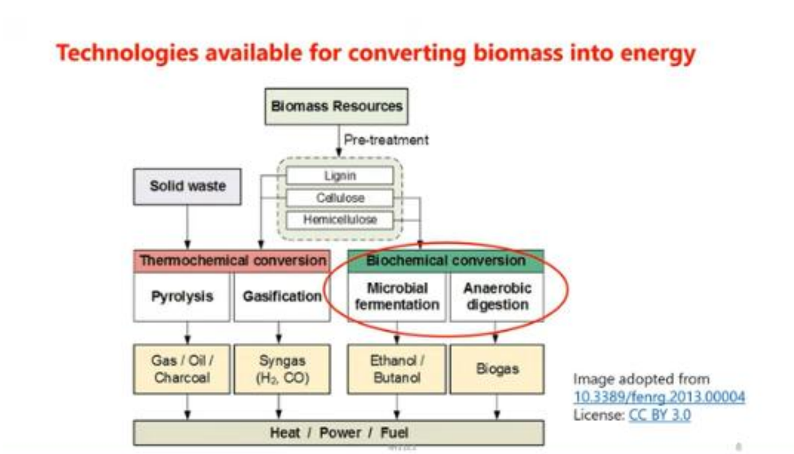
Then we have a large portion of waste generated from agricultural produce. We commonly term them as agricultural waste, which may be solid biomass, lignocellulosic material, wet biomass, or drupe endocarp. All these—for example, solid biomass—provide the scope for producing bioethanol from cellulosic material, often referred to as second-generation biofuels. Wet biomass, particularly organic waste manure, is used for biogas production. Then the drupe endocarp—like olive, eastern black walnut, peach, coconut, etc.

Can be used for producing bioelectricity. Then we have oil crops, which directly produce oil, such as rapeseed, corn, sunflower, soybean, coconut, palm, and cotton. These vegetable oils can be directly converted into biodiesel. So, we can see that there are various feedstocks from which we can produce biofuel, but these processes involve the action of microbes. That's why we call them microbial biofuels.



Now, what are the technologies for converting the biomass that we just discussed in the earlier slide into energy? So, we have these diverse biomass resources. Which we need to pretreat. Then we also have solid waste, which may be directly used for thermochemical conversion through the processes of pyrolysis and gasification. We can also use this biomass, which is obtained by treatment, for thermochemical conversion.

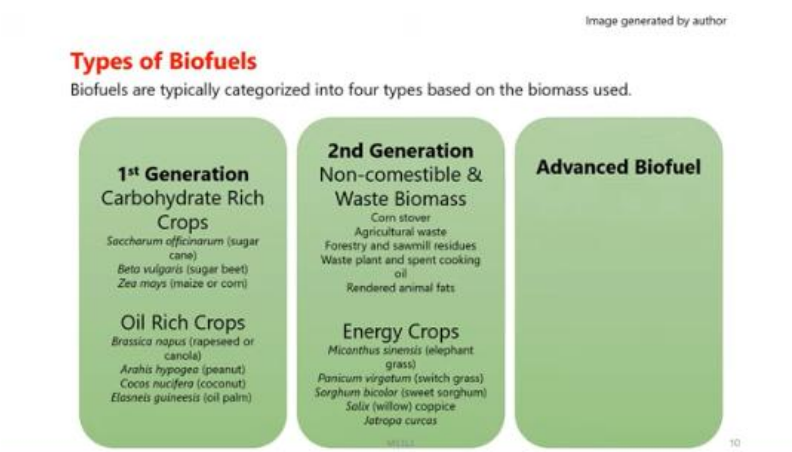
Through the process of pyrolysis, we can obtain gas, oil, and charcoal, and through gasification, we can produce syngas. And we have lignin, cellulose, and hemicellulose obtained by pretreatment. These can be used for biochemical conversion, either through microbial fermentation or anaerobic digestion, producing ethanol, butanol, and biogas, respectively, all of which are used as power or fuel sources. In our lecture here, we will mostly focus on the biochemical conversion part. So, let us have a brief definition of biofuels.



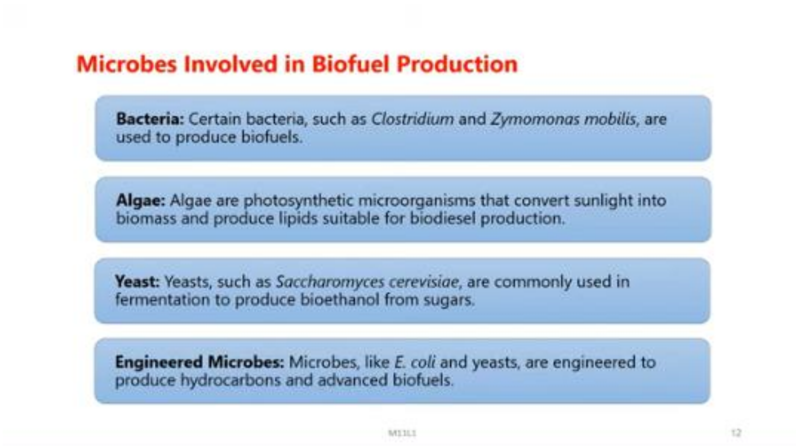
So, we can see that they can be so diverse depending on the biomass. Biofuels are fuels produced by biological agents from biomass, which could be solid, liquid, or gaseous. They are primarily used in transportation as an alternative to non-renewable fossil fuels derived from petroleum. Biofuels offer a solution as fossil fuel supplies are declining rapidly. Economic and industrial growth in developing countries has increased fossil fuel consumption, further highlighting the need to consider biofuels as a sustainable replacement.

So, we can categorize biofuels into four different types based on the biomass used. They can be first-generation, biofuels like carbohydrate-rich crops, sugarcane for example, Saccharum officinarum, and then sugar beet, beet of Algeria, said GMAs or corn. Then we have the oil-rich crops like Brassica napus, then we have the peanut, Arachis hypogea, and then Cocos nucifera (coconut), and we may also use the oil palm. Then second-generation biofuels are the non-combustible and waste biomass.

Corn stover, agricultural waste, forestry and sawmill residue, waste plant and spent cooking oil, then rendered animal fats are some examples of these second-generation biofuels. Then we have the energy crops like Miscanthus sinensis, then we have Panicum virgatum, or we may have sweet sorghum or Jatropha curcas. Then we have advanced biofuels, or third-generation biofuels, like microalgae, cyanobacteria, oleaginous microbes, and fourth-generation biofuels, which are genetically engineered microbial cell factories. Microbiofuels are renewable energy sources derived from microorganisms. Bioethanol is produced by Saccharomyces cerevisiae fermentation and is widely used as a gasoline additive to enhance combustion and reduce emissions.

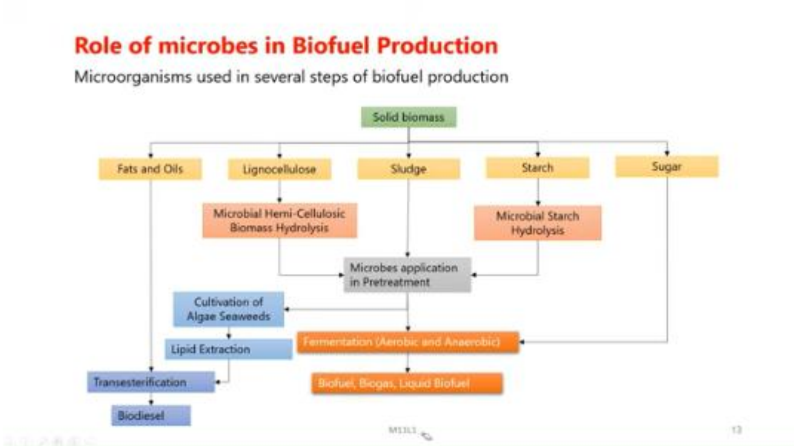


Butanol is synthesized by Clostridium bacteria and offers higher energy content and better engine compatibility than ethanol. Biodiesel is made by transesterifying triacylglycerides from oils or fats into fatty acid methyl esters (FAMEs) by some oleaginous microbes. The microbes involved in biofuel production can be bacteria like Clostridium and Zymomonas mobilis, which produce various biofuels, or algae, which are photosynthetic microorganisms that convert cellulose into biomass and produce lipids suitable for biodiesel production. Then we have yeast like Saccharomyces cerevisiae, which is used in the fermentation process to produce bioethanol from sugars. Then we may have engineered microbes like E. coli and yeast, which are engineered to produce hydrocarbons and advanced biofuels.



What is the role of microbes in biofuel production? Microorganisms are used in several steps of biofuel production. For example, we may have solid biomass, which may be further divided into fats and oils, lignocellulose, sludge, starch, and sugar. These fats and oils can be transesterified. Lignocellulose microbial hemicellulose biomass hydrolysis is done.

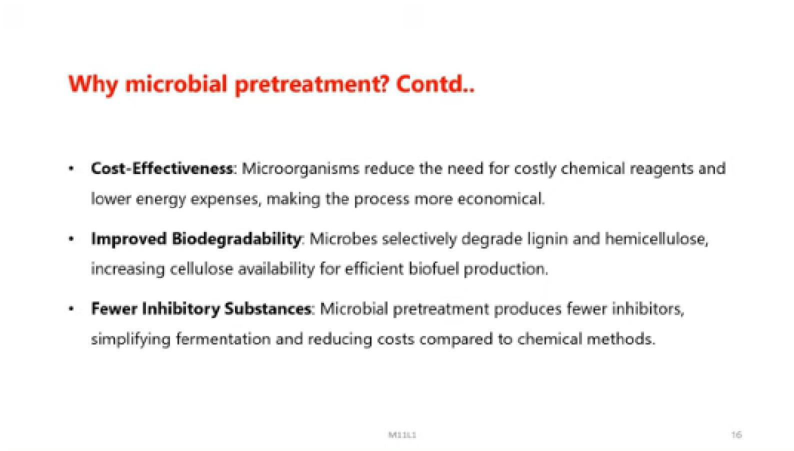
Similarly, starch hydrolysis is also done. These undergo microbial pretreatment. Then we may use them for the cultivation of algae and seaweeds, from which we extract lipids that can be further transesterified. Some parts of these pretreated lignocellulose, lignin, and starch can also be directly fermented by both aerobic and anaerobic microbes, which give rise to biofuels, biogas, and liquid biofuels. We may obtain sugar directly, which can also be fermented to produce biofuels.



So, the pretreatment of solid waste by microbes is very, very important, particularly solid waste from agricultural, municipal, and forest resources, which often contain hemicellulose, lignin, and cellulose, which are difficult to biodegrade. Pretreatment is required to prepare this biomass for fermentation. Biological pretreatment with microorganisms, especially white rot fungi like Phanerochaete chrysosporium, offers a sustainable, cost-effective alternative to chemical methods. Microwave pretreatment is preferred because it is sustainable. It is eco-friendly, relies on natural processes without producing harmful by-products or hazardous waste.

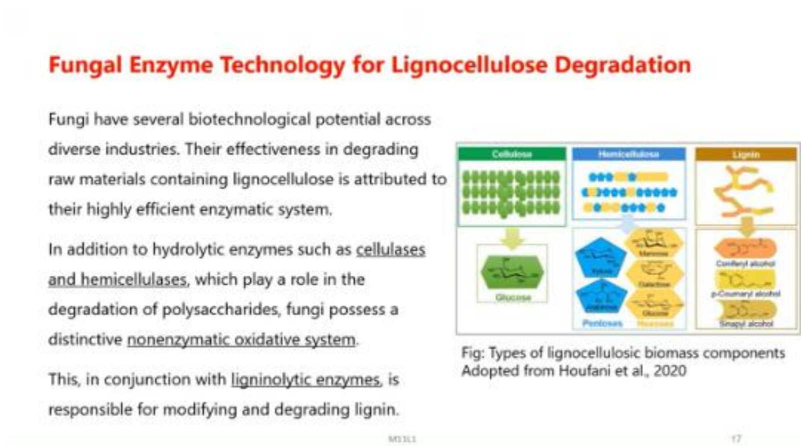
So, there is reduced chemical use or minimal use of synthetic chemicals that have less environmental impact and also reduces the cost involved. Then, it also utilizes lower energy because these microbial processes operate at ambient conditions. Therefore, it requires less energy than thermal or chemical methods. These are cost-effective methods. It does not involve costly chemical reagents, and the energy expenses are also cut down, making the process much more economical.

And then there is improved biodegradability due to microbes, which selectively degrade lignin and hemicellulose, increasing cellulose availability for efficient biofuel production. There are fewer inhibitory substances when microbial pretreatment is done. It simplifies the fermentation process and reduces cost compared to chemical methods. Let us now discuss fungal enzyme technology for lignocellulose degradation. Fungi have several biotechnological potentials across diverse industries.



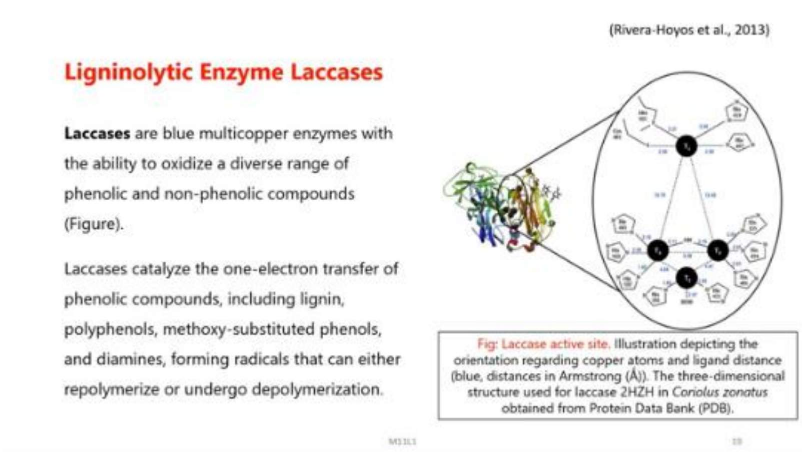
Their effectiveness in degrading raw materials Containing lignocellulose is attributed to their highly efficient enzymatic systems, in addition to hydrolytic enzymes such as cellulases and hemicellulases, which play a role in the degradation of polysaccharides. Fanzai possesses a distinctive non-enzymatic oxidative system. Here, you can see the illustrative representation of holocellulose, hemicellulose, and lignin cellulose, mostly consisting of glucose units. When it is broken down, we can obtain glucose.

Hemicellulose contains xylose and arabinose, which are pentose sugars. It also contains hexose sugars: mannose, galactose, and glucose. They may be arranged in different permutations and combinations. Then we have lignin, which consists of coniferyl alcohol, p-coumaryl alcohol, and sinapyl alcohol as its constituent units. These, in conjunction with lignolytic enzymes, are responsible for modifying and degrading lignin.



Lignin degradation by lignolytic enzyme systems is one of the most important steps in this process. This is primarily an oxidative process, often linked to secondary metabolism or conditions of limited carbon and high nitrogen or sulfur availability. It is rarely utilized as a sole carbon and energy source. Basidiomycete white-rot fungi are the primary agents for lignin degradation in nature. These fungi produce key lignolytic enzymes like laccases, manganese peroxidases, lignin peroxidases, and versatile peroxidases.

So, these lacquers are blue multi-copper enzymes with the ability to oxidize a diverse range of phenolic and non-phenolic compounds. These illustrations depict the orientation regarding copper atoms and the ligand distance, the blue distances in Armstrong's, the three-dimensional structure used for lacquers in Coriolis and Jonathas which are obtained from PDB. They catalyzed the one electron transfer of phenolic compounds including lignin, polyphenols, methoxy substituted phenols and diamines forming radicals that can either repolymerize or undergo depolymerization. They exist as monomeric, dimeric or tetrameric glycoproteins typically containing four copper atoms per monomer distributed across three redox heights named T1, 2 and 3. In their resting state all four copper ions are in the plus two oxidation state as you can see in the figure.



The majority of lacquers are produced by white rot fungi secreted into the medium by the mycelium of filamentous fungi. Some examples of fungi known for producing lacqueages with high activity includes trematase pubescence, coriolis hirsutus and others. So, here you can see the lacquered catalytic cycle, substrates are oxidized by the Cu T1 center and electrons are transferred by highly conserved motif, His-Cis-His to the T2 and T3 copper centers, this is where the reduction of molecular oxygen to water takes place. Let us now discuss about the hemicellulose degradation by hemicellulolytic enzymes, the hydrolysis of hemicellulose

(Rivera-Hoyos et al., 2013)

They exist as monomeric, dimeric, or tetrameric glycoproteins, typically containing four copper atoms per monomer, distributed across three redox sites named T1, T2, and T3. In their resting state, all four copper ions are in the +2 oxidation state **(Figure)**.

The majority of laccases are produced by white-rot fungi, secreted into the medium by the mycelium of filamentous fungi. Examples of fungi known for producing laccases with high activity include *Trametes pubescens*, *Coriolus hirsutus*, and others.

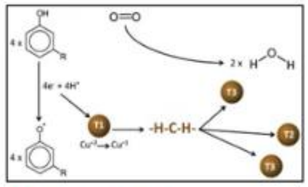


Fig: Laccase catalytic cycle. Substrates are oxidized by the Cu-T1 center and electrons are transferred by a highly conserved motif: His-Cys-His (HCH) to the T2 and T3 copper centers. This is where the reduction of molecular oxygen to water takes place.

requires the coordinated action of multiple enzymes that target various components within the hemicellulose matrix. So, here we have different enzymes with their EC number and the substrates corresponding to these enzymes and the various functions listed in these tables. For example, beta-menosidase which is the EC number of 3.2.1.25 The substrate for these menocytes is mannose containing oligosaccharides. They hydrolyze beta D menocytic linkages breaking down mannons and mannons containing oligosaccharides.

Then we have the beta xylosidases which hydrolyze beta D xylosidic bonds in xylan or xylo oligosaccharides thereby releasing xylos. Then we have the beta xylosidases malanases, which hydrolyze beta-D-mannan into smaller oligosaccharides or monosaccharides. Then we have the beta-gylanase, which cleaves internal beta-1,4-glycosidic bonds within the gylan chain, producing shorter oligosaccharides. So, here this figure shows enzymatic hydrolysis involved in the breakdown of cellulose and hemicellulose biomass.

Hemicellulose degradation by hemicellulolytic enzymes

The hydrolysis of hemicellulose requires the coordinated action of multiple enzymes that target various components within the hemicellulosic matrix.

Enzyme	Enzyme Commission (EC) Number	Substrate	Function
β-Mannosidases	EC 3.2.1.25	Mannose-containing oligosaccharides (e.g., β-D-mannose)	Hydrolyze β-D-mannosidic linkages, breaking down mannans and mannan-containing oligosaccharides.
β-Xylosidases	EC 3.2.1.37	Xylose-containing oligosaccharides (e.g., β-D-xylose)	Hydrolyze β-D-xylosidic bonds in xylan or xylooligosaccharides, releasing xylose.
β-Mannanases	EC 3.2.1.78	Mannan (β-D-mannan)	Hydrolyze β-D-mannan into smaller oligosaccharides or monosaccharides.
β-xylanase	EC 3.2.1.8	Xylan (a polysaccharide made of xylose)	Cleaves internal β-1,4-glycosidic bonds within the xylan chain, producing shorter oligosaccharides.

So, here we have these hemicellulose where the hydrolysis begins with an endoglucanase attack as you can see here, then these oligomers are available for the exoglucanase action where This is cleaving this. Then we have this crystalline region where the cellulobios are released. So, these basically the beta glucoside is this will finally release the glucose. Then here again we can see the action of endogylanases taking place that releases the gylobios and beta-gylocidases will thereby release the gylos and the action of beta-mananases will give rise to mannooligomers which will be acting upon beta-manlocidases to release the mannose.

So, this is in brief the enzymatic hydrolysis in the breaking down of cellulose and hemicellulose biomass. Fungal degradation of cellulose relies on a suite of glycoside hydrolyzed enzymes each with distinct catalytic roles. Cellulases possess a carbohydrate binding module that facilitates binding to crystalline cellulose. Based on their mode of action and specificity, cellulases are categorized into three primary classes. Endoglucanases, exoglucanases and beta-glucosidases having distinct EC numbers and they act on cellulose, beta-1,4-glucan and cellulobios, beta-1,4-glucosidic bone.

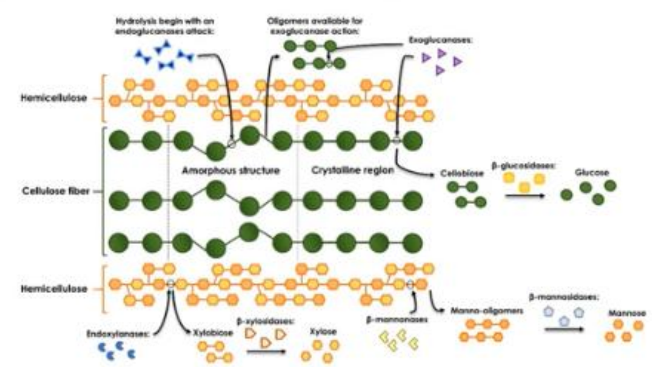
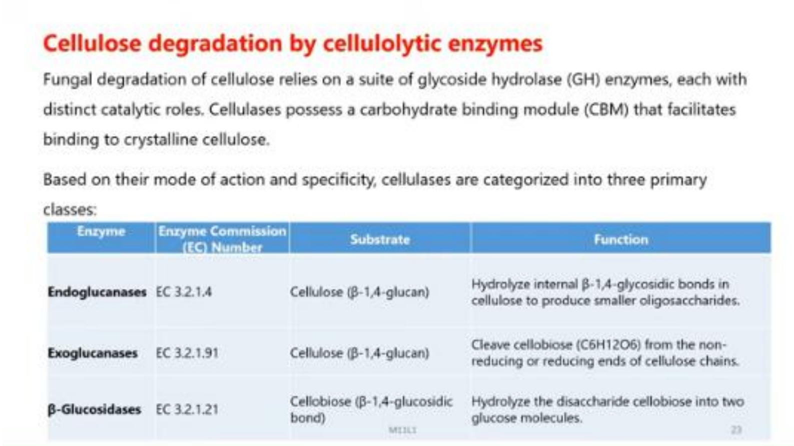


Figure: Enzymatic hydrolysis involved in the breakdown of cellulose and hemicellulose biomass (Adopted from Araújo et al., 2023)

And the functions are listed corresponding to endoglucanases. It hydrolyzes internal beta-1,4-glycosidic bonds in cellulose to produce smaller oligosaccharides. Exoglucanases cleave cellobioses from the non-reducing or reducing ends of cellulose chains. Beta-glucosidases hydrolyze the disaccharide cellobiose into two glucose molecules.



Cellulose degradation by cellulolytic enzymes

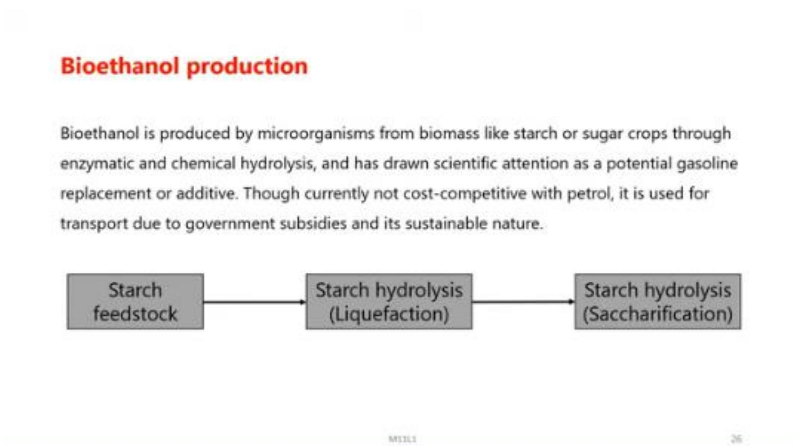
Fungal degradation of cellulose relies on a suite of glycoside hydrolase (GH) enzymes, each with distinct catalytic roles. Cellulases possess a carbohydrate binding module (CBM) that facilitates binding to crystalline cellulose.

Based on their mode of action and specificity, cellulases are categorized into three primary classes:

Enzyme	Enzyme Commission (EC) Number	Substrate	Function
Endoglucanases	EC 3.2.1.4	Cellulose (β-1,4-glucan)	Hydrolyze internal β-1,4-glycosidic bonds in cellulose to produce smaller oligosaccharides.
Exoglucanases	EC 3.2.1.91	Cellulose (β-1,4-glucan)	Cleave cellobiose (C6H12O6) from the non-reducing or reducing ends of cellulose chains.
β-Glucosidases	EC 3.2.1.21	Cellobiose (β-1,4-glucosidic bond)	Hydrolyze the disaccharide cellobiose into two glucose molecules.

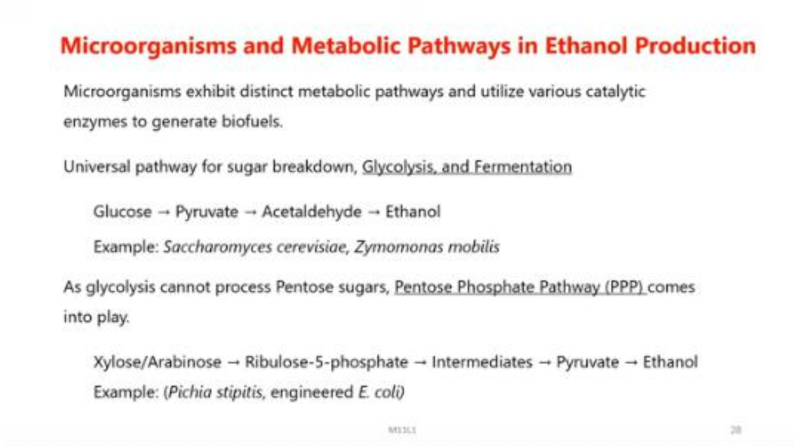
Let us now move to section 2, where we will discuss some of the major biofuels, how bioethanol is produced, how biodiesel is produced, and how biogas and biohydrogen are produced. So, as we can see, the major biofuels include bioethanol, biodiesel, biogas, and hydrogen gas. Bioethanol is produced by microorganisms from biomass like starch or sugar crops through enzymatic and chemical hydrolysis and has drawn scientific attention as a potential gasoline replacement or additive, though currently not cost-competitive with petrol. It is used for transport due to governmental subsidies and its sustainable nature. So, for the production of bioethanol, we need starch feedstock, which is hydrolyzed.

The process is also known as liquefaction. Then we proceed to the saccharification step, whereby we obtain fermentable sugars that are acted upon by yeast to produce bioethanol. Saccharomyces cerevisiae is preferred for bioethanol production due to its tolerance to high ethanol concentrations and inhibitors. Bacteria like Escherichia coli and Zymomonas mobilis are other commonly used microbes for ethanol production. Saccharomyces cerevisiae and Zymomonas mobilis are commonly used microbes, but neither can ferment xylose, a monosugar in lignocellulosic biomass hydrolysate.

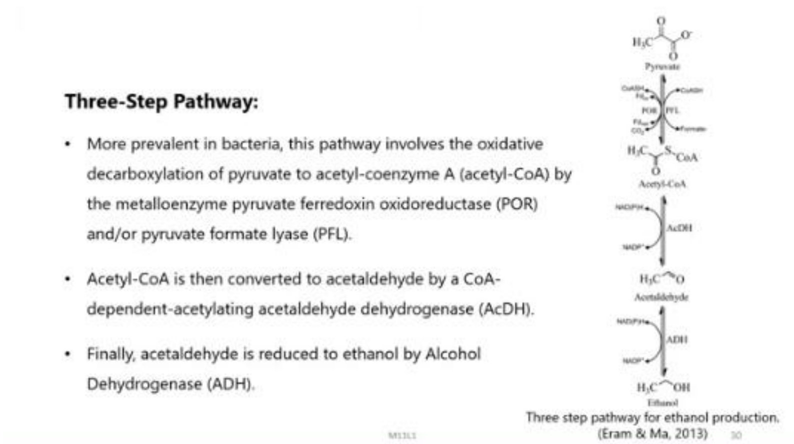


Efforts to genetically engineer Saccharomyces cerevisiae to ferment xylulose have shown promise, with genes from Pichia stipitis enabling xylulose conversion to ethanol. However, no engineered strain has reached commercial production yet. These microorganisms exhibit distinct metabolic pathways and utilize various catalytic enzymes to generate biofuels. The universal pathway for sugar breakdown, primarily glycolysis and fermentation, involves glucose conversion to pyruvate, then acetaldehyde, and finally to ethanol with the help of yeast and bacteria. Since glycolysis cannot process pentose sugars, the pentose phosphate pathway comes into play.

Xylose and arabinose are converted to ribulose 5-phosphate, which is then converted to many intermediates and finally to pyruvate, and ultimately to ethanol. In this case, Pichia stipitis and engineered E. coli have been used. Some pathways for the production of acetaldehyde and pyruvate, intermediates in the central metabolism of carbohydrates, can undergo conversion to acetaldehyde, a precursor ultimately reduced to ethanol through two distinct pathways. One is a two-step pathway in this process. Pyruvate undergoes non-oxidative decarboxylation to form acetaldehyde and carbon dioxide, catalyzed by pyruvate decarboxylase.



Subsequently, acetaldehyde is transformed into ethanol through the action of alcohol dehydrogenase. Then there is a three-step pathway, which is more prevalent in bacteria. This pathway involves the oxidative decarboxylation of pyruvate to acetyl coenzyme A by the metalloenzyme pyruvate ferredoxin oxidoreductase or pyruvate formate lyase. Acetyl coenzyme A is then converted to acetaldehyde by a coenzyme-dependent acetylating acetaldehyde dehydrogenase. Finally, acetaldehyde is reduced to ethanol by alcohol dehydrogenase.



Let us now have a discussion on the next important biofuel: biodiesel. Biodiesel is a diesel-like liquid derived from biological materials, typically made of fatty acid alkyl esters. It is an environmentally friendly alternative to diesel fuel, attracting attention for use in diesel engines. While alkaline catalysis is common for commercial production, enzymatic transesterification offers benefits like fewer processing steps and easier glycerol separation, though high enzyme costs are a limiting factor. Immobilizing fungal mycelium and expressing lipase on yeast cells helps reduce enzyme costs to a large extent.

The production process involves the transesterification of oil, and sometimes we also start with methanol, ethanol solvent, immobilized lipase enzymes or potassium hydroxide, which is a chemical. These are all mixed and used in the transesterification process. Then we proceed with phase separation, and the byproduct glycerol is separated out. Through this phase separation, we obtain crude biodiesel.

We then wash this crude biodiesel, dry it, and finally obtain the biodiesel. The feedstock used in biodiesel production, particularly microbial feedstock, includes bacteria, yeast, fungi, and microalgae with high lipid content, with oleaginous bacteria being less used compared to microalgae and yeasts. Among bacteria, Rhodococcus species are well studied for their versatile substrate growth and lignin degradation potential. Using algae for biodiesel is promising due to their easy cultivation, diverse metabolic activities, and high fatty acid content. Algal species like Dunaliella, Tetraselmis, and Chlorella are identified as potential feedstocks.

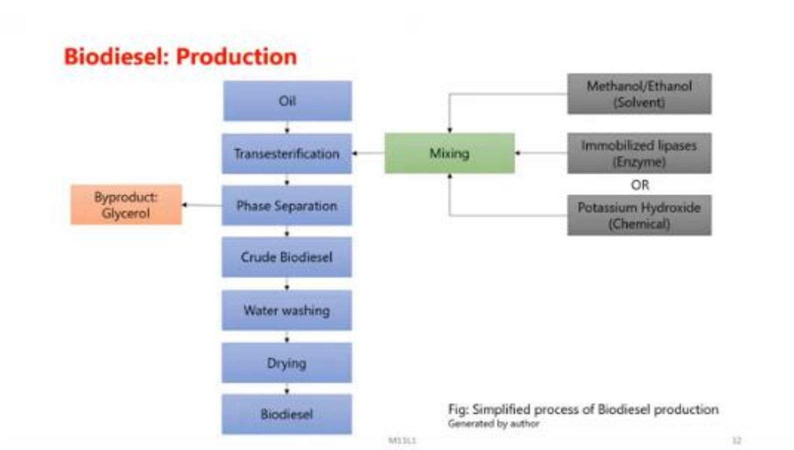
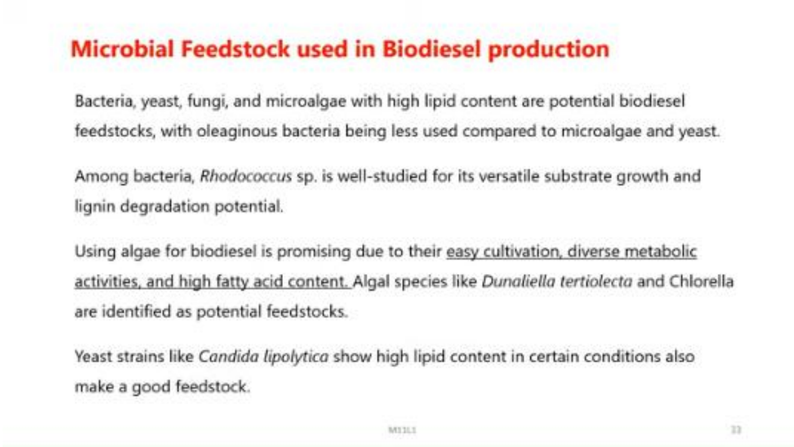
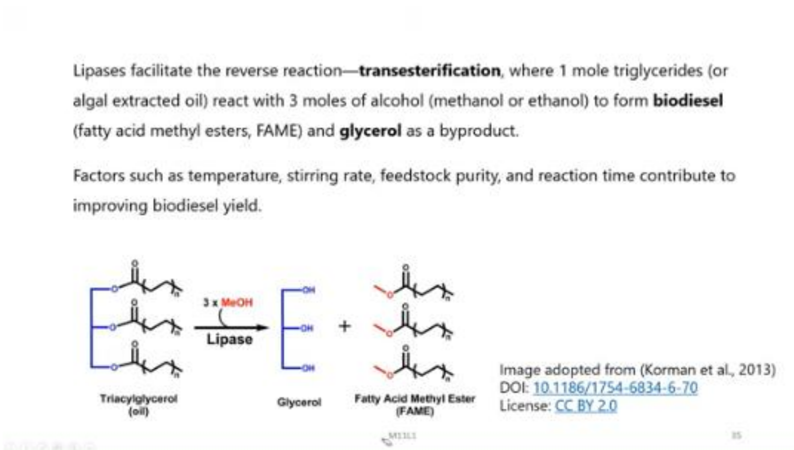


Fig: Simplified process of Biodiesel production
Generated by author

E-strains like Candida lipolytica show high lipid content in certain conditions and they are also potentially very good feedstock. The transistification is the most important step in this whole process. The production of biodiesel from algae involves obtaining its lipid fraction by either mechanical process such as microwave assisted pyrolysis extraction, ultrasonic assisted extraction or chemical methods involving organic solvents like chloroform, petroleum ether. Acetone, Methanol and Hexane as well as techniques like Soxhlet and supercritical fluid extractions. Following the extraction of Algal lipids the next crucial step is the transesterification of these lipids into biodiesel.

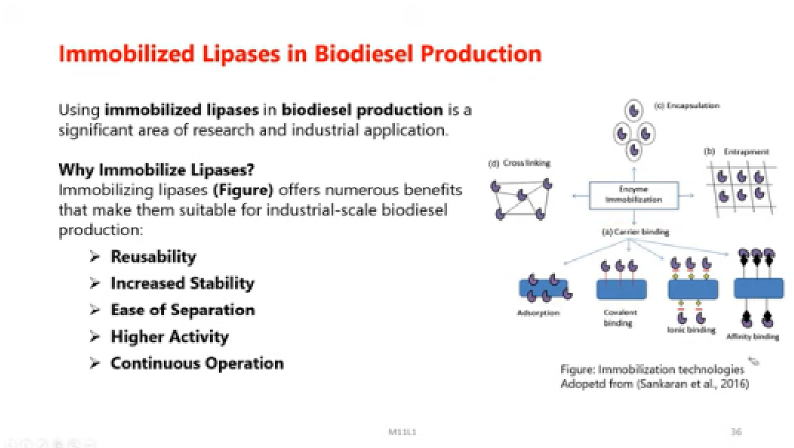


Lipases facilitate the reverse reaction transesterification where one mole triglycerides reacts with three moles of alcohol, methanol or ethanol to form biodiesel, fatty acid methyl stress and glycerol as a byproduct as you can see in this reaction. Factors such as temperature, stirring rate, feedstock purity and reaction time contribute to improving biodiesel yield. Sometimes we will use immobilized lipases in this catalytic reaction. Using immobilized lipases in biodiesel production is a significant area of research and industrial application.

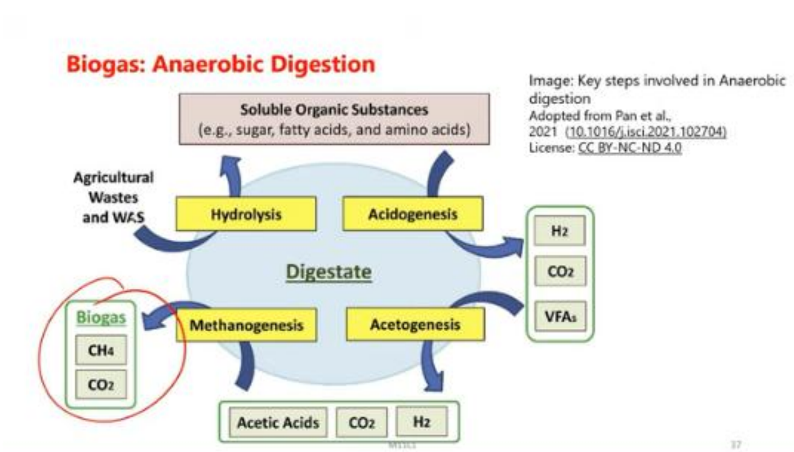


Immobilizing offers numerous benefits that make them suitable for industrial scale biodiesel production. The first utility is that we can reuse the enzyme. Immobilization increases the stability of the enzyme, disease of separation of the enzyme the catalyst from the product immobilize enzyme so higher activity and we can use it for continuous operation so there are various methods of immobilization due to limitation of scope and time we will not go into details of those so some of these can be binding them to carriers by simple adsorption or covalent binding or ionic binding or by affinity binding methods

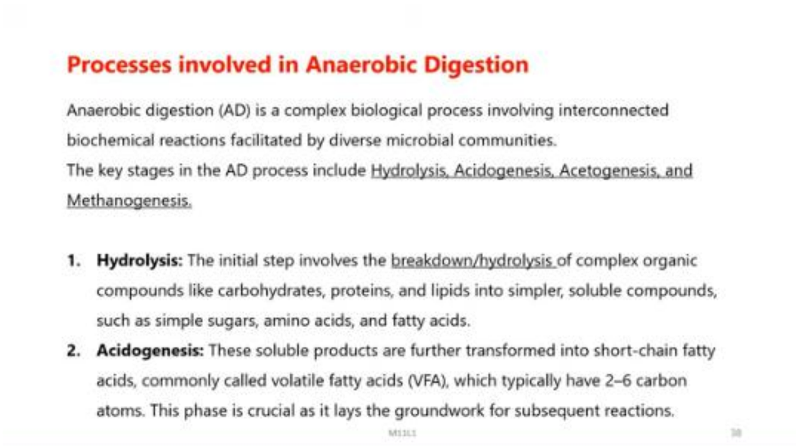
Another method is to encrypt them into a certain matrix or encapsulate them into small particles which may be micro particles then also cross linking the lipases with one another.



Let us now discuss the production of biogas, particularly anaerobic digestion. So, here we have this digester which we will be using for the production of biogas. Particularly, we have agricultural waste which we use for hydrolysis reactions that produce soluble organic substances like sugar, fatty acids, and amino acids, which are used for carrying out acidogenesis to produce hydrogen, carbon dioxide, and volatile fatty acids, which are further subjected to acetogenesis reactions. And these products of the acetogenesis reactions are further treated for carrying out methanogenesis, which produces biogas. So, this is in brief, the process of biogas production by anaerobic digestion, which is a complex biological process involving interconnected biochemical reactions facilitated by diverse microbial communities.



The key stages include hydrolysis, acidogenesis, acetogenesis, and methanogenesis, as already shown in the diagram. Hydrolysis is the initial step that involves the breakdown of complex organic compounds like carbohydrates, proteins, and lipids into simpler soluble compounds such as simple sugars, amino acids, and fatty acids, followed by acidogenesis, where these soluble products are further transformed into short-chain fatty acids, commonly called volatile fatty acids, which typically have two to six carbon atoms. This phase is crucial as it lays the groundwork for subsequent reactions. Then, the third is acetogenesis, where these volatile fatty acids produced undergo further anaerobic oxidation, resulting in the production of acetic acid along with the release of carbon dioxide and hydrogen gas.



These steps serve as an intermediary stage before the final conversion of these compounds into methane. In the final methanogenesis, we have the conversion of the produced gases, particularly acetate, and a combination of hydrogen and carbon dioxide into methane. This conversion occurs through two primary pathways. One is acetoclastic methanogenesis, where acetate is directly transformed into methane and carbon dioxide.

Then there is hydrogenotrophic methanogenesis. Hydrogen, in combination with carbon dioxide, is utilized to produce methane. The culmination of these various steps results in the production of biogas, primarily composed of methane and carbon dioxide, which can be harnessed as a renewable energy source. Some of the microbes used in anaerobic digestion are hydrolytic bacteria for the hydrolysis stage, which includes Clostridium, Bacteroides, Bacillus, and Actinomyces. Then, the acidogenic bacteria involved in acidogenesis include Escherichia coli, Clostridium, Lactobacillus, and Streptococcus.

3. Acetogenesis: The VFAs produced undergo further anaerobic oxidation, resulting in the production of acetic acid, along with the release of CO_2 and hydrogen gas (H_2). This step serves as an intermediary stage before the final conversion of these compounds into methane.

4. Methanogenesis: The final stage involves the conversion of the produced gases, particularly acetate and a combination of hydrogen and CO_2, into methane (CH_4). This conversion occurs through two primary pathways:
Acetoclastic Methanogenesis: Acetate is directly transformed into methane and CO_2.
Hydrogenotrophic Methanogenesis: Hydrogen, in combination with CO_2, is utilized to produce methane.

The culmination of these steps results in the production of biogas, primarily composed of methane and CO_2, which can be harnessed as a renewable energy source.

Then, for the acidogenesis stage, we have acidogenic bacteria like Syntrophomonas, Syntrophobacter, and Clostridium. And the methanogenic archaea carry out the methanogenic reactions. These are mostly Methanobacterium, Methanospirillum, Methanosarcina, and Methanosaeta. Now, let us discuss the production of biohydrogen, which is produced by bacteria and algae and is mainly generated through anaerobic fermentation and photolysis of water. Photosynthetic microbes such as cyanobacteria, photosynthetic bacteria, and green algae, as well as non-photosynthetic bacteria like anaerobic and nitrogen-fixing bacteria, are key producers of hydrogen.

Microbes in Anaerobic Digestion

1. Hydrolytic Bacteria *(Hydrolysis Stage)*
- *Clostridium spp.*
- *Bacteroides spp.*
- *Bacillus spp.*
- *Actinomyces spp.*

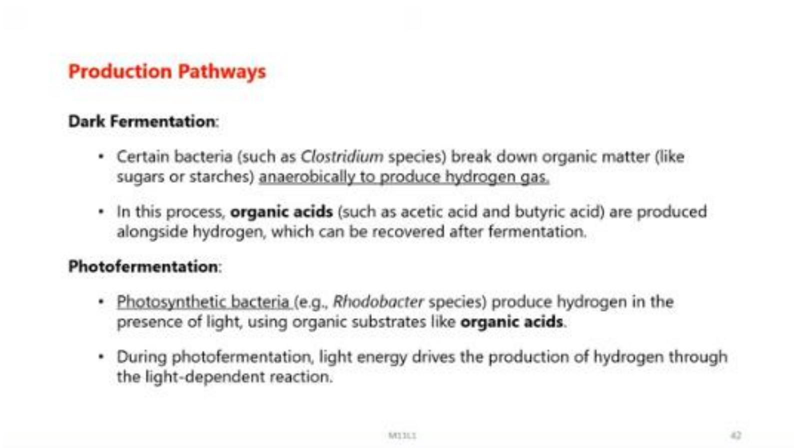
2. Acidogenic Bacteria *(Acidogenesis Stage)*
- *Escherichia coli*
- *Clostridium butyricum*
- *Lactobacillus spp.*
- *Streptococcus spp.*

3. Acetogenic Bacteria *(Acetogenesis Stage)*
- *Syntrophomonas spp.*
- *Syntrophobacter spp.*
- *Clostridium thermoaceticum*

4. Methanogenic Archaea *(Methanogenesis)*
- *Methanobacterium spp.*
- *Methanospirillum spp.*
- *Methanosarcina spp.*
- *Methanosaeta spp.*

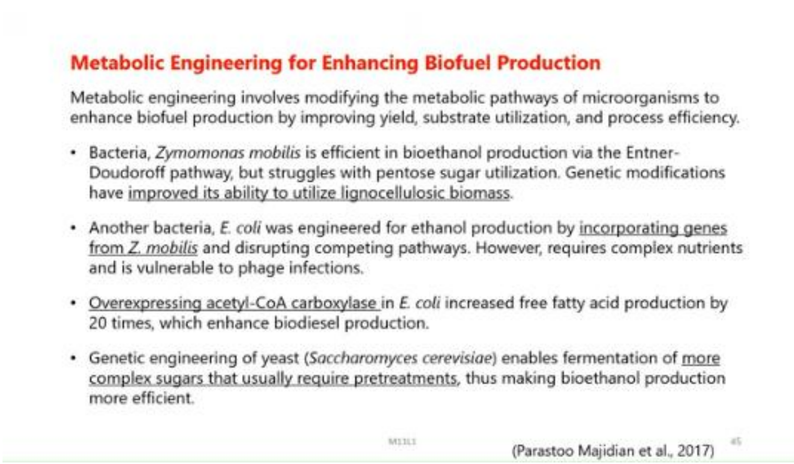
Non-photosynthetic and halophilic photosynthetic bacteria can produce hydrogen. The co-culture of Rhodobium marinum and Vibrio fluvialis produces hydrogen comparable to the bacterial community, highlighting the significance of these bacteria in hydrogen production from starches. The production pathways for biohydrogen involve dark fermentation, where certain bacteria like Clostridium break down organic matter such as sugars or starches anaerobically to produce hydrogen gas. In this process, organic acids such as acetic acid and butyric acid are produced alongside hydrogen, which can be recovered after fermentation. Then, in photofermentation, photosynthetic bacteria like Rhodobacter species produce hydrogen in the presence of light using organic substrates such as organic acids.

During photofermentation, light energy drives the production of hydrogen through the light-dependent reaction. Then there is biophotolysis, where microalgae or higher plants utilize sunlight to split water molecules into hydrogen and oxygen in a process similar to photosynthesis. This method is still under research, as it requires efficient methods to optimize and harvest hydrogen from algae. Then we have microbial electrolysis cells, which we will be discussing in the next lecture. Briefly, in MEC, bacteria are used to reduce protons in water and generate hydrogen gas.



This is an electrochemical process where bacteria interact with electrodes in a cell that applies a small electrical current, facilitating the production of biohydrogen. Let us now move to section 3, where we will briefly discuss metabolic engineering for enhancing biofuel production, genetic engineering of lipid metabolism in microalgae, sustainability and environmental benefits of biofuels, and cell engineering in microbial biofuel production. Metabolic engineering involves modifying the metabolic pathways of microorganisms to enhance biofuel production by improving yield, substrate utilization, and process efficiency. Bacteria like Zymomonas mobilis are efficient in bioethanol production via the Entner-Doudoroff pathway but struggle with pentose sugar utilization. Genetic modifications have improved its ability to utilize lignocellulosic biomass.

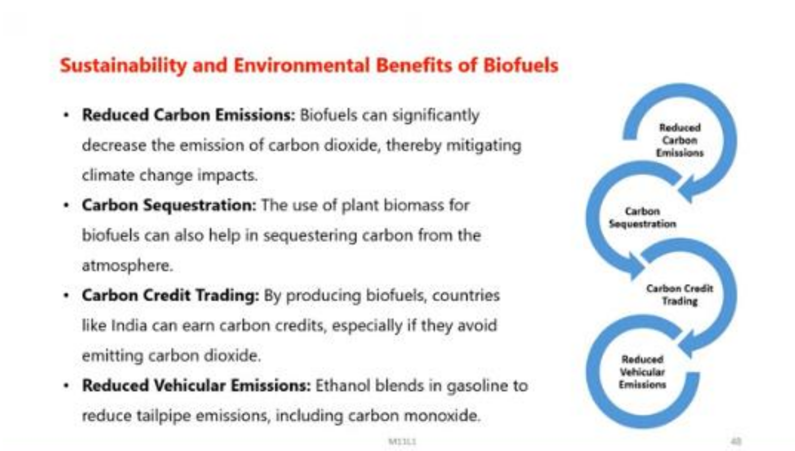
Another bacterium, E. coli, was engineered for ethanol production by incorporating genes from Z. mobilis and disrupting competing pathways. However, it requires complex nutrients and is vulnerable to phage infections. Overexpressing acetyl-CoA carboxylase in E. coli increased free fatty acid production by 20 times, which enhanced biodiesel production. Then there are attempts at genetic engineering of Saccharomyces cerevisiae to enable fermentation of more complex sugars that usually require pre-treatments, thus making bioethanol production more efficient. There are also attempts at genetic engineering of lipid metabolism in microalgae.



Enhancing lipid production by overexpressing certain genes has been shown to increase lipid content in both plants and microalgae. For instance, overexpressing G3 phosphate dehydrogenase in Brassica napus resulted in a 40% increase in lipid production. Blocking competing pathways is another approach in microalgae. Blocking starch production pathways, for example in Chlamydomonas reinhardtii, can divert resources toward triacylglycerol accumulation, especially under stress conditions like nitrogen deprivation, thus increasing lipid content. Modifying lipid catabolism is also a strategy.

Reducing lipid catabolism by inactivating genes involved in fatty acid degradation can increase lipid accumulation. However, this often results in growth inhibition, as the breakdown of lipids is also crucial for energy production. Modifying lipid characteristics by genetic modification, which aims to change the fatty acid profile—such as shifting chain lengths or unsaturation—can enhance biofuel suitability. Overexpression of thioesterase genes from Umbellularia californica in microalgae can produce short-chain fatty acids ideal for biodiesel, while others aim to produce short-chain fatty acids for gasoline or jet fuel production. Biofuels benefit us in many ways, particularly from a sustainability and environmental perspective. Biofuels help us reduce carbon emissions.

They can significantly decrease the emission of carbon dioxide, thereby mitigating climate change impacts. They help in carbon sequestration, particularly the use of plant biomass for biofuels, which can sequester carbon from the atmosphere. Additionally, they can help by offering carbon credit trading through biofuel production. Countries like India can earn carbon credits, especially if they avoid emitting carbon dioxide. There will also be reduced vehicular emissions, with internal blends in gasoline reducing tailpipe emissions, including carbon monoxide.



However, there are many challenges in microbial biofuel production. It suffers from low yield and productivity. Limited biofuel production rates in engineered microbes make it economically unfeasible at large scales. The cost of raw materials, high cost of feedstocks like lignocellulosic biomass and waste oils, is due to complex processing requirements. Then there are the technical challenges, where genetic stability—particularly in engineered strains—may lead to the loss of biofuel production traits over time.

There are also challenges like scaling up, as it is difficult to translate lab-scale processes to large industrial systems. Additionally, environmental concerns arise, such as competing land use—biofuel crops may compete with food production, raising sustainability issues. Furthermore, the sustainability of feedstocks is a concern, as large-scale feedstock production may have environmental impacts, such as water use and soil depletion. So, with this, we come to the end of this lecture. Thank you for your kind attention.