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Fakulta chemicko-technologická

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Výroba biolíhu z lignocelulózových materiálů
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ANOTACE

Tato bakalářská práce se zaměřuje na lignocelulózových biolihu a jeho výrobu. V první části jsou představeny různé druhy používané lignocelulózových biomasy a jejich složení. Dále se práce zaměřuje na technologický proces výroby bioetanolu a využití různých mikroorganismů pro výrobu paliva.

KLÍČOVÁ SLOVA

Druhá generace, Biomasa, Lignocelulóza, Biolihu, Výroba, Předúprava, Fermentace, Mikroorganismy

TITLE

The production of Bioethanol from Lignocellulosic materials

ANNOTATION

This bachelor thesis focuses on lignocellulosic bioethanol and its production. The first part introduces the different kinds of lignocellulose biomass used and their composition. Furthermore, the thesis focuses on the current technological process of bioethanol production and the utilization of different microorganisms for fuel production.

KEYWORDS

Second-generation, Biomass, Lignocellulose, Bioethanol, Production, Pretreatment, Fermentation, Microorganisms

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
CBP	Consolidated bioprocessing
DMBQ	2,6-Dimethoxybenzoquinone
EISA	Energy Independence and Security Act of 2007
HMF	Hydroxymethylfurfural
HVO	Hydrotreated vegetable oil
IEA	International Energy Agency
ILs	Ionic liquids
RD	Renewable diesel
ROS	Reactive oxygen species
SHF	Separate hydrolysis and fermentation
SSCF	Simultaneous Saccharification and Co-Fermentation
SSF	Simultaneous saccharification and fermentation

INTRODUCTION

The 21st century is suffering from climate change and has seen an increase in the demand to find a sustainable alternative source of energy especially for industries such as the transportation and heating industries. As the negative impact of fossil fuels on today's climate is also a major concern, this alternative energy should be associated with a significant reduction in greenhouse emissions and environmental pollution.[1]

Biofuels such as bioethanol and biomethanol are an alternative energy sources due to their environmental friendliness. As a renewable energy source, they have little to no impact on the balance of the atmosphere's air contents, this is due to the negligible amount of carbon dioxide and a considerably low level of sulphur emission. Biofuels can therefore be a way to reduce or remove completely the world's dependency on unsustainable energy resources [2].

We can characterize biofuels based on their biomass origin as 1st, 2nd, and 3rd generation biofuels. 1st generation fuels are produced from biomass that is also considered a food source.

2nd generation biofuels are produced from a type of non-food biomass known as lignocellulosic biomass. These include discarded biomass from agricultural and food processing systems. Lastly, 3rd generation biofuels are produced from aquatic biomass. [3]

Bioethanol is of great importance to the transportation industry worldwide. its production from biomass addresses the need to reduce not only the consumption of fossil fuels but also the need to reduce environmental pollution. Bioethanol can be generally produced from agricultural or forestry raw materials like simple sugars, starch, and lignocellulose, of which lignocellulose raw materials are the most promising.[4]

The thesis focuses on the production of lignocellulosic bioethanol as an alternative biofuel. One advantage of lignocellulosic bioethanol is that it helps address the threat on food security that sugar and starch-based bioethanol poses. Lignocellulose being the most available biomass on earth also makes lignocellulosic bioethanol production a good biofuel source for countries rich in agricultural and forestry waste [1],[5]

1 BIOETHANOL

Bioethanol chemically referred to as ethyl alcohol ($\text{CH}_3\text{-CH}_2\text{-OH}$) or as grain alcohol is a clear biodegradable liquid that can be produced from various types of biomasses with help of different conversion technologies. Production of bioethanol largely involves the fermentation of C_6 sugars (mostly glucose) from starchy crops such as corn and rice. Bioethanol has been in use since the early 1900s, but due to how expensive production costs were compared to other fuels, it was overlooked. The mass production of bioethanol was only initiated during the 1970s in Brazil and the United States [10] and these countries still account for a majority of today's world production of bioethanol. It is believed that by 2050, liquid bioethanol will likely be on top of the 'biofuel ladder' due to its effectiveness in replacing fossil fuels within the transportation sector. In comparison with currently used fossil fuels bioethanol has the following advantageous characteristics:

- A higher octane value.
- Wider flammability range
- A higher evaporating temperature
- A reduced level of harmful particle emission
- A lower cost of production
- More eco-friendly.[6]

1.1 First generation biofuels

Production of first-generation bioethanol, which is designed for road vehicles, uses food feedstock hence, they appear unjustifiable for commercial use due to the food versus fuel argument. Sugarcane is among the common raw materials used in the production of first-generation bioethanol. Brazil is the largest single producer of sugar cane with over 27% of global production and one of leading countries using it to produce first generation bioethanol. The production process using sugarcane is relatively simple. First the sugar cane is crushed in water to generate sucrose, which is then purified to give raw sugar or ethanol. [11] The use of food raw materials has expressed serious concern about the socio-economic and environmental effects brought on by large-scale production. The production of first-generation biofuels competes with the agricultural industries for water and arable land and at the same time contributes to the depletion of resources. The increase of food prices such as cereals, is also an indirect consequence of large-scale production.[12]

1.2 Second generation biofuels

Second-generation bioethanol production may not be ahead in development as first-generation production. However, when it comes to the availability of raw materials, the second-generation production has huge potential if implemented on a large scale. For example, Malaysia, the world's second largest producer of palm oil, generates an excess amount of palm oil waste, which accounts for 85.5% of the total national agricultural waste. The traditional methods for waste treatment, such as landfill, incineration and composting, are not sustainable enough due to the limited space and toxic emission. Palm oil waste has a high organic and oil content, making it a viable replacement for current fossil fuels. [13]

1.3 Third generation biofuels

The third-generation bioethanol is defined as ethanol produced from the use of marine organisms such as microalgae and macroalgae. These have very distinctive growth yield when compared to other biomass sources in first- and second-generation production. Lipid and carbohydrate content in microorganisms is a main factor when it comes to algal ethanol production, for example in species such as *Chlorella*, which may contain as much as 60 to 70% of lipid content are highly targeted for this process. The advantage of using algae as alternative biomass is that it is easy to cultivate in a wide range of aquatic environments, requires less land usage and has a high-level carbon dioxide (CO₂) absorption. The problems associated with algal biomass are linked to the need for favourable conditions. This entails a constant availability of large volumes of water, normally at industrial levels.[11] The shortage of water resources for algal cultivation contributes to the unstable industry of third generation bioethanol production.

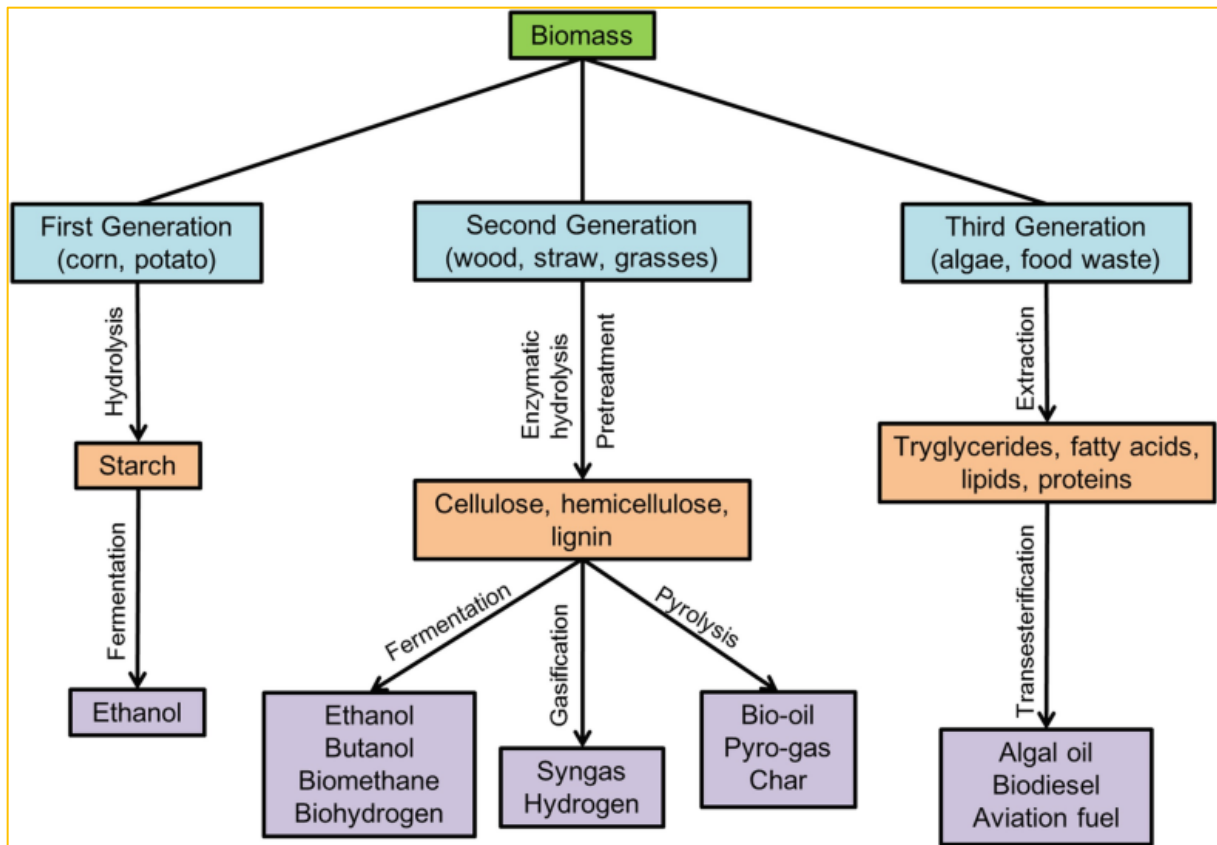


Figure 1. An illustrated Structure of production steps of first-, second-, and third-generation biofuels.[14]

2 LIGNOCELLULOSIC BIOMASS AND IT'S STRUCTURE

Lignocellulosic biomass is a natural renewable resource, a complex component of plant structure and the most abundant neutral carbon source in the planet that has the potential to be used in the production of biofuels.[7] In general lignocellulosic biomass can be broken up into cellulose (30–60%), hemicelluloses (20–40%), and lignin (15–25%),and small portion of extractives and ashes .Forestry, agricultural and agro-industrial wastes materials are major sources of lignocellulosic biomass. These include materials such as, corn cobs, sugarcane bagasse, wastepaper, brewer's leftover grains, grass, and stalks, leaves, straws, stems, husks, shells.[8]

2.1 Lignocellulose structure

Lignocellulose is mainly made up of two carbohydrate polymers, cellulose and hemicellulose, and a non-carbohydrate polymer, lignin. These are all part of a complex structure of the cell wall in which the polymers hemicellulose and lignin surround the cellulose skeleton. [8]

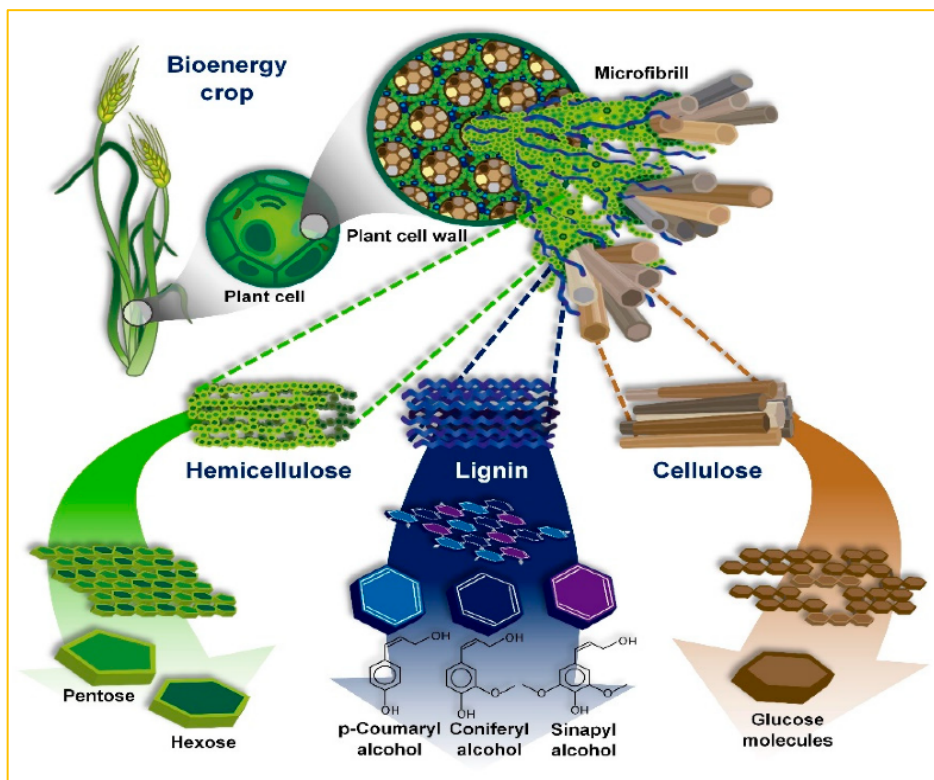


Figure 2. An illustrated Structure of lignocellulosic biomass and its biopolymers.[9]

Cellulose is the major component of plant cell structure with a high molecular weight. It is a linear homo-polymer consisting of repeated cellobiose (β -1,4 glycosidic linking of two D-pyran-glucose). [9] cellulose molecules are aggregated together in the form of micro-fibrils, in

which crystalline regions alternate with amorphous regions. The crystalline region contains linear molecules of cellulose bonded laterally by hydrogen bonded. The amorphous region allows easy disintegration of the cellulose by hydrolysis. Because of the crystalline region containing strong hydrogen bonds, this region of the cellulose is more recalcitrant to enzyme and microbial degradation.[37]

Hemicellulose is a linear and branched heterogeneous polymer typically made up of various pentose and hexose sugars such as arabinose, galactose, glucose, mannose and xylose - as well as other components such as acetic and glucuronic acids.[8]

Lignin is a highly branched complex structure that comprises three phenolic alcohol units as its monomers namely p-coumaroyl alcohol, coniferyl alcohol and sinapyl alcohol. The oxidative polymerisation of these alcohols is what leads to the formation of lignin. Its function is to provide rigidity and strength to the cell wall and protect the structural polysaccharides from microbial degradation. Lignin does not participate in bioethanol production, but it is a source of essential aromatic products [9]

The composition of cellulose, hemicellulose, and lignin vary from one plant species to another. In addition to this, the ratios of these polymers may differ even in a single plant species depending on the age, growth stage and other conditions. In most cases if not all, cellulose tends to maintain its status as the dominant polymer with content value ranging from 35-50%, this is followed by hemicellulose which accounts for 20–35% of the plant cell wall. The third component which is lignin accounts for 10–25% of lignocellulose dry mass.[8]. The contents of these polymers in some lignocellulose wastes are shown in Table 1.

Lignocellulose Biomass	Composition (%weight)		
	Cellulose	Hemicellulose	Lignin
<i>Barley straw</i>	33.8	21.9	13.8
<i>Corn cobs</i>	33.7	31.9	6.1
<i>Rice straw</i>	36.2	19.0	9.9
<i>Sugarcane bagasse</i>	40.0	27.0	10.0
<i>Wheat straw</i>	32.9	24.0	8.9
<i>Rye straw</i>	37.6	30.5	19.0
<i>Sunflower stalks</i>	42.1	29.7	13.4

Table 1. The contents of cellulose, hemicellulose, and lignin in common lignocellulose wastes [8]

3 BIOCHEMICAL CONVERSION OF LIGNOCELLULOSIC BIOMASS

The main steps in the conversion of lignocellulosic biomass into ethanol are hydrolysis of cellulose and hemicellulose, fermentation, separation of lignin residue and, lastly recovery and the purification of the ethanol product. Hydrolysis of lignocellulose biomass into fermentable sugars is problematic because it is highly recalcitrant to degradation by microbial attack such that an essential pretreatment step is included in the process.[15]

3.1 Pretreatment

Pretreatment is an essential step that breaks down the lignin structure and enhances hydrolytic enzyme's ability to access the cellulose by disrupting its crystalline structure. The aim of the pretreatment is also to enhance the availability of fermentable sugars during the hydrolysis step.[4] The changes that lignocellulosic biomass undergo during the pretreatment process can be shown schematically in the Figure.3

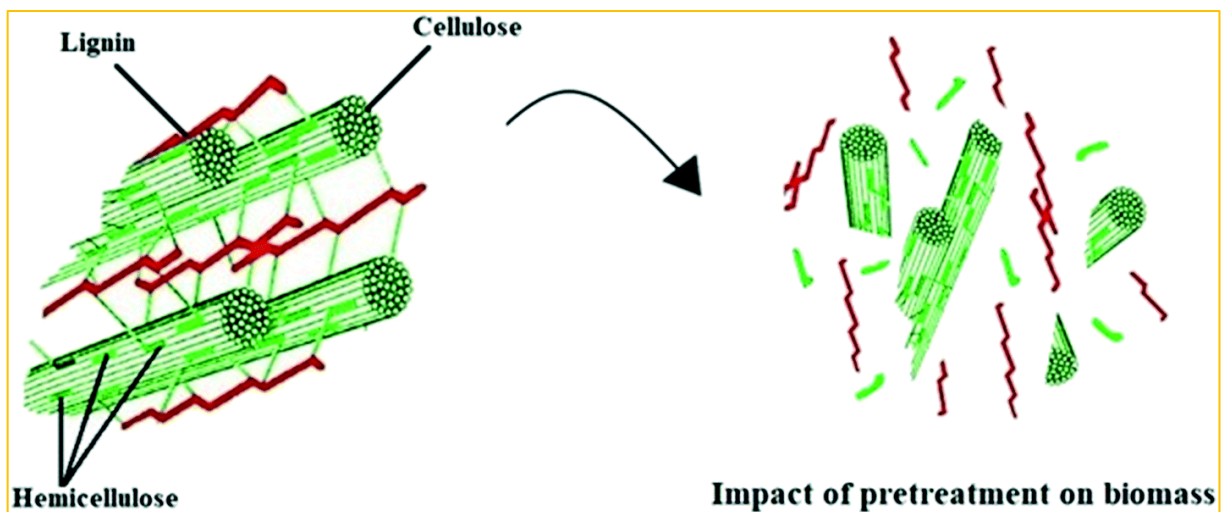


Figure 3. Illustration of lignocellulose pretreatment [16]

Despite being a crucial step in the bioconversion of lignocellulose biomass, the pretreatment of biomass is said to be the second most costly step and ways in which to make it more cost effective is still being developed. There are several factors to take into consideration for an ideal lignocellulose pretreatment [15]it should: Enhance enzymatic attack on cellulosic fibre, avoid the degradation hemicelluloses and cellulose, avoid the formation of inhibitors during hydrolysis and fermentation, reduce the energy demand, reduce the cost of size reduction for feedstocks and construction of pretreatment reactors, and lastly produce less residues.[4][17]

Pretreatment methods can be classified into four kinds: physical, Physico-chemical, chemical, biological or a combination of all the mentioned. The choice of pretreatment method relies greatly on the properties of the lignocellulose biomass since each lignocellulose biomass has its own Physico-chemical properties [15]

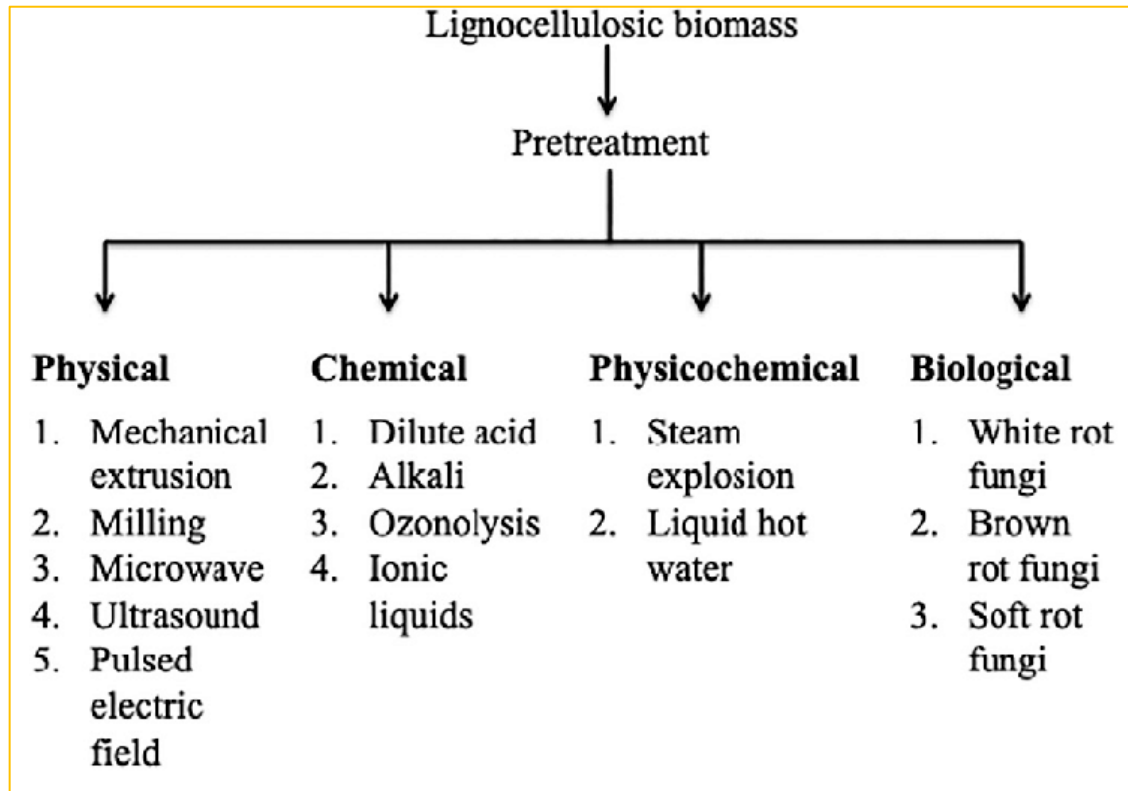


Figure 4. Classifications of lignocellulose pretreatment methods [36]

3.1.1 Physical pretreatment

Physical pretreatment of lignocelluloses typically involves mechanical operations such as grinding, microwaving, pyrolysis, milling and chipping. The aim is to reduce the crystallinity of the cellulose fibres in the biomass and make it easier to access hydrolysable polymers by increasing the pore size and surface area of lignocellulosic biomass.

3.1.1.1 Mechanical comminution

This method of biomass pretreatment is carried out by cutting, grinding or milling the biomass into smaller particles. Mechanical Comminution has its advantages as it participates in the overall bioconversion of lignocellulosic material into biofuels without generating any of toxic

by-products. The procedure of disintegrating and shearing is certainly energy demanding thus the need to maintain the balance between efficiency and energy consumption. In order to achieve this optimum energy use, proper selection of size-reduction equipment must be carried out. The selection should take into account the following mechanical properties such as, the feed rate of material, the particle size being worked on, amount of moisture and finally machine characteristics [18]

3.1.1.2 Microwave

A team of researchers from Kyoto University, Japan made early discoveries of microwave pretreatment on lignocellulosic biomass. Since then, many researchers have shown that microwave pretreatment a promising approach in the conversion of lignocellulosic biomass into useful bioproducts. Microwave pretreatment utilises thermal and non-thermal effects which directly interact with the biomass, thereby accelerating chemical, physical, and biologic reactions. The main advantages of the microwave process are that the procedure can be done with ease, heating capacity is increased, the processing time is shortened, inhibitor generation is minimal, and energy requirements are low. [19][20]

3.1.1.3 Pyrolysis

Pyrolysis is the chemical decomposition of lignocellulosic biomass at very high temperature (which can be between 500 and 800°C) in the absence of an oxidizing agent. The rapid heating of biomass results in the production of organic vapor composed of cellulose, hemicellulose, and lignin polymers fragments. These vapours can then be condensed to produce a bio-oil. The nature of the bio-oil produced profoundly depends on the conditions at which pyrolysis takes place namely temperature, heating rate, pressure, environment, catalyst used etc. [21]

3.1.2 chemical pretreatment

3.1.2.1 Alkali pretreatments

Alkali pretreatment uses alkaline solutions such as Sodium, potassium, calcium and ammonium hydroxides to remove lignin and a part of the hemicelluloses by inducing depolymerization and disruption of lignin-carbohydrate linkages. Of these pretreatment agents sodium hydroxide was found to be the most effective. The conditions of alkali pretreatment depend on the type and composition of biomass used for pretreatment. The most important parameters affecting pretreatment of lignocellulosic biomass are the type and concentration of alkali, biomass loading, temperature as well as pretreatment time. [22]

3.1.2.2 Acid pretreatment

Acids are used to pretreat lignocellulosic biomass. The acid pretreatment method is based on the susceptibility of the glucosidic bonds between hemicellulose and cellulose to acid. The breakdown of the glucosidic bonds leads the transformation of polysaccharides into oligomeric and monomeric sugars. This type of pretreatments can utilize concentrated or diluted acid for which concentrated acid is less attractive for bioethanol production as this steer into the formation of inhibiting compounds. Most used acids are mineral acids (e.g., H_2SO_4 , HCl , HNO_3 , and H_3PO_4) and organic acids (e.g., CH_2O_2 , $\text{C}_2\text{H}_4\text{O}_2$, $\text{C}_3\text{H}_6\text{O}_2$ and $\text{C}_4\text{H}_4\text{O}_4$). [22][23]

3.1.2.3 Organosolv

Methanol, acetone, ethanol, and ethylene glycol are all organic solvents that can be used during organosolv pretreatment method. These organic solvents under specific temperature and pressure conditions can extract lignin in the presence of salt catalysts, acid or base. Cellulose fibres are therefore exposed when lignin is extracted which makes it easier for hydrolysis to be carried out.

3.1.2.4 Ionic liquids (ILs) pretreatment

Ionic liquids are salts, made up of large organic cations and small inorganic anions, which at relatively low temperatures exist as liquids. The disruption of non-covalent interactions among biomass polymers of cellulose, hemicellulose and lignin occurs due to Ionic liquids competing for hydrogen bonding with lignocellulosic components. Examples of ionic liquids

that have been made use of are 1-Ethyl-3-methylimidazolium diethyl phosphate-acetate, 1-butyl-3-methylimidazolium-acetate, and cholinium amino acids. [18]

3.1.3 Physico-chemical pretreatment

Physico-chemical pretreatment include well known methods such as liquid-hot water (LHW), steam explosion (SE), ammonium fibre explosion (AFEX) whose main go is to enhance hydrolysis.

3.1.3.1 Steam-Explosion

Steam-Explosion pretreatment is one of the commonly used options, it combines both chemical and physical techniques to break the lignocellulosic biomass structure. This pretreatment method exposes the biomass to high pressures and temperatures for a short duration of time followed by sudden reduction of pressure to atmospheric or lower levels. The result is a disruption of the structure of the fibrils increasing the accessibility of the cellulose to the enzymes during hydrolysis.

3.1.3.2 Liquid hot-water (LHW) pretreatment

This hydrothermal process involves heating of biomass in liquid water at elevated temperatures (160°C to 240°C) and high pressure over a time period ranging from a few mins to an hour. The goal of this process is to promote disintegration and separation of the lignocellulosic Matrix.

3.1.3.3 Ammonium fibre explosion (AFEX)

The ammonia fibre explosion process is a Physico-chemical process like steam explosion pretreatment, in which liquid (anhydrous) ammonia is mixed with the biomass material under high pressures and moderate temperatures (60–200°C) and then followed by a rapid pressure drop. This results in disruption of lignin-carbohydrate bonds, hydrolysis of hemicellulose and ammonolysis of glucuronic bonds, and in addition the partial decrystallization of cellulose.[18]

3.1.4 Biological pretreatment

Biological pretreatment utilizes naturally found microorganisms such as white-rot, brown-rot, and soft-rot fungi as well as bacteria to modify the composition or structure of the lignocellulosic biomass prior to enzymatic hydrolysis. The microorganisms secrete hydrolytic enzymes (E.g., hydrolase) and ligninolytic enzymes (E.g., peroxidase and laccases) which target and degrade lignin. The resulting extracellular alteration and degradation of lignocellulose causes the cell wall structure to open and expose the biopolymers to hydrolysis. This method offers many advantages over physical, chemical, and physicochemical pretreatment methods as it is considerably more environmentally friendly, cost effective (requires low energy and no chemicals) and occurs under mild environmental conditions. [24]

3.2 Hydrolysis

Once the pretreatment process is completed, the carbohydrate polymers in lignocellulosic biomass need to be converted into fermentable sugars via a vital stage known as hydrolysis. Hydrolysis can be described as the cleaving of a molecule by introducing a water molecule. Various hydrolysis methods are available but, in this chapter, we will focus on the most applied methods, chemical hydrolysis and enzymatic hydrolysis. [4] Other methods such as gamma-ray or electron-beam irradiation microwave irradiation can be used but are considered commercially unimportant.

Lignocellulosic hydrolysis produces many products which can be used for other purposes other than Bio-oil production [25]

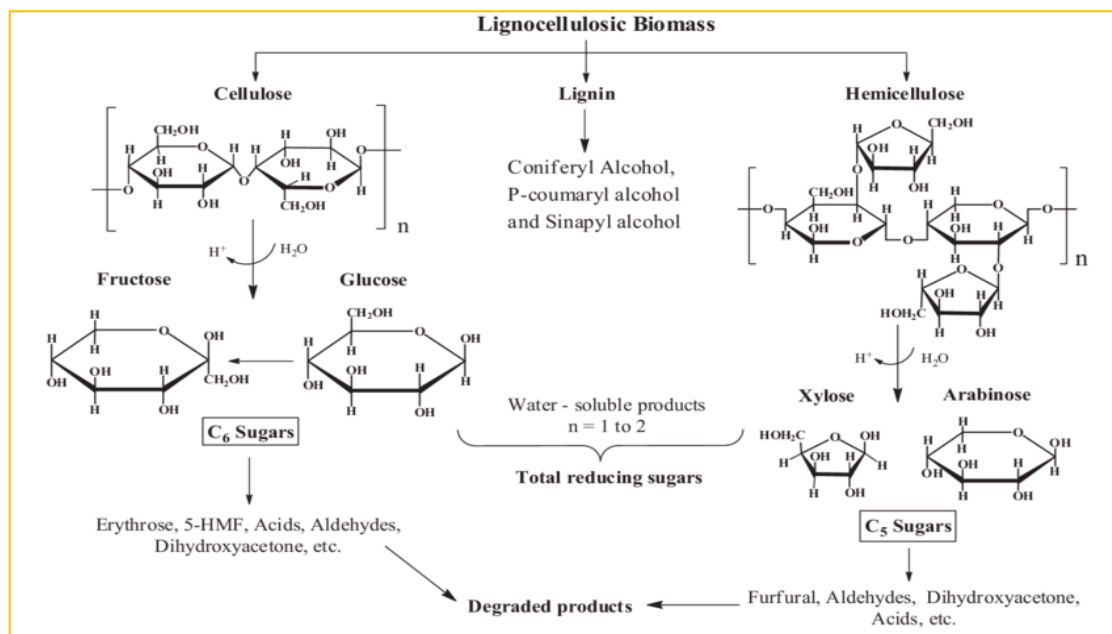


Figure 5. Reaction pathway of lignocellulosic biomass hydrolysis [26]

3.2.1 Enzymatic hydrolysis

This phase in bioethanol production from lignocellulosic materials involves the cleaving of cellulose and hemicellulose using enzymes. Cellulose chains are made up of glucans, whilst hemicellulose consist of several different polymers of sugars such as mannan, xylan, glucan, galactan, and arabinan. Different hexoses and pentoses are the key products of hemicellulose and cellulose hydrolysis by enzymes.

Different species of bacteria for instance *Clostridium*, *Cellumonas*, *Thermomonospora*, *Bacillus*, *Bacteriodes*, *Ruminococcus*, *Erwinia*, *Acetovibrio*, *Microbispora*, and *Streptomyces*, and fungi such as *Trichoderma*, *Penicillium*, *Fusarium*, *Phanerochaete*, *Humicola*, and *Schizophillum spp.*, are able to produce the necessary cellulases and hemicellulase for enzymatic hydrolysis.

3.2.1.1 Cellulose degradation

Enzymatic hydrolysis of cellulose can be summarised into 3 steps:

- Adsorption of cellulase enzymes on the surface of cellulose,
- Biodegradation of cellulose into fermentable sugars,
- Cellulose desorption - the release of the enzyme from the surface of the cellulose.

Cellulase or cellulolytic enzymes refer to enzymes that actively participate in the degradation of cellulose into glucose, they can collectively be grouped into three distinct classes of enzymes: exo-1,4- β -D-glucanases (EC 3.2.1.91), endo-1,4- β -D-glucanases (EC 3.2.1.4), and β -glucosidases (EC 3.1.1.21). Endoglucanase and exoglucanase act synergistically to separate cellobiose off the cellulose chains. Exoglucanase are divided into cellobiohydralase I (CBH I), which hydrolyse the reducing end of the cellulose chain, and cellobiohydralase II (CBH II) which hydrolyses the non-reducing end. The internal *O*-glycosidic bonds of the cellulose chain are hydrolysed by the endoglucanase, this results in the formations of differently sized oligomers. The cellobiose is then further cut or hydrolysed by β -glucosidase into the desired glucose.[27]

Different microorganisms can produce cellulase, however cellulases of *Trichoderma reesei* or *T. viride* have by far been studied and characterized the most. Resistance to chemical inhibitors and stability under the enzymatic hydrolysis conditions are among the advantages of the cellulase of *Trichoderma*. The main disadvantages are the suboptimal levels and low β -glucosidases activity.[29]

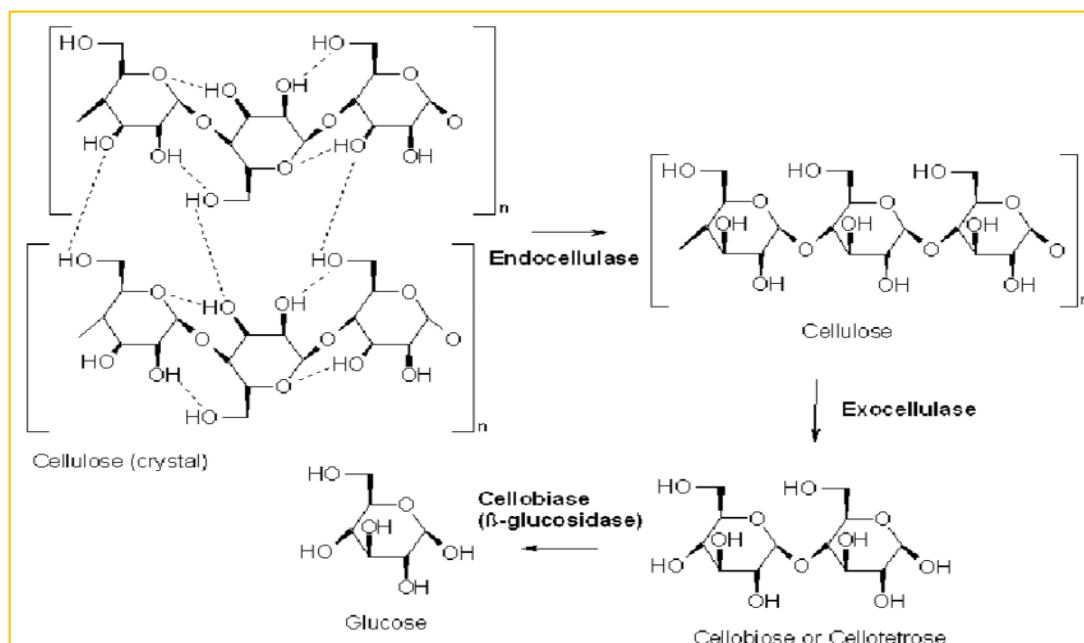


Figure 6. Cellulose conversion reactions [28]

3.2.1.2 Hemicellulose degradation

Hemicellulolytic enzymes or hemicellulase are specific for the degradation of hemicellulose. These include endo-1,4- β -xylanase (EC 3.2.1.8) that is responsible for the full hydrolysis of internal bonds in the xylan chain producing xylooligomers, exo-1,4- β -xylosidase (EC 3.2.1.37) that further hydrolyses xylooligomers into xylose. Endo-1,4- β -mannase (EC 3.2.1.78) that is responsible for the hydrolysis of internal bonds in the mannan chain resulting in the formation of mannan oligomers. The exo-1,4- β -mannosidase (EC 3.2.1.25) hydrolyses the formed mannooligomers and mannobiose into mannose.

Hemicellulose side groups are hydrolysed by α -L-arabinofuranosidase (EC 3.2.1.99), endo- α -1,5-arabinanase (EC 3.2.1.99) and α -glucuronidase (EC 3.2.1.139), these enzymes take away the arabinose and 4-O-methyl glucuronic acid substituents, from the xylan.

α -galactosidase (EC 3.2.1.22), endo-galactanase (EC 3.2.1.89), acetyl xylan esterase (EC 3.1.1.72), acetyl mannan esterase (EC 3.1.1.6), and ferulic and *p*-cumaric acid esterases (EC 3.1.1.73) are also responsible for the hydrolysis of hemicellulose side groups.[27]

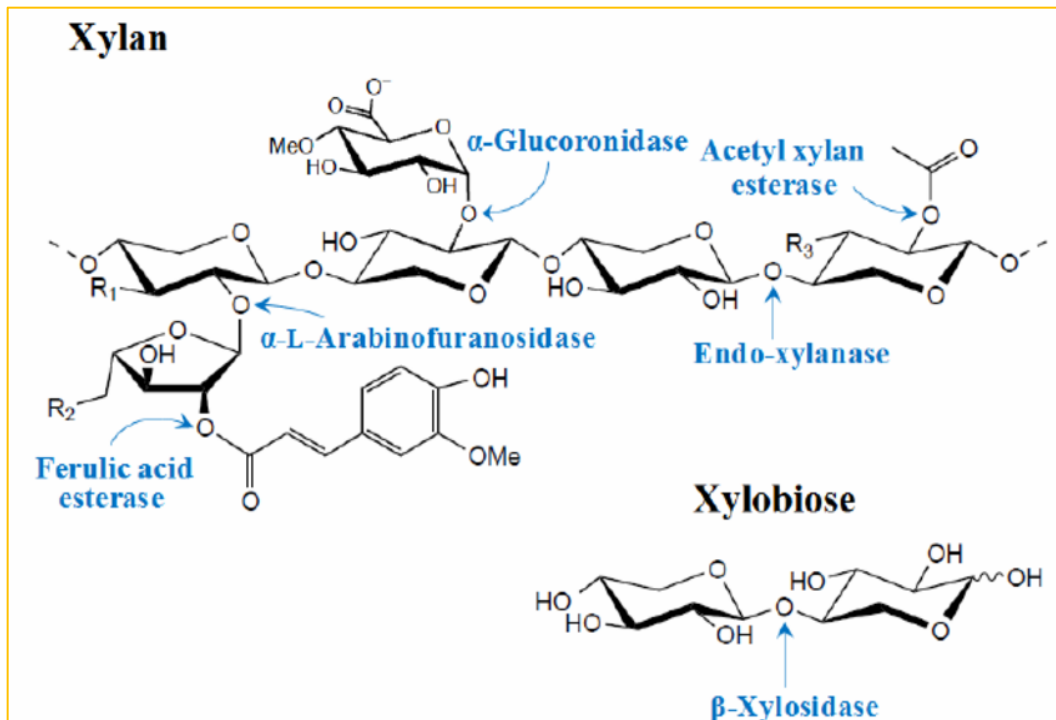


Figure 7. Xylan degradation [33]

3.2.1.3 Factors affecting enzymatic hydrolysis

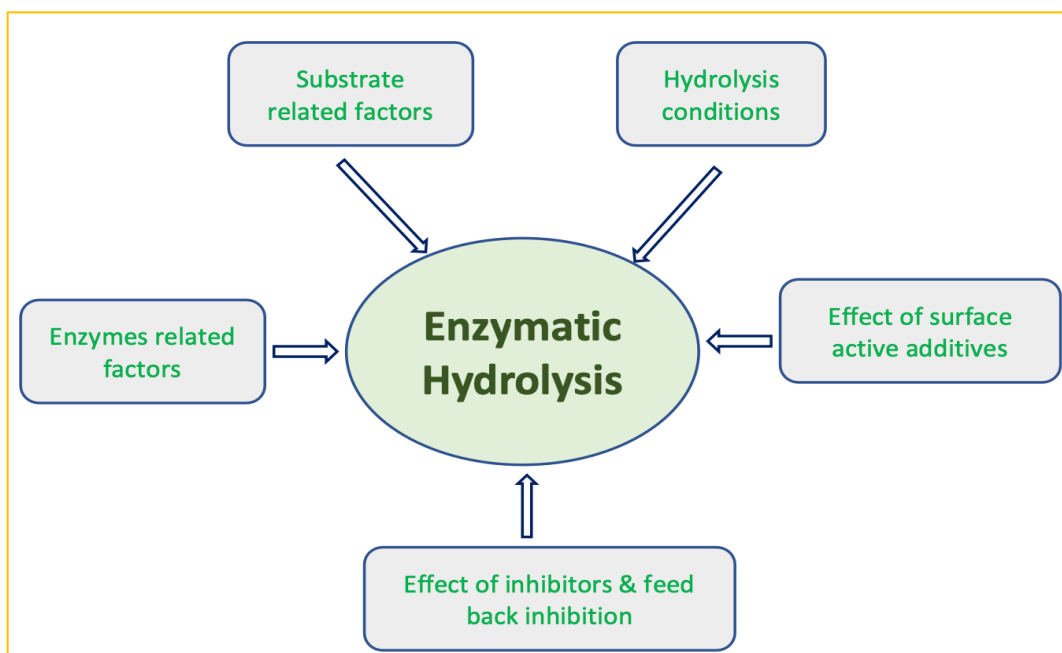


Figure 8. Summary of factors influencing enzymatic hydrolysis [30]

3.2.1.3.1 Substrate concentration and composition

Substrate concentration (solid load) and substrate quality affect the hydrolysis yield and rate of enzyme activity. A high solid load can directly instigate substrate inhibition which in turn lowers the rate of hydrolysis. The ratio of total enzyme to total substrate determines the degree of inhibition. Obviously, applying more cellulase to a certain extent would increase the rate and yield of hydrolysis. However, the cost of the process would as a result significantly rise. [29]

Lignin presence is an important factor limiting enzymatic hydrolysis of lignocellulosic biomass. Lignin acts as protective layer for cellulose and hemicelluloses and is resistant to lignocellulose biomass degradation. This makes the cell wall somewhat impermeable to cellulases and hemicellulase which would require an increase in enzyme applied.

Enzymatic hydrolysis is also greatly affected by structural features of cellulose (particle size, cellulose crystallinity, degree of polymerization and accessible surface area). Pretreatments of lignocellulosic biomass enables enzymatic hydrolysis to run smoothly.[30]

3.2.1.3.2 Enzyme based factors

Loading of enzymes depends on pretreatment choice, substrate type and its properties. In substrates with higher lignin content, it is necessary to utilize a higher loading of enzymes. Enzyme combinations also determined enzyme loading, for example in the presence of xylanase, cellulase loading would be lower.

Synergism is defined as “the ratio of the rate or yield of product released by enzymes when used together to the sum of the rate or yield of these products when the enzymes are used separately in the same amounts as they were employed in the mixture” [31]. In Cellulases synergy has been observed between different cellobiohydrolases, between endo and exo-glucanases; between endo-glucanases; between cellobiohydrolases, endo-glucanases and β -glucosidases. With respect to hemicellulase, homeosynergy, heterosynergy and anti-synergy are identified as the three types of synergy. Homeosynergy is the synergy between main-chain cleaving enzymes, while heterosynergy describes the synergy between main-chain and side-chain cleaving enzymes. Antisynergy is the term used when the action of one enzyme inhibits the action of another, for example when a main-chain cleaving enzyme requires a substituent and a side-chain enzyme eliminates that substituent.[30][32]

3.2.1.3.3 Factors influenced by inhibitors and feedback inhibition

The pretreatment and hydrolysis of biomass produces a wide range of products which can impact the enzymes negatively. Compounds such as furfural, 5-hydroxymethyl furfural, ρ -coumaric acid, hydroxycinnamic acid and phenolic compounds are generated during pretreatments. These compounds in hydrolysates limit the efficiency of bioethanol production from reducing sugars during fermentation. Cellulase and hemicellulase inhibition during hydrolysis occurs when the concentration of products (monomer sugars, disaccharides and oligosaccharides) is high. For example, cellulases are inhibited by higher concentrations of cellobiose while β -glucosidase is inhibited by higher amounts of glucose. [29].

3.2.1.3.4 Effect of surface-active additives

Addition of surfactants during the hydrolysis of lignocellulosic biomass can alter the biomass' structure making it more accessible to enzymes. The use of non-ionic surfactants such as fatty acid esters of sorbitan polyethoxylate and polyethylene glycol are among the most effective surfactants. Enhancement effects are due to adsorption of surfactants to lignin, which reduce unproductive binding of enzymes to lignin and results in positive interaction between enzymes and substrates. Careful selection of surfactants should be considered as some may negatively impact fermentation. [29].

3.2.1.3.5 Hydrolysis conditions

Conditions such as temperature and pH greatly influence the hydrolysis of lignocellulosic biomass. Enzymes require a specific temperature and pH for optimal activity. Under unfavourable conditions enzyme denaturation may occur or in other cases a higher dose of enzymes may be required to attain the same level of hydrolysis as if it were in optimal conditions. The optimum temperatures and pH for enzymatic hydrolysis are usually reported to be in the range of 40 - 50 °C and pH 4 – 5.[29][30]

3.2.2 Chemical hydrolysis

In as early as 1819 Braconnot proved that when linen is first dissolved in concentrated sulphuric acid (H_2SO_4), then diluted in water and further heated, it can be converted into fermentable sugars. The concept of chemical hydrolysis offers an alternative hydrolysis method that predominantly involves acids. The exposure of lignocellulose biomass to concentrated or dilute acid results in degradation of cellulose and hemicellulose polymers.[4] The table below shows a summary of advantages and disadvantages of the individual chemical hydrolysis processes.

	Concentrated acid hydrolysis	Dilute acid hydrolysis
Advantages	<ul style="list-style-type: none"> - Occurs under low temperatures - High sugar yield 	<ul style="list-style-type: none"> - Low acid consumption - Shorter reaction time
Disadvantages	<ul style="list-style-type: none"> - High acid consumption - Equipment corrosion - High investments costs - High energy demand for acid recovery - Longer process time (2-6hrs) 	<ul style="list-style-type: none"> - Occurs under high temperatures - Low sugar yield - Equipment corrosion - Formation of inhibitors

Table 2. Advantages and disadvantages of concentrated and dilute acid hydrolysis [29]

3.2.2.1 Dilute acid hydrolysis

Dilute acid hydrolysis put forward by Mel Parsons in 1856, is the oldest and probably the most applied technology for converting cellulose into bioethanol. It can be either used as a pretreatment method which can be followed by enzymatic hydrolysis or as a hydrolysis method. The process is performed under high temperature and pressure conditions, with a reaction time between seconds and minutes. Sulphuric acid (H_2SO_4) of about 1% concentration in a continuous flow reactor is used. Hemicellulose and cellulose differ in resistance to degradation and dilute acid hydrolysis takes account of this by involving two stages. The first stage is conducted under temperatures of about $162^{\circ}C$, this is to maximise the hemicellulose yield. Under mild conditions and acid concentration of around 0.7% H_2SO_4 ,

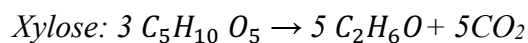
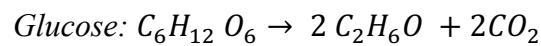
pentoses are extracted from hemicellulose. The second stage caters to the recalcitrant cellulose and is performed under harsher conditions (215°C; 0.4% H₂SO₄) in the recovery of hexoses. One known setback of this method is the low sugar yield. [4][29][34]

3.2.2.2 Concentrated acid hydrolysis

The principle of concentrated acid hydrolysis is that crystalline cellulose can be completely dissolved in 72% sulphuric acid or 42% hydrochloric acid or 77–83% phosphoric acid at a lower temperature with longer reaction times. In comparison to dilute acid hydrolysis, concentrated hydrolysis is reported to have a higher sugar yield of about 90%. Furthermore, it can be performed under lower temperatures which makes it more advantageous than dilute acid hydrolysis. However, this method is considerably more expensive as it would require, costly alloys or specialised non-metallic (e.g., ceramic) constructions crucial for the dilution and heating of the highly concentrated acid. Without the use of these non-metallic constructions, concentrated acid will severely corrode the equipment. Concentrated acid hydrolysis is energy demanding when it comes to acid recovery. The high costs of this method have largely contributed to the reduced interest to further pursue this process on a commercial level.[4][29]

3.3 Fermentation of Hydrolysates

Fermentation is a biological process carried out by microorganisms which secrete enzymes that catalyse the conversion of simple sugars into compounds such as acids and alcohols. The fermentation of the two common hydrolysates is performed according to the following two reactions under the action of microbes:



The process of fermenting lignocellulose or second-generation hydrolysates is much more complex than that of first-generation feedstock (such as sugarcane and molasses). Reasons being that the formed hydrolysates contain a wider range of toxic compounds that can influence the metabolic activity of the fermentation microorganism; the composition and concentrations of these inhibitors depend on the type of lignocellulose raw material and the choice of pretreatment and hydrolysis methods. Secondly pentose sugars, dominantly xylose, is contained in the hydrolysates of hemicellulose. Efficient fermentation requires the use of a fermenting microorganism that can utilize both pentose and hexose sugars, not only this it has to be tolerant to potential inhibitors and produce ethanol from the hydrolysates with a high yield whilst is of remaining safe for humans. [35]

The commercially used microorganisms for ethanol production *Saccharomyces cerevisiae* commonly known as Baker's yeast and the bacteria *Zymomonas mobilis*, are able to efficiently ferment hexoses into bioethanol, but are incapable of fermenting xylose as they do not contain the genes for expression of xylose reductase and xylitol dehydrogenase .[29] Yeasts, such as *Pichia stipitis*, *Candida shehatae*, and *Candida parapsilosis*, can naturally metabolize xylose into xylitol by the action of xylose reductase and utilises xylitol dehydrogenase , to convert xylitol to xylulose. Therefore, fermentation of xylose can be performed by combining good ethanol producers with microorganisms naturally utilizing pentoses. [39]

3.3.1 Fermentation techniques

Ethanol conversion can occur by following several schemes where fermentation and hydrolysis are carried out in the same reactor or separately. These techniques are attempts at increasing the efficacy of bioethanol production.[39]

3.3.1.1 Separate hydrolysis and fermentation (SHF)

SHF process is the oldest technique and currently the most used in the production of Lignocellulosic bioethanol. This is a two-step process where hydrolysis and fermentation reactions are carried out in separate reactors (two stroke reactors). In the first step the pretreated biomass is enzymatically degraded into glucose and xylose. The second step is the fermentation of these formed monomer sugars into ethanol. This technique has an advantage of offering optimum conditions for both hydrolysis and fermentation, as discussed earlier enzymatic hydrolysis occurs between 40-50°C and microorganisms that perform the conversion into ethanol require temperatures between 30-37°C. SHF technique has a major drawback, which is primarily the accumulation of glucose and cellulose in the hydrolysis reactor inhibiting the β -glucosidase and cellulase activity. As indicated, the technique requires two separate reactors to function adequately, thus would require an increase in investment to achieve this separate process.[38][39][40]

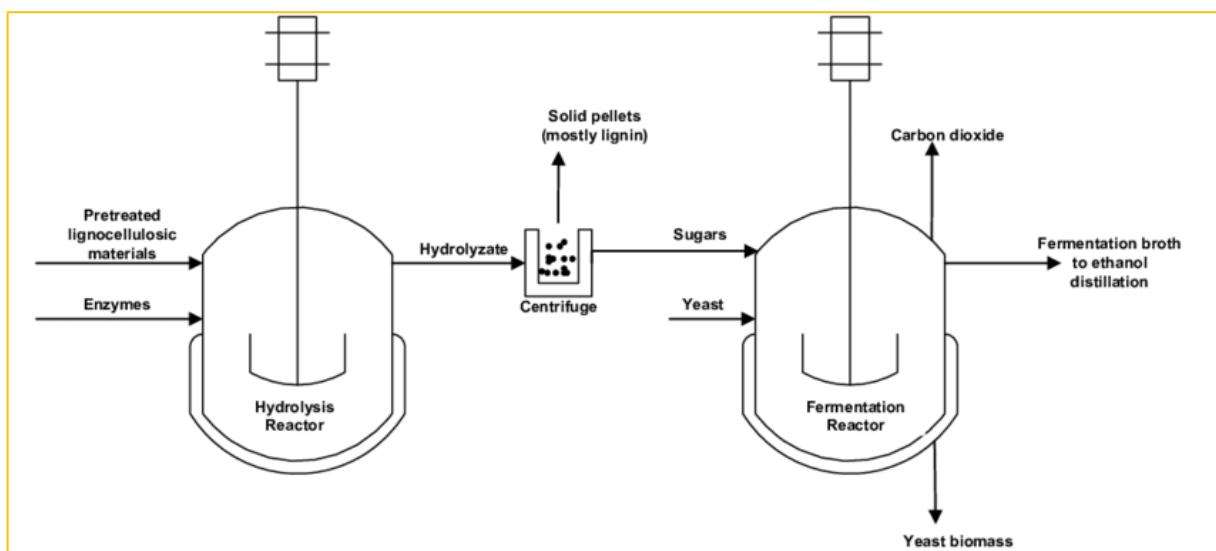


Figure 9. Schematic depiction of Separate hydrolysis and fermentation (SHF) technique. [42]

3.3.1.2 Simultaneous saccharification and fermentation (SSF)

SSF technique combines the saccharification (enzymatic hydrolysis) and fermentation of the released sugar monomers. The two processes are performed in a single reactor at the same time. The use of a single reactor is much more economically advantageous compared to the previous technique. Another advantage is that the released sugars are fermented as soon as they are produced, thus diminishing their accumulation that would inhibit enzyme activity. The main drawback arises from the difficulty in optimization of the process which directly affects the enzymatic yield. For instance, cellulosic enzymes require temperatures around 40-50°C and the yeast require a significantly less temperature range in order to perform at their best. The use of thermotolerant microbial strains would be required to produce ethanol under high temperatures, as the protein engineering of enzymes to lower their optimum temperature would be practically difficult. Yeast strains of *Kluyveromyces*, *Saccharomyces* and *Fabospora* genera have been identified as thermotolerant and capable of producing a substantial amount of ethanol. [39][41]

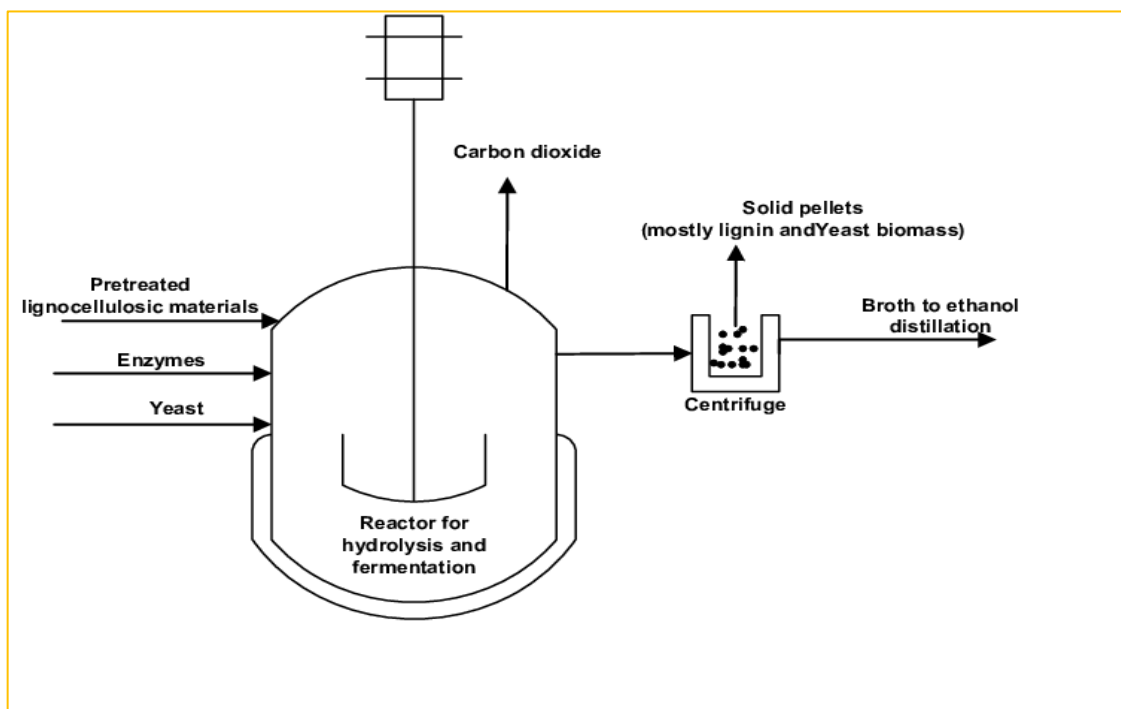


Figure 10. Process flow of Simultaneous saccharification and fermentation (SSF) technique [42]

3.3.1.3 Simultaneous Saccharification and Co-Fermentation (SSCF)

SSCF involves processes for both pentose (xylose) and hexose (glucose) hydrolysates, in fact the SSCF process is considered to be an improvement to SSF. The hydrolysis and fermentation of both sugars is performed simultaneously in the same reactor. SSCF is recommended when there is a noticeably significant amount of xylose found after hydrolysis is performed. As a result, Microorganisms capable of assimilating both glucose and xylose sugars with a high yield would be required. Genetically engineered *S. cerevisiae* and *Z. mobilis* are generally used to co-ferment both sugars.[39]

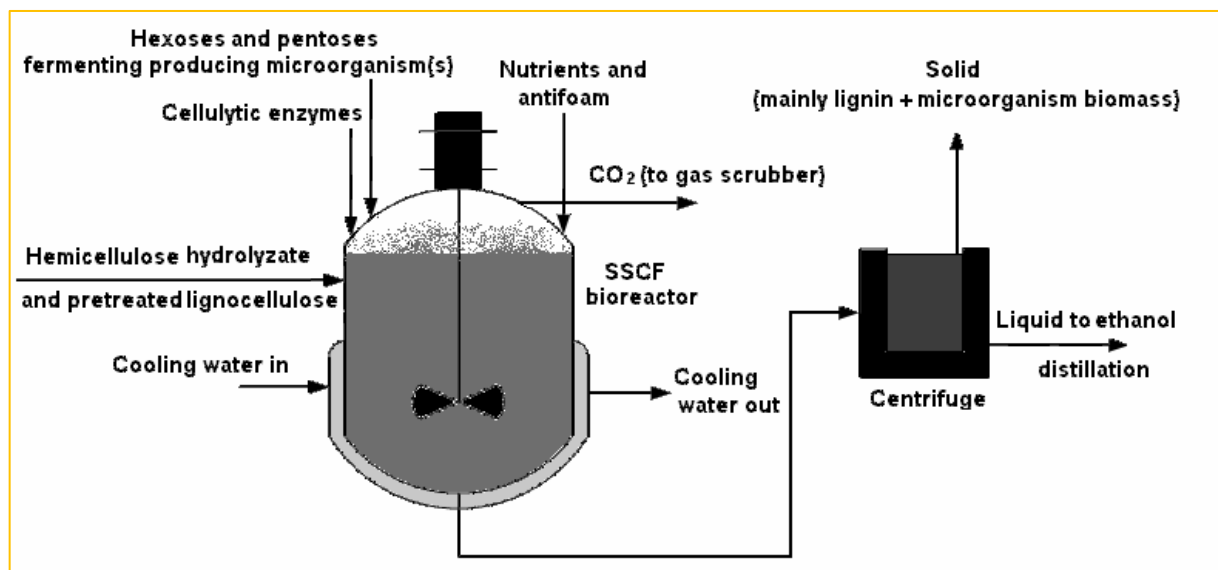


Figure 11. Schematic overview of Simultaneous saccharification and Co-fermentation (SSCF) technique [43]

3.3.1.4 Consolidated bioprocessing (CBP)

Consolidated biomass processing (CBP), initially known as direct microbial conversion, is a technique that with the use of a single organism or their consortium, combines the generation of cellulase and hemicellulase, cellulose hydrolysis, and ethanol fermentation in one reactor. CBP is a less energy-intensive and potentially low-cost approach for producing lignocellulosic ethanol and other essential industrial products. CBP seems the logical endpoint in the evolution of second-generation bioethanol production.

There are a number of natural microorganisms (such as *Clostridium thermocellum* and *Caldicellulosiruptor*) that have the ability to convert biomass directly to ethanol. These bacteria are anaerobic strains that can breakdown sugars and hydrolyse cellulose to generate ethanol. Via modern genetic modification techniques, additional CBP microorganisms are being studied and developed.

The main disadvantages are low bioethanol yields, caused by by-product formation (acetate, lactate), low tolerance of the microorganism to bioethanol, and limited growth in hydrolysate broth. [44]

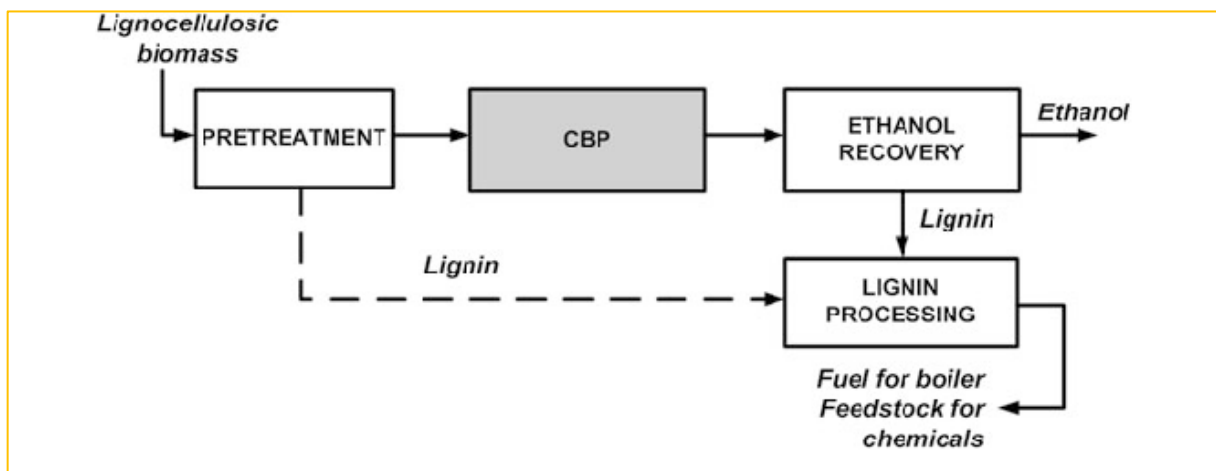


Figure 12. Schematic representation of consolidated bioprocessing technique [45]

3.4 Ethanol Purification

The second-generation process produces bioethanol in a diluted form. The composition of ethanol retrieved after fermentation is around 0.75 % and 5%, therefore water contained, and other impurities need to be removed to achieve a fuel-grade product. In cases where products are more volatile than water, recovery by distillation is often the preferred technology. The basic principle is based on the different volatilities of components in the mixture. The boiling point of ethanol (78.3°C) is lower than the boiling point of water (100°C), therefore ethanol evaporates before water when the mixture is heated and can be separated by condensing the formed ethanol vapor. Distillation initially yields only about 37 wt. % ethanol which is further concentrated in a rectifying column to a concentration just below the azeotrope (95%).

Anhydrous bioethanol is a required condition in which ethanol can be useful as a commercial fuel. Anhydrous ethanol, with 99.5% ethanol and with a 0.5% water content, may be produced via advanced distillation techniques for example adsorption distillation, azeotropic distillation, diffusion distillation, extractive distillation, membrane distillation, and vacuum distillation.

The primary issue with distillation is the likely presence of impurities with identical boiling points to ethanol in the obtained distillate. [46][47]

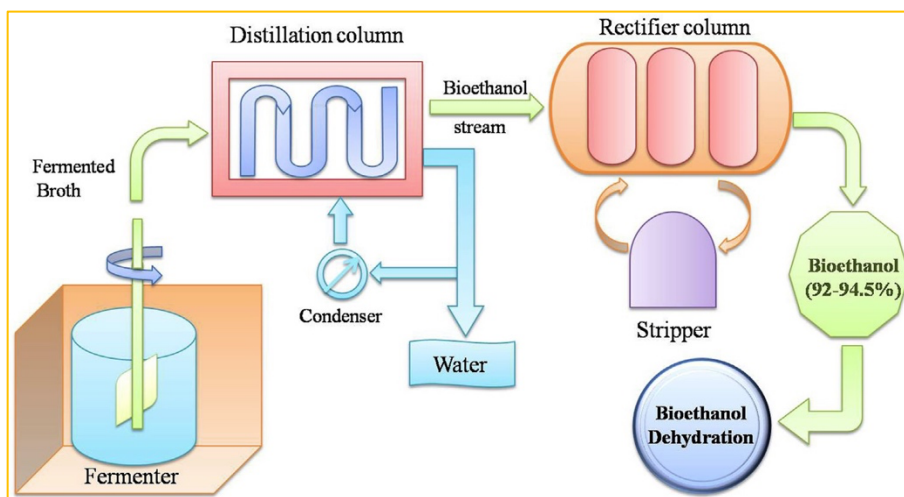


Figure 13. Ideal continuous distillation system [48]

3.5 Enzymatic and Microbial Biocatalyst Inhibitor formation

The hydrolysis and pretreatment of biomass generate a range of inhibitors under severe conditions or during prolonged periods. Pretreatment may convert sugars into phenolic compounds and other aromatics, aliphatic acids, furan derivatives (furfural and HMF) and inorganic ions. Some inhibitors may arise from extractives of lignocellulose biomass or even from equipment utilised (e.g., copper, nickel, chromium, and iron). Pretreatment and hydrolysis methods vary in pressure, pH, duration, chemicals added etc and based on these parameters, inhibitor formation vary. Maintaining a pH of around 7 during pretreatment may reduce or avoid the generation of inhibitors.[49][51]

Biomass composition plays a more important role in the formation of inhibitors. As discussed in earlier chapters different biomass contain varying amounts of cellulose, hemicellulose, and lignin. Most organic inhibitors are derived from hemicellulose and lignin. For example, softwoods contain more lignin than hardwoods, as a result it is more likely that aromatic inhibitors will be generated during the pretreatment of softwoods.[50]

Classification	Inhibitor Examples
Aromatic Compounds	<ul style="list-style-type: none"> - Phenolic compounds <ul style="list-style-type: none"> - coniferyl aldehyde, - vanillin - syringaldehyde - Non-phenolic compounds <ul style="list-style-type: none"> - benzyl alcohol, - cinnamaldehyde - benzoic acid - Benzoquinones <ul style="list-style-type: none"> - p-Benzoquinone - 2,6-Dimethoxybenzoquinone (DMBQ)
Aliphatic Acids	<ul style="list-style-type: none"> - Acetic acid, - Formic acid - Levulinic acid
Sugar-derived aldehydes	<ul style="list-style-type: none"> - 5-hydroxymethylfurfural (HMF) - Furfural
Inorganic Compounds	<ul style="list-style-type: none"> - Copper, - Nickel, - Chromium - Iron,

Table 3. Inhibitors found during lignocellulosic biomass pretreatment [52]

The toxicity of inhibitors considers different factors, and conditions such as the pH and temperature of hydrolysates. Furthermore, the toxicity of inhibitors is different for every microorganism since every microbe is capable of different levels of tolerance against different inhibitors. The concentration of the inhibitor in the hydrolysate also is also an additional factor of inhibitors toxicity.[52]

3.5.1 Aromatic compounds

During acid catalysed hydrolysis or pretreatment, many different aromatic compounds are derived from lignin. Some may be derived from hemicellulose or the extractives of lignocellulosic biomass. Aromatic inhibitors are divided into three subgroups phenolic compounds, non-phenolic compounds, and benzoquinones. Phenolic compounds (such as coniferyl aldehyde, vanillin and syringaldehyde) contain inhibitors, with at least one hydroxy-group on their aromatic ring. The subgroup of non-phenolic compounds, contain inhibitors of phenolic origin but are no longer classified as phenols. Some examples of non-phenolic aromates are benzyl alcohol, cinnamaldehyde and benzoic acid. The subgroup benzoquinones, contains inhibitors such as p-Benzoquinone and 2,6-Dimethoxybenzoquinone.[52]

The effects of aromatic compounds on microbes can be partly related to their functional groups and their hydrophobic properties. Both microorganisms and cellulase may be inhibited by phenolics and other aromatic compounds. Due to their hydrophobic properties and lower molecular weight, phenolic compounds inhibit microorganisms by penetrating their cell membranes and damaging internal structures. This damages its natural protection against external influences and changes its overall function. Enzymatic inhibition is as a result of the formation of aromatic compounds' complex around cellulases. This induces precipitation and deactivation of cellulases.[51][53]

3.5.2 Aliphatic acids

Weak organic acids, derived from different parts of lignocellulosic biomass, are found in pretreated hydrolysates. These short-chained acids can hinder microorganisms' (such as yeasts and fermenting bacteria) cell growth. A few examples of aliphatic acids are formic, acetic, lactic and levulinic acids. Acetic acid is primarily a degradation product of acetyl-groups located in lignin and hemicellulose. The formation of acetic acid does not depend on the chemicals used in the pretreatment process. However, an increase in temperature and

duration of pretreatment increases the concentration of acetic acid. Formic acid is a degradation product of furfural and HMF, similarly to acetic acid, the concentration of formic acid increases with the temperature and duration pretreatment. Other aliphatic acids are lactic acid and levulinic acid. Lactic acid is a degradation product of sugars during an alkaline pretreatment method, while levulinic acid is formed by degradation of HMF. This occurs when pretreatment method is primarily acidic.

The above-mentioned acids are weak acids that affect the natural homeostasis of cell membranes. The toxic effect is attributed to the undissociated acid molecule which is liposoluble and can easily diffuse through cell membrane. The neutral cytosolic pH causes the undissociated acid molecule to dissociate, thus acidifying the intracellular pH. This may lead to cell death or may cause a microorganism to focus most of its energy (in the form of ATP) in an attempt to return to natural homeostasis [51][52].

3.5.3 Furan aldehydes

Furan aldehydes, furfural and HMF are products of the dehydration of hexose and pentose sugars which are both present in hemicellulose and cellulose. Furfural which originates from pentose (such as D-xylose and L-arabinose) is predominantly produced when the pretreatment process occurs under acidic conditions, with an increased process duration, temperatures under 150 °C. HMF is derived from degradation of hexoses (such as d-mannose, d-glucose, and d-galactose). It is formed under similar conditions as Furfural formation.

Both furfural and HMF affect the growth of yeast which subsequently decreases ethanol production. Furan derivatives affect yeast growth by inhibiting microbial enzymes, such as alcohol dehydrogenase and other glycolytic enzymes. This results in the reduction of ATP production which is important for yeast growth. Furthermore, furfural instigates the accumulation of reactive oxygen species (ROSEs) in yeast which results in the damage of cellular organelles such as mitochondria, vacuole membranes, actin cytoskeleton and nuclear chromatin.[51][53]

3.5.4 Inorganic compounds and other inhibitors

Another group of inhibitors, Inorganic ions such as copper, nickel, chromium and iron, are present in lignocellulose hydrolysates and have been demonstrated to inhibit microorganisms. They often originate from the pretreatment equipment, chemicals added during pretreatment,

conditioning and hydrolysis and also from the mineral part (ash) of the lignocellulosic biomass. Under acidic conditions, the pretreatment equipment usually releases metal ions.

Sugars generated from lignocellulosic biomass can also be inhibitory. During hydrolysis cellulases have been shown to be inhibited by oligomeric and monomeric pentose sugars, glucose and cellobiose. To avoid this kind of inhibition it is important to monitor the concentration of these sugars in the recovered hydrolysates.

Ethanol produced during fermentation inhibits microorganisms' viability, growth and glucose transport systems. Some ethanologenic microorganisms can tolerate ethanol at certain concentration levels. These include *S. cerevisiae* and *Zymomonas mobilis* that can tolerate concentrations up to 18 and 12%, respectively.[49]

4 Overview of global biofuel trends

Bioethanol the most common biofuel represents approximately 82% of the total biofuel produced today. Its main producers are the United States and Brazil, they collectively had an annual production of 59.7 billion litres in 2020 and 34.4 billion litres in 2019.

In 2018, only 3.7% of transportation fuel needs were covered by renewable energy. Most of it powered by biofuels (93%) and the rest covered by renewable electricity. Environmental policies such as the Energy Independence and Security Act of 2007 (EISA), Paris Agreement and the European Green Deal could make significant contributions to the widespread use of biofuels in achieving the 80-95% reduction target for greenhouse gas emissions by 2050.

Many countries, such as the United States, Brazil, EU and China, have launched biofuel programs to reduce the use of fossil fuels in transportation, with a global share of biofuels in this sector by 2050. It is expected to reach 17%. [56][58].

The idea of full commercial scale production of second-generation biofuels has been around for decades, but only became reality between the years 2014 and 2015 when several facilities began operation. As at now, there are around 67 second generation biofuel facilities worldwide with about 35% located in the U.S. A. Its value worldwide in 2020 was estimated to be approximately US\$ 23.9 billion.

Another significant biofuel is renewable diesel (RD), in some cases called “second-generation biodiesel,” “green diesel,” or “HVO” (hydrotreated vegetable oil). This biofuel is chemically comparable to petroleum diesel (i.e., composed primarily of paraffins) but can be yielded from a renewable feedstock containing triglycerides and greasy acids through different forms such as hydrotreating, gasification and pyrolysis .In Comparison to biodiesel, its properties permit its utilization in conventional engines either as a pure fuel or an additive. [57][59]

Biofuels can provide other benefits to society besides reducing greenhouse gas emissions. It can, diversify the fuel supply within the transportation industry and can provide a reliable alternative to the current transportation industry. On the other hand, it may also (based on the geographical location and the availability of resources) promote biofuel feedstock, and in the long run strengthen domestic agricultural and farmland sector. Biofuels are furthermore a source of economic development and employment in rural regions.[61]

Opportunities for biofuel industries can be noticed within Africa’s domestic market. Since the 2010s, the demand for transport fuels has grown yearly at an estimated 5% - 6%. Sugarcane is

the main biomass used in both first- and second- generation biorefineries. In Africa, there are currently eight second-generation biorefineries in operation, with two of them producing considerable amounts of second-generation bioethanol. In response to population and income increments, as well as deforestation increase close to urban areas, a total of 11 African countries created biofuel mandates and planned biofuel targets in 2016. However, alternative energy sources require immense economic investment and technical power to operate, and this creates a major setback for these countries mostly comprising of developing countries.[55] [54]

Since the emergence of COVID-19 in China in late 2019 and its rapid spread across the world, demand for fuels declined sharply due to minimal economic activities, which directly impacted prices. In addition, competition among major fuel producers further pushed down the prices. According to the International Energy Agency (IEA), global production dropped 11.6% (146 billion litres of ethanol and biodiesel). This was the first annual production decline seen in in two decades. As pandemic restrictions begun to lift in 2021, biofuels production started to recuperate, reaching an estimated volume of 151.3 billion litres. For the year 2022, the IEA is estimating biofuel production to reach 158.6 billion litres.

Unfortunately, the recent war in Ukraine conveys an uncertainty of the estimated goal for the year 2022. Russia's invasion of Ukraine has generated a considerable, and potentially long-lasting economic impact on the global supplies of both food, raw materials and energy.

[60][61]

5 Conclusion

While on the surface the overall concept of biofuels is attractive from an environmental perspective, however an increasing criticism on the sustainability of many first-generation biofuels has raised attention to the potential of second-generation biofuels. Studies have shown that the benefits at the end of the production process first-generation biofuels can be outweighed by increased emissions. This issue is not particularly significant when it comes to second-generation bioethanol. Exclusive dependency on first-generation bioethanol has proven to be unsustainable in the long term, as it has led to land use competition between fuel and food production. Second-generation bioethanol can contribute to the reduction or eventually the elimination of this problem, thus representing a valid alternative to fossil fuels. The possibility of using waste residue as biomass for the process further highlights its suitability, as it allows to generate energy from waste products only. This creates the potential to provide benefits such as consuming waste residues and making use of abandoned land whilst promoting rural development.

However, the technology has not been perfected to meet all large-scale refineries' requirements and therefore further advances need to be carried out to improve its viability. The main issues complicating the expansion of second-generation bioethanol production is that each stage subsequently leads to inhibitor formation and is economically non-competitive due to high costs brought about by a necessary pretreatment procedure and use of genetically modified Microbes. There is a need to develop innovative technologies that can promote a large-scale level production of lignocellulosic bioethanol while minimizing the fore-mentioned drawbacks.

The parameters to take into account when analysing the viability of second-generation bioethanol range from purely economical to environmental. In conclusion, second-generation bioethanol production has the potential to be a truly successful route, but more innovations and studies need to be conducted for it to become a practical and exclusively relied on energy source.

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