

Biofuel Production from Lignocellulosic Biomass: A Hope for Future

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ABSTRACT

Biofuels have emerged to be a great alternative not only due to depleting fossil fuels and their elevated costs but also due to the increasing emission of carbon dioxide and other greenhouse gases. Lignocellulosic biomass is promising because of their energy efficiency and ease of availability at low cost, as non-edible agricultural waste. The presence of structural complexity and composition, biomass offers resistance to the whole process of biofuel production. Due to this, several pretreatment methods, such as physical, chemical as well as biological among others, have been introduced by scientists that are efficient in nature. Saccharification involves enzymatic hydrolysis of cellulose and gives high yield at low cost, which is then followed by fermentation. Different combined strategies of the pretreatment method and microorganisms for the saccharification and fermentation can lead to great process efficiency and output. This review illustrates several pretreatment methods, their various advantages and disadvantages, and different ways of saccharification and fermentation for biofuel production.

Keywords: Biofuel, second generation, lignocellulosic biomass, pretreatment, saccharification, fermentation

GENERATIONS OF BIOFUELS

Recently, the latest concerns regarding the change in climatic conditions due to the emission of greenhouse gases and the lowering of fossil fuel deposits have forced people to move to other forms of renewable energies and various sustainable energy [1]. It is estimated that at least 27.0TW of energy demand will exist by 2050 due to a growing population as well as an increase in the average income even though better technologies for efficient use of energy will be introduced [2].

Biofuels can be described as any type of fuel produced from organic substances, such as lignocelluloses[3].

Biofuels can be produced from various resources. When they are obtained from the food-based crops such as sugar-based and starch-based substrates, then these are called first-generation biofuels [4,5], i.e., biomass which is edible in nature [6]. But there is a debate regarding the food v/s fuel for the synthesis of first-generation biofuels by using feedstocks. Hence, it led to the introduction of the second-generation biofuels that are considered an important source for fulfilling the increased demand for fuels in transport [7-9].

Second-generation biofuels use lignocellulosic biomass for the production of biofuels, which can not only reduce carbon emission but also increase energy efficiency which in turn can reduce the dependency on fossil fuels [4,9-11]. Lignocellulosic substrates are found abundantly as a low-cost source for fuel production. The different sources from which the lignocellulosic wastes can be obtained are industrial wastes, forestry wastes, agricultural residues, domestic wastes and municipal solid wastes [12-14].

The third and fourth generation uses algae and engineered algae for the production of biofuels respectively. The overall cost in the production of fuels from algae requires a high cost since the harvesting requires various steps that add on to the cost. Moreover, the requirement of infrastructure and mixing and separation cost is very high. Thus, lignocellulosic biomass is the only feedstock that is cheap that can meet the growing demands and help in reducing the environmental problems [15,16]. The major steps for the production of second-generation biofuels are substrate preparation, that includes a reduction in size and further pretreatment, saccharification, fermentation, and product separation and purification [17].

BIOMASS FOR BIOFUEL PRODUCTION

Biomass is present as a clean and sustainable resource. It has the ability to substitute some amount of non-renewable petrol and fossil feedstock for fuel production. Biomass, which stores solar energy in chemical energy form, is produced by photosynthesis of carbon dioxide and water. An estimate of 100 billion tonnes of biomass is produced per year globally [18]. Since lignocellulose is non-digestible by humans and several animals [19], it is an available resource present abundantly, if handled properly. The IEA (International Energy Agency) suggests that “bioenergy can provide 10% of primary energy supply globally by 2035 and biofuels can substitute approximately 27% of fuels used for transportation by 2050, globally” [18].

The unsustainability of the first-generation ethanol and limited production capability, on a large scale, has led to the introduction of second-generation biofuels.

The exploitation of biomass can help with two major global issues: the increasing fuel demand, and the severe impact of fossil fuel depletion on the climatic and environmental changes [20,21].

Lignocellulosic biomass refers to organic materials, like agricultural crop residues, grass, wood, etc. The lignocellulose comprises hemicellulose, lignin, and cellulose. Lignin is present on the outer cell wall that gives extra hardness and resistance to the biomass. Cellulose is present below the lignin layer and is attached alongside hemicellulose in a random structure [22,23]. Cellulose is a linear crystalline polymer of repeating glucose monomers. Hemicellulose is a branched amorphous polymer comprising five sugar monomers, that is, D-xylose, D-galactose, D-glucose, L-arabinose, and D-mannose, out of which xylose is present in a large quantity. Lignin is a polymer of the amorphous nature of aromatic allylic alcohols [24]. It takes up to 15-20 wt.%, while cellulose and hemicellulose contribute to 30-40 % and 20-25%, respectively [25].

“Biomass recalcitrance” was first defined by Lynd et al. as the resistance of the lignocellulosic biomass and its components to the destruction carried out either by microbes or enzymes [12]. The factors were further explained later as: i) the epidermis layer especially the cuticles, ii) vascular bundles’ arrangement, iii) an amount of the sclerenchymatous tissue, iv) the extent of lignification, v) the structural complexity and the heterogeneity of cell wall components, vi) an inhibitors that follow fermentation and exist in cell wall naturally or are produced during

conversion, and vii) difficulties for the enzymes acting on to an insoluble substrate [26]. Such structural complexity leads to four major unit operations i.e., pretreatment, hydrolysis, fermentation, and distillation.

| Material | Cellulose ^a | Hemicellulose | Lignin | Ash | Extractives |
|-----------------------|------------------------|---------------|--------|-----------|-------------|
| Algae (green) | 20-40 | 20-50 | - | - | - |
| Cotton, flax, etc. | 80-95 | 5-20 | - | - | - |
| Grasses | 25-40 | 25-50 | 10-30 | - | - |
| Hardwoods | 45 ± 2 | 30 ± 5 | 20 ± 4 | 0.6 ± 0.2 | 5 ± 3 |
| Hardwood barks | 22-40 | 20-38 | 30-55 | 0.8 ± 0.2 | 6 ± 2 |
| Softwoods | 42 ± 2 | 27 ± 2 | 28 ± 3 | 0.5 ± 0.1 | 3 ± 2 |
| Softwood barks | 18-38 | 15-33 | 30-60 | 0.8 ± 0.2 | - |
| Cornstalk | 39-47 | 26-31 | 3-5 | 12-16 | - |
| Wheat straw | 37-41 | 27-32 | 13-15 | 11-14 | - |
| Newspaper | 40-55 | 25-40 | 18-30 | - | - |
| Chemical pulp | 60-80 | 20-30 | 2-10 | - | - |
| Sorghum stalks | 27 | 25 | 11 | - | - |
| Corn stover | 38-40 | 28 | 7-21 | 3.6-7.0 | - |
| Coir | 36-43 | 0.15-0.25 | 41-45 | 2.7-10.2 | - |
| Bagasse | 32-48 | 19-24 | 23-32 | 1.5-5 | - |
| Rice straw | 28-36 | 23-28 | 12-14 | 14-20 | - |
| Wheat straw | 33-38 | 26-32 | 17-19 | 6-8 | - |
| Barley straw | 31-45 | 27-38 | 14-19 | 2-7 | - |
| Sorghum straw | 32 | 24 | 13 | 12 | - |
| Sweet sorghum Bagasse | 34-45 | 18-28 | 14-22 | - | - |

Table 1: Different Lignocellulosic Biomass Materials and their Composition [27,28,29].

Pretreatment can partially remove lignin and hemicellulose, reduce cellulose crystallinity, and increase the porosity (accessible surface area) of the biomass [16,17]. Even though this step is the most expensive one in biofuel production, it has the capacity to improve the process efficiency that is further carried out after the pretreatment step and decrease the cost through further investigations [30,31].

PRETREATMENT

Cellulose is tightly bound such that it is impervious to water as well as enzymes. Hemicellulose act as a connector. It connects lignin and cellulose, but is hydrolyzed either via enzymes like hemicellulase or dilute acid or base. Lignin is covalently bound to hemicellulose and cellulose [32]. The major target of the pretreatment step is to increase the yield of the sugars liberated during hydrolysis [30]. Pretreatment methods are divided into mechanical, physicochemical, biological, and chemical methods. A combination of these methods can also be applied. These pretreatment methods can fractionate, solubilize, hydrolyze and separate the components of lignocellulose. Some of the processes are microwave, milling, dilute and concentrated acid hydrolysis, enzymatic pretreatments, Sulphur dioxide, alkaline hydrolysis, LHW, AFEX, irradiation, steam explosion, organosolv procedure, ozonolysis, supercritical CO₂ and its explosion, and wet oxidation.

Physical Pretreatment

Size reduction is the first step followed for the ethanol production through milling, chipping or grinding. All these procedures improve the efficiency of downstream process. A high moisture content and smaller particle size will result in higher energy consumption [33], however these methods help in decreasing crystallinity, and the degree

of polymerization of cellulose. Moreover, there is an increase in the specific surface area and biomass digestibility [34,35].

Since there is an increased energy consumption, it's difficult to be economically feasible and applied at an industrial scale. Therefore, extrusion seems to be a novel technique. The treatment is done at a temperature of more than 300°C, following which mixing and shearing is done. This leads to the defibrillation, fibrillation and shortening of the fibers. The extruder is advantageous, such that it can supply rapid heat transfer and mixing along with high shear [35]. Pulse electric field is another method that requires voltage between 5.0-20.0kV/cm for short time periods in nanoseconds to milliseconds that further leads to porosity of the cell membrane. This helps the agents to penetrate and break the cellulose to its monomers. are suitable [36].

| | | | | | | | |
|-------|-----|-------------------------|--|---|--|--|------|
| S.No. | 11. | Alkaline Pretreatment | 0.5g Ca (OH) ₂ , 55 °C temp. for 4weeks, enzyme 15FPU/g cellulose | Yield of glucose 93.2% and xylose 79.5% | [47] | | |
| | 12. | Alkaline Pretreatment | Aqueous ammonia, ammonia recycled percolation method | Reduced lignin content by 70–80% | [48] | | |
| | 1. | 13. | Steam Explosion | 205 °C temp. for 3–10min | Yield of xylose 10.3 g/100 dry chips with 3min | [49] | |
| | | 14. | Steam Explosion | SO ₂ -catalyzed steam explosion, 200 °C temp. for 5min, 4% SO ₂ (w/w) | Over all 77% ethanol yield | [50] | |
| | 2. | 15. | Steam Explosion | 180–210 °C temp. for residence time (4,8or12min), 0.25% or 0.5%(w/w) H ₂ SO ₄ | 55.6g glucose and xylose per 100g of raw material at 200°C for 4 or 8min using 0.5% H ₂ SO ₄ | [51] | |
| | | 16. | Steam Explosion | 190–240 °C temp. with impregnation by water or H ₂ SO ₄ solution | Ethanol yield (7.2g of ethanol/100g of raw material) is obtained with water impregnated at 240 °C | [52] | |
| | 3. | 4. | 17. | Steam Explosion | 3.53 MPa steam pressure for 5 min | 215 ml of methane/g of exploded bamboo | [53] |
| | | | | | | | |
| | 5. | 18. | Steam Explosion | 190 °C temp. for 10min, 0.2% H ₂ SO ₄ | Recovery of glucose 102% and xylose 96% | [54] | |
| | | 19. | Steam Explosion | 190–210 °C temp. for 2–8 min | 75–90% of xylose content | [55] | |
| | 20. | Liquid Hot Water | Temperature 160 °C for 20min | Yield of 74% arabinose and 54% xylose | [56] | | |
| | 21. | Liquid Hot Water | Temperature (200–230 °C) with different pressure and time | Yield of 11.0 and 14.7g/L ethanol with 200 and 230°C treatment | [57] | | |
| | 22. | Liquid Hot Water | Temperature (170and 200 °C), residence time (0and 40 min), solid concentration (5 and 10% (w/v)), pressure in reactor (30bar), enzymatic hydrolysis using commercial cellulases | Sugar recovery (53% of content in raw material) and enzymatic hydrolysis (EH) yield (96% of theoretical) | [58] | | |
| | 23. | Liquid Hot Water | Different process conditions | Recovery of 71.2% hemicelluloses-derived sugars (HDS) at 184 °C for 24min, whereas 214 °C for 2.7min led to a maximum enzymatic hydrolysis (EH) yield of 90.6% of theoretical | [59] | | |
| | 24. | Ammonia Fiber Explosion | Temperature of 90 °C, ammonia: dry corn stover mass ratio of 1:1, moisture content of 60% (dry weight basis), residence time of 5min | Yield of 98% theoretical glucose, ethanol yield was increased up to 2.2 times over untreated samples | [60] | | |
| | 25. | Ammonia Fiber Explosion | Temperature 160 °C, 2:1(w/w) ammonia to biomass loading, 233% moisture (dry weight basis) and 5min reaction time, 15FPU/ (g of glucan) of cellulose and 64 p-NPGU/ (g of glucan) of beta-glucosidase, xylanase and tween-80 as supplementation | 95% glucan and 81% xylan conversions were achieved after 168 h enzymatic hydrolysis | [61] | | |

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|-----|---------------------------|--|---|------|
| 26. | Ammonia Fiber Explosion | Aqueous ammonia for soaking, period of 10–60 days at room temperature and atmospheric pressure, spezyme CP enzyme, simultaneous saccharification and fermentation with <i>S. cerevisiae</i> (D5A) | About 55–74% lignin removed, but retained nearly 100% of the glucan and 85% of xylan. 77% of ethanol yield based on glucan and xylan content | [62] |
| 27. | Ammonia Fiber Explosion | Aqueous ammonia hydroxide (30%) with different liquid-solid ratios (5 and 10 ml/g) for either 5 or 10 days | 40–50% delignification, cellulose content remain unchanged and hemicelluloses content decreased by approximately 50% | [63] |
| 28. | CO ₂ Explosion | Pretreatment with supercritical CO ₂ (SC- CO ₂) at 3100 and 4000 psi. | Yield of 84.772.6 and 27.373.8% sugar at 3100 psi and 165 °C for 30min. SC- | [64] |
| 29. | CO ₂ Explosion | Application of supercritical fluid to the hydrolysis of cellulose by the enzyme cellulose at pressure of 160atm for 90min at 50 °C temperature. | Glucose yield was 100% at supercritical conditions | [65] |
| 30. | CO ₂ Explosion | Treatment with supercritical CO ₂ pressure with enzymatic hydrolysis | Accessible surface area increases, glucose yield by as much as 50%. | [66] |
| 31. | CO ₂ Explosion | Underpressurized CO ₂ at 35 °C for a controlled time period | More glucose (50%) is produced with increase of pressure compared to without the pretreatment | [67] |
| 32. | Ionic Liquids | Using ionic liquid (IL), 1-n-butyl-3-methylimidazolium chloride | The enzymatic hydrolysis rates were 50-fold higher for regenerated cellulose as compared to untreated cellulose | [68] |
| 33. | Ionic Liquids | Using ionic liquid 1-butyl-3-methylimidazolium chloride, water used as control | Hydrolysis rate of wheat straw and SEWS reached at 70.37 and 100% with ionic liquids, while with water rate was 42.78 and 68.78%. | [69] |
| 34. | Ionic Liquids | Using ionic liquid 1-ethyl-3-methylimidazolium diethyl phosphate, temperature 30°C for 30min fermentation with <i>S. cerevisiae</i> | Yield of reducing sugar reached at 54.8% after being enzymatically hydrolyzed for 12 h, ethanol production was 0.43 g/g glucose within 26 h | [70] |
| 35. | Ionic Liquids | Using ionic liquid 1-ethyl-3-methylimidazolium acetate, <i>Trichoderma viridae</i> cellulase | About 40% of lignin removed, cellulose crystallinity index dropped below 45, resulting in 490% of the cellulose in wood flour to be hydrolyzed by cellulase | [71] |
| 36. | Organosolv Method | Temperature, time, catalyst dose and ethanol concentration using a composite (180 °C, 60min, 1.25% H ₂ SO ₄ and 60% ethanol) enzyme loading (20 filter units of cellulose/g cellulose) | About 82% of cellulose was recovered as monomeric glucose for 24h, ~85% was recovered after 48h hydrolysis | [72] |
| 37. | Organosolv Method | Bioorganosolv pretreatment by ethanolysis and white rot fungi for 2–8 weeks | Ethanol yield 0.294 g/g of ethanolysis pulp (74% of theoretical) and 0.176 g/g of beech wood chips (62% of theoretical), yield was 1.6 time higher than fungal pretreatment | [73] |
| 38. | Organosolv Method | Temperature 195 °C, 5min, pH 2.0 and acetone: water 1:1 of ratio | Yield of 99.5% ethanol | [74] |
| 39. | Organosolv Method | Pulp with lignin 6.4–27.4% (w/w), temperature of 48h, enzyme loading of 40 filter paper units/g cellulose | About 490% conversion with 48h | [75] |
| 40. | Ozonolysis | Ozonated wheat and rye straw under room condition | Yields of up to 88.6 and 57% compared to 29 and 16% in nonionized wheat and rye straw | [76] |
| 41. | Ozonolysis | Oxidative ozone pretreatment with anaerobic sludge digestion | Solubilization of 19 and 37% of the solids lead to high methane recovery | [77] |
| 42. | Wet Oxidation | Temperature 200 °C for 10min at neutral pH | Highest yield of about 79% of theoretical in 72 h | [78] |
| 43. | Wet Oxidation | Alkaline and acidic wet oxidation (WO) (195 °C, 15min and 12 bar oxygen) enzyme cellulases added at 50 °C | About 22, 29 and 83% of the theoretical ethanol yield with enzyme loading of 73, 76 and 43.5% FPU/g cellulose. | [79] |

Table 2: Different Pretreatment Methods Applied at Certain Conditions and their Result.

Liquid Hot Water Pretreatment

In Liquid Hot Water treatment (hydrothermal pretreatment), pressure is applied at high temperature to allow the water to remain in the liquid phase. A temperature range of 160-240°C is applied at a pressure greater than 5MPa is generally applied [80]. These optimum conditions are capable to release a large amount of hemicellulosic sugar fractions most prominently in oligomeric form, thus helping in the decrease of the undesired products [30,81,82]. Recovery of these hemicellulosic sugars at maximum and maximum yield of enzymatic hydrolysis can be obtained at different time and temperature conditions [59], thus these two factors are most crucial for such recovery and the yield [58]. A two-step pretreatment: soaking at 80°C for 5-10min continued by 195°C for 6-12min has reportedly given recovery of 70% hemicellulose while 93-94% cellulose when performed on wheat straw [83].

This method has certain advantages: there is lack of nonspecific degradation of the polysaccharides and there is better pH control. Moreover, inhibitors formation is less and the pentose recovery is higher. But this method requires a large amount of water and higher energy and is not that feasible at commercial level [82,84].

Chemical Pretreatment

Acids like H₂SO₄ that are inorganic in nature, are considered for wheat straw pretreatment. The amount of acid used determines whether the process is concentrated or diluted acid hydrolysis. For the concentrated acid approach, a highly concentrated acid is added to the biomass at ambient temperature that leads to a high yield of sugars. This approach does not require further enzymes for the saccharification, while in the dilute acid approach, an acid concentration of about 0.5%-1% H₂SO₄ at high temperature is applied, which is favorable to convert cellulose to glucose. When wheat straw was treated with 72% H₂SO₄ (w/v) for 30min. The temperature is set a 30°C. 11.1g of monomeric sugars were obtained from 18.8g material (dry) which accounted for 59% of the maximum theoretical value [85].

The optimum concentration of 0.75% (w/v) was set for the maximum carbohydrate yield in the dilute acid pretreatment method. The effect of the temperature was studied which indicated that only at the highest temperature of 180°C does the formation of furfural occurs [86].

Thus, for the higher concentration of acids, a shorter time duration is taken and vice versa.

Another study on the pretreatment of the dilute mineral and inorganic acid onto the wheat straw was done at varying temperatures of 130, 150 and 170°C for 30min. It was observed that at the highest temperature, and after enzymatic hydrolysis, the yield of glucose came out to 98% and 96%. The acids were sulfuric acid and maleic acid. The maximum xylose yield was obtained at 150°C using sulfuric acid and at 170°C using maleic acid [87].

The method is suitable for producing a high sugar yield and for increased hemicellulose hydrolysis. But the high cost of energy input as well as the corrosive nature of acids are the factors due to which further studies need to be done [35,88,89].

Dilute bases are also utilized for the pretreatment process. Sodium, potassium, calcium, and ammonium hydroxides are generally used, although sodium hydroxide has been studied the most [90,91]. The method

employs the disruption of ester bonds and the glycosidic side chains due to which the certain alteration in the structure of lignin is observed, swelling of the cellulose and its partial decrystallization occurs and partial hemicellulose solubilization is also found. Even though the accessibility to the hemicellulose degrading enzymes becomes easier and the lignin removal is efficient, this method is not efficient at commercial levels [46,92].

Both the acid and alkali method requires thorough washing before the fermentation process is commenced.

Ionic liquids seem to be promising due to their lower mp (melting point) less than 100°C, negligible Vp (vapor pressure), a high chemical as well as thermal stability, high polarity, broad liquid range, and a good solvation property [14,93]. ILs dissolve cellulose which helps in its enzymatic hydrolysis and is made of a large cation and a smaller anion [93]. But the formation of the inhibitor and difficult reusability and recyclability, and high costs of ILs (e.g. Imidazolium salts) are the reasons due to which further studies need to be conducted [36].

Deep Eutectic Solvents (DESs) possess similar properties and physical behavior with that of ionic liquids and are also made from nonionic species [36,94,95].

In the alkaline/oxidative pretreatment method, hydrogen peroxide is used along with NaOH. Peracetic acid (C₂H₄O₃) may also be used instead of H₂O₂. The process is worked at a milder temperature. When 1% H₂O₂ is used at a pH of 11.5 at 25°C for 18-24h, more than 50% of the lignin is solubilized along with most of the hemicellulose. These values are higher as compared to an alkali treatment alone. In the presence of H₂O₂, the enzymatic hydrolysis of the treated wheat straw enhanced, wherein about 100% conversion is achieved at a pH of 11.5 [96].

AFEX method

In Ammonia Fiber Explosion (AFEX) method which is a basic thermal pretreatment method, the lignocellulose is dipped into liquid NH₃ at increased pressure (250-300psi) and temperature (60-100°C) for a fixed duration (30-60min) after which the pressure is released rapidly. No inhibitors are created through AFEX and the disruption causes more accessibility to the enzymes, and small particle size does not influence its efficiency [81,82,13,34]. But since the ammonia at a large amount, the cost is very high, and the method is not that suitable for lignin content [34,35].

Steam Explosion

One of the methods is steam explosion methods which are also widely used for the wheat straw. The biomass, whose size is previously reduced, is heated rapidly under high steam pressure for a fixed duration and then that pressure is spontaneously reduced which leads to explosive decompression of the materials. The temperature applied is in the range of about 160-230°C for several seconds [97,98]. It was observed that maximum delignification occurs at 210°C and 1-2 min while maximum solubilization of the cellulose-rich solid fraction and the maximum glucose production obtained during enzymatic hydrolysis occurs at 230°C and 1min [99]. The effect of the steam explosion on sulfuric acid (0.2%) in the impregnation liquid before the pretreatment step was studied for the wheat straw. The highest total yield of 102% glucose and 96% xylose was obtained at 190°C for 10min.

Obtaining such yield under the same conditions is unusual. This makes wheat straw a very suitable material for bioethanol production [54].

This method is feasible for the industries and gives a high sugar yield but, the formation of inhibitors occurs and the cost is huge [100,101].

Wet Oxidation

In the process of wet oxidation, high-pressure air or oxygen (120-480psi) is used with lignocellulose at a temperature above 120°C [102]. At 10bar of oxygen pressure, maximum delignification of about 65% as well as 50% hemicellulose solubilization occurred. It was possible at 170°C and within 10min. The residue which is left showed maximum convertibility of cellulose (85% w/v) to glucose after enzymatic hydrolysis [103].

Almost all of the hemicellulose i.e., 96%, was solvable when pretreated with O₂ and no carbonate (Na₂CO₃) involvement. But the formation of furfural decreased by more than 10fold with the addition of alkali, irrespective of the presence or absence of oxygen. However, the delignification process was affected significantly in the presence of oxygen (60% in comparison to 11% in the absence of oxygen) [104].

The process was optimized at 185°C, 15min. and 12 bar oxygen pressure. It was observed that temperature was a critical process parameter compared to time and oxygen pressure [102].

Ozonolysis

In the ozone pretreatment method, it was found that 50% of the lignin reduced was enough for the enzymatic hydrolysis. Studies quoted that, “around 75% of the cellulose was digested within 24h after treatment, as opposed to the 20% in the untreated wheat straw. This was obtained due to the delignification and the treated cellulose had decreased degree of polymerization” [105]. When the ozone pretreatment was studied at room temperature, it was found that moisture content was the most significant variable and a controlling parameter for the values below 30%. Enzymatic hydrolysis yield of up to 88.6% was achieved in comparison to 29% in non-ozonated wheat straw. This method degrades the aromatic rings of lignin but this is expensive due to large amount of ozone requirement [76].

Biological Pretreatment

Several fungi are also used for the selective degradation of hemicellulose and lignin. White-rot fungi are found to be the most efficient microbe. Laccase and peroxidase also lead to the degradation of lignin [106].

| S.No. | Microorganism used | Biomass used | Effects | Refs |
|-------|--------------------------------------|------------------------------------|---|-------|
| 1. | <i>Punctularia</i> sp. TUFC20056 | Bamboo culms | 50% of lignin removal | [107] |
| 2. | <i>Irpex lacteus</i> | Corn stalks | 82% of hydrolysis yield | [108] |
| 3. | Fungal consortium | Straw | Seven-fold increase in hydrolysis | [109] |
| 4. | <i>P. ostreatus/P. pulmonarius</i> | <i>Eucalyptus grandis</i> saw dust | Twenty-fold increase in hydrolysis | [110] |
| 5. | <i>P. chrysosporium</i> | Rice husk | - | [111] |
| 6. | Fungal consortium | Corn stover | 43.8% lignin removal/ seven-fold increase in hydrolysis | [112] |
| 7. | <i>Ceriporiopsis subvermispora</i> . | Wheat Straw | Minimal cellulose loss | [113] |
| 8. | <i>Ceriporiopsis subvermispora</i> | Corn stover | 2-3-fold increase in reducing sugar yield | [114] |
| 9. | Fungal consortium | Plant biomass | Complete elimination of use of hazardous chemicals | [115] |

Table 3: Various Biological Microbes Involved for the Biological Pretreatment of Biomass

Several aerobic bacteria as well as fungi such as *Trichoderma reesei*, *Acidothermus cellulolyticus* and *Aspergillus niger*, synthesize certain enzymes in order to degrade the lignocellulose. *Aspergillus niger* and *Aspergillus awamori* pretreatment gave better outcome for the yield of sugars as well as ethanol after the fermentation step [116].

The capital cost is less and the chemicals are not involved. The energy requirement is also less and milder conditions are suitable, with improved productivity. The drawbacks include slower hydrolysis rate and increased operational cost at industrial levels [35,117,118].

| S. No. | Different Combinations | Biomass used | Refs. |
|--------|---|-------------------------------|-------|
| 1. | Biological + Liquid hot water (LHW) pretreatment | <i>Populus tomentosa</i> | [119] |
| 2. | Biological + ultrasound + H ₂ O ₂ | Rice hull | [120] |
| 3. | Biological + 0.25% H ₂ SO ₄ | Water hyacinth | [121] |
| 4. | Biological + Steam explosion | Plant Biomass | [122] |
| 5. | Biological + alkali/ultrasound | Birch saw dust/ pine saw dust | [123] |
| 6. | Biological + Ammonia fiber explosion (AFEX) | Rice straw | [124] |

Table 4: A Combination of Strategies for Biological Pretreatment of Lignocellulosic Biomass

SACCHARIFICATION

Saccharification in simpler terms is known as cellulose hydrolysis, i.e., conversion of cellulose to glucose. The total cost of the acid and alkali pretreatment is high due to which enzymatic hydrolysis is preferred. They give high yield; production of ethanol is cost effective since it's a mild process and has low maintenance cost [27].

Enzymatic hydrolysis is suited with most pretreatment methods but the materials toxic for the enzymes must be eliminated when the chemical treatment is performed [125].

Cellulases are the class of enzyme that catalyze the hydrolysis of cellulose. Usually fungi are used commercially for their production because most bacteria, that are well suited for the production, are anaerobes and the growth rate is very less.

For the hydrolysis process, three enzymes are involved. They are endoglucanase, exo-glucanase, and β -glucanases. These work synergistically. The role of endoglucanase is to attack the region where there is low crystallinity and thus create free chain ends. Exo-glucanase then further remove the cellobiose unit from the free chain ends, thus

| S.No. | Pretreatment Method | Enzymes Mixture | Enzyme Source | Saccharification conditions | Sugar Yield (mg/g DM) | % maximum theoretical | Refs. |
|-------|---|---|--|-----------------------------|-----------------------|-----------------------|-------|
| 1. | Dilute H ₂ SO ₄ impregnation + SE | Cellulase β -glucosidase | <i>T. reesei</i> <i>A. niger</i> | 40°C pH 5.0 96h | 612 | 99.6 | [54] |
| 2. | 0.75% (v/v) H ₂ SO ₄ , 121°C | Cellulase β -glucosidase xylanase | <i>T. reesei</i> <i>A. niger</i> <i>T. longibrachiatum</i> | 45°C pH 5.0 72h | 565 | 74 | [89] |
| 3. | 2.15% (v/v) H ₂ O ₂ , 35°C | Cellulase β -glucosidase xylanase | <i>T. reesei</i> <i>A. niger</i> <i>T. longibrachiatum</i> | 45°C pH 5.0 120h | 672 | 96.7 | [45] |
| 4. | Fine grinding + wet oxidation | Cellulase β -glucosidase | <i>T. reesei</i> <i>A. niger</i> | 50°C pH 5.0 24h | 638 | 92 | [128] |

Table 5: Enzymatic Hydrolysis of Pretreated Wheat Straw and its Sugar Yield.

degrading the molecule further. These are then cleaved by β -glucanase thus giving glucose monomers. It is important for β -glucanase to act else cellobiose remains an end-product inhibitor of various cellulases [126,127].

The factors that affect this saccharification process are: concentration of the substrate, loading of enzymes, temperature control and saccharification time [125].

The glucose later on inhibits β -glucanase therefore the substrate concentration matters. Most of the cellulases shows activity at 45-55°C temperature with pH range of 4-5 [126]. Enzyme loading depends in the pretreatment method, concentration of the raw material as well as its type.

The action of these cellulolytic enzymes occurs via steps of adsorption, biodegradation and desorption. The enzymes' activity decreases during the process of hydrolysis and it said that the adsorption of cellulase on the

cellulose irreversibly, is somewhat responsible for their deactivation. Though, the surfactants addition may better the enzymatic activity for the conversion to sugars [129]. Tween 20, a nonionic surfactant, is observed to be most efficient for the enhancement of the hydrolysis process [130,131]. Its addition increased the enzymatic saccharification yield after the wheat straw was pretreated with H₂SO₄ from 488 to 520mg/g [86].

Use of other enzymes along with the cellulases can also increase the hydrolysis rate, such as, cellulases and hemicellulase. The best results for sugar yield were found when cellulase, xylanase and feruloyl esterase (FAE), were used in conjugation at 50°C that resulted in 81% of maximum glucose recovery.

FERMENTATION

The monomeric sugars that are produced, i.e., hexoses and pentoses after the enzymatic saccharification are further processed through fermentation for the bioethanol production with the help of certain microorganisms [132].

The process efficiency can be increased by studying different integrated methods of hydrolysis and fermentation.

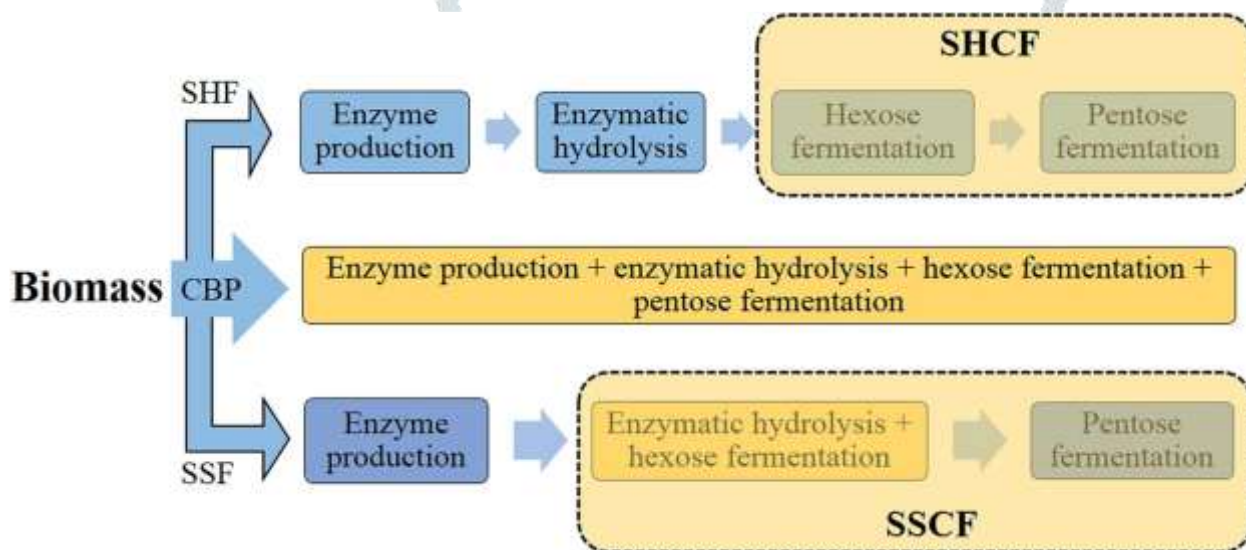


Fig. 1: Different methods of hydrolysis and fermentation. SHCF denotes Separate Hydrolysis and Co-Fermentation [133].

Separate Hydrolysis and Fermentation (SHF)

As the name suggests, this process is two-step process in which both the hydrolysis and fermentation occurs separately. Firstly, the monomers are produced by the degradation of lignocellulose and then later on the fermentation occurs for ethanol production from these sugars [134,135].

SHF can be used when the microbes used operate at different conditions of pH and temperature for carrying out the process of hydrolysis and fermentation [136], though the concern is regarding the inhibition of the enzymes due to sugar accumulation.

Simultaneous Saccharification and Fermentation (SSF)

SSF differs in a way that both the processes can be carried out simultaneously in a single bioreactor. This helps ease the concerns of accumulating sugars since as soon as sugars are formed via hydrolysis, they are fermented to ethanol. There is an ease in the operations, with less equipment's requirement and thus, the chance of contamination is less due to the presence of ethanol [134,137,138]. This process leads to a compromise between the operating and optimum condition of the enzymatic hydrolysis and the fermenting microbe [136].

The only drawback is the difficulty in setting optimized conditions for both the enzymes and microorganisms. Enzymes may best show its activity at 50°C, while microbe may work in the range of 28-37°C for ethanol fermentation. Therefore, the thermotolerant strains are required [139].

Simultaneous Saccharification and Co-Fermentation (SSCF)

This combination is for the microbial breakdown of the sugars that are released during the pretreatment of the lignocellulosic biomass and its hydrolytic processes. Mixed cultures of yeast can also be used but hexose conversion is faster as compared to the pentose sugars for ethanol production. It is due to faster growth of microbes utilizing C6 sugars. Another possibility of using a single microbe for both hexose and pentose assimilation is there for a higher conversion as well as ethanol yield [80]. There is low cost involved, with less chances of contamination and shorter operation time as well as less inhibitors formation [141].

Consolidated Bioprocessing (CBP)

CBP or Direct Microbial Conversion (DMC) includes all the reactions required for the biomass transformation into ethanol. The difference is in the presence of only a single microbe for the enzyme production, hydrolysis of biomass and its further fermentation. Thus, no further cost for enzyme production is involved with whole biomass being utilized for the release of monomers and no part of it being involved in the production of cellulases. The biggest advantages lie in the compatibility of hydrolysis and fermentation processes. The thermophilic microbes can work at higher temperatures and can directly utilize lot of inexpensive biomass feeds, thus giving it an edge over yeasts.

The only problem arises in their low bioethanol tolerance (<2% v/v), creating a hurdle for industrial application. But still, thermotolerant strain, *K. marxianus*, a yeast, has been engineered for the co-display of the enzymes, endoglucanase as well as β -glucanase on the surface of the cells. It can grow at extreme temperature of up to 48°C and give a yield of 0.47g/g (ethanol/consumed carbohydrate) from the β -glucans. Another approach is using the mixed cultures such that both saccharification and fermentation can be carried out simultaneously [134].

CONCLUSION

Globally, millions of tons of agricultural waste are produced, annually, which can be utilized for the production of biofuels. Non-renewable fossil fuels are becoming scarcer and costlier. They are playing a role in increased greenhouse gas emissions and global warming. Second generation biofuels have the potential to be an alternative that can essentially replace fossil fuels in the near future. The methods are being developed by the scientists to generate biofuels efficiently that are cost effective in nature. Because of the availability of the biomass throughout the world, designing a common method for the production of biofuel is essential which is not only cost effective but also efficient in nature. Several cost-effective pretreatment methods are being designed, although further research and studies needs to be done to overcome different challenges posed by complete methodology on the whole that leads to the production of biofuels. Consolidated bioprocessing and SSCF are certain emerging technologies that have shown improvements in the biofuel production. Genetically modified strains are also being developed that can work at increased temperatures for more ethanol yield. More efforts are required for the integration of these technological advancements and scientific approaches, for obtaining a generic method for feedstock versatility present globally, at a greater level for their commercial production. This can lead to an optimal second-generation biofuel production.

Conflict of interest

Authors declare that no conflicts of interest exist.

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