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Biomass breakdown: A review on pretreatment, instrumentations and methods

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1. ABSTRACT

Enzymatic breakdown of lignocellulosic biomass for liquid fuel production is a viable alternative to fossil fuels, due to its renewable and environmental friendly nature. Naturally, plants protect their cell wall polysaccharides by giving limited access to the cell wall degrading enzymes. Lignocellulose breakdown requires proper pretreatments that disrupt the close inter-component association between the constituents of the plant cell wall. For efficient biomass conversion, the choice of the correct pretreatment is important for removing the barriers to enhance access to microbial enzymes. Among the pretreatment methods available, biological pretreatment is a promising approach for biomass degradation as there are no inhibitors generated. Another significant area that needs attention is the development of methods that can qualitatively and quantitatively determine the degradation of biomass and product generation. More technological advancement would be required in the field of pretreatment technology and fermentation processes to make the whole process economical. Here, we review the recent developments in the field of lignocellulosics, role of various pretreatments, instruments & methods and role of microbial enzymes in biomass degradations.

2. INTRODUCTION

The development of clean renewable energy as an alternative to fossil fuels has attracted great attention worldwide (1-4). Energy demand is continually increasing due to rapid increase in population & heavy industrialization. To meet this growing energy demand there is an urgent need to explore alternative energy resources, particularly biomass due to its renewable nature and availability (5). Biomass is an important energy source that provides about 13% of the World energy consumption (7) & its conversion into biofuel is a significant choice for utilizing renewable energy globally (8, 9). According to International Energy Agency report, biofuels as transport fuel have the potential to fulfill more than a quarter of World energy demand by 2050 (6). Moreover, increasing biofuel production from second & third-generation feedstocks may help to decrease GHG emission because of its carbon neutral nature (10). The production of 2nd generation biofuels from lignocellulosics seems promising since there is a plentiful organic material in nature. Therefore, utilization of lignocellulosic biomass would help in promoting rural economy, enhancing energy security, and decreasing greenhouse gas emissions (11).

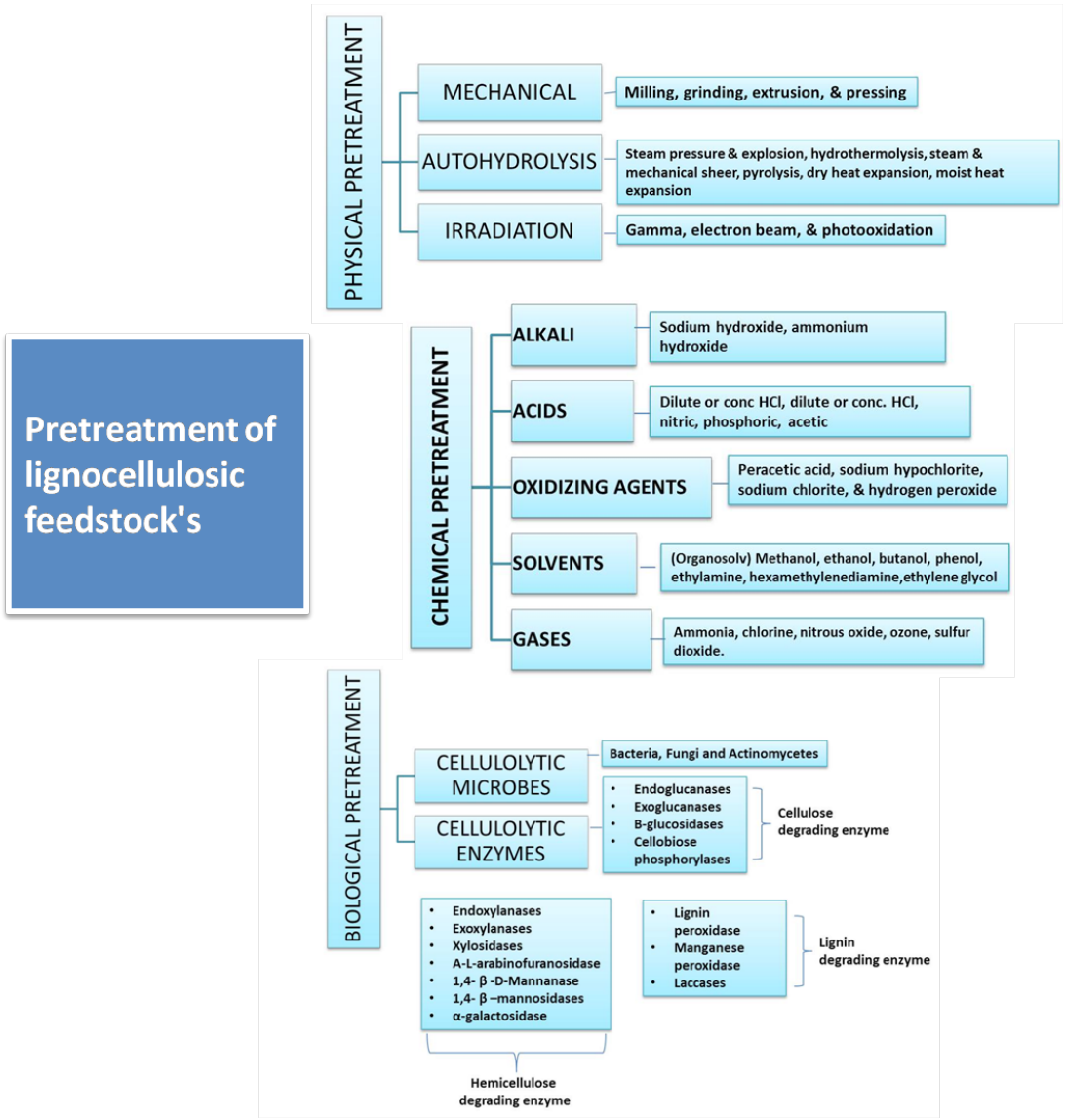

Figure 1. Various pretreatment methods of lignocellulosics

Lignocelluloses in plant biomass is a renewable, abundant and relatively cheap mixture of organic materials, containing polysaccharides (~75% dry weight) and lignin (~25% dry weight) (1, 12). The most important biomass resources are energy crops (22–1,272 EJ), agricultural residues (10–66 EJ), forestry residues (3–35 EJ), wastes (12–120 EJ) and forest (60–230 EJ) (8). Hence, lignocellulosic materials like agricultural wastes and industrial wastes are important feedstock's for bioethanol production as they are abundant, cheap & easily available. The production of biofuel from agricultural and industrial waste can prove to be a boon but there are several key challenges and restrictions in proper utilization of biomass for energy production (13). The success of bioenergy production depends fundamentally on the intrinsic recalcitrance of biomass and the range of enzymes associated in biomass degradation. Among the various components of biomass (cellulose, hemicelluloses and lignin), in particular, there is an increase interest in understanding the degradation of lignin as it can comprise up to 30% of plant biomass and must be removed before the cellulose and hemicellulose can be accessed. There are different pretreatment methods developed and used by researchers to enhance the biomass degradation and some of them are presented in the Figure 1. Although no single method is currently available and economically feasible that can efficiently convert whole biomass into bioethanol (14). Presently, researchers have been working on the screening, isolation and characterization of well-adapted microbial communities capable of degrading lignocellulosic biomass from natural habitat for lignocellulolytic enzymes production

Table 1. Biomass terminologies

Lignocellulosic plant biomass		Components[1]
Cellulose		Linear polysaccharide made up of hundreds to more than ten thousand β-1,4 linked units of glucose. Complete depolymerization of cellulose yields just one product, glucose.
	Microfibrils	Crystalline, non-soluble and makes enzymatic saccharification challenging. Cellulose chain aggregate into microfibrils with the help of hydrogen bonding and van der waals interactions
Hemicellulose		Non-cellulose polysaccharides with variable quantity within same plant species. Enzymatic degradation is easier as compared to cellulose.
	Xylan	Grasses and angiosperms
	Mannan	Gymnosperms
	Xyloglucan	Angiosperms
	Glucomannan	It is hemicelluloses components in the cell wall. It is a straight chain polymer mixed β-1,4-linked mannose/glucose backbone substituted with α-1,6-linked galactose and with some mannose residues O-2/O-3 acetyl-esterified.
Lignin		Hydrophobic heteropolymer composed of three monolignols, coniferyl alcohol, sinapyl alcohol and p-coumaryl alcohol. Lignin is crosslinked with carbohydrates by either ether or ester linkages in lignocellulose biomass via e.g. arabinose-ferulic acid or glucuronic acid.
Enzymes	**Cellulases**	Hydrolyze the β-(1,4) bond in cellulose to produce sugar monomers.
	Hemicellulases	It hydrolyzes hemicelluloses, such as the β-(1,4) bond of the xylan main chain or any of the various linkages in the side chains
	Glycoside hydrolase	It hydrolyzes a glycosidic bond between carbohydrate and non-carbohydrate moiety and or two adjacent saccharide groups.
	Endo-1,4-β-glucanases	Cleave the internal bonds in the cellulose chain randomly.
	Exo-1,4-β-glucanases	Also known as, cellobiohydrolases. In cellulose structure, it cuts the reducing or non-reducing end.
	β-glucosidases	Convert cellobiose into glucose.
	Cellulosomes	It is multi-enzyme complexes composed of numerous functional domains produced by many cellulolytic microorganisms that degrade cellulosic substrate.
Biomass to Ethanol	**Hybrid hydrolysis &fermentation (HHF)**	This process allows the staging of the saccharification and fermentation steps, frequently intended to let a high temperature enzyme treatment then by a lower temperature fermentation step.
	Simultaneous saccharification & fermentation (SSF)	It involves hydrolysis of cellulose and other cell wall polysaccharides into sugars with fermentation.
	Consolidated bioprocessing (CBP)	Single microorganism is able for the production of cellulolytic enzymes and converting soluble sugars into ethanol. In CBP whole process of cellulase production, substrate hydrolysis and fermentation complete in a single step.

Adapted from (97, 98). [1]Three main components of polymers: Cellulose, hemicellulose, and lignin along with some minor components (proteins, lipids, pectin, soluble sugars and minerals)

(15). This article reviewed the recent developments in the field of lignocellulosics, role of various pretreatment methods and role of microbial enzymes in biomass degradation. Here, in this article we reviewed the role of various instruments in analysis of the deconstruction of biomass.

3. COMPOSITION OF BIOMASS

Biomass is important and one of the largest primary energy resource in the world after coal and crude oil (16). Biomass is primarily produced by capture of solar energy by green plants through the process of photosynthesis which is stored energy in the form of cell wall material. Lignocellulose, which comprises the plant cell wall, is the Earth's most abundant renewable source of convertible biomass (17). Bioenergy generated by biomass is having high-energy content and have compatibility with prevailing petroleum-based transportation infrastructure, which helps to support their desirability as a fuel source (18). Common terms used in biomass and bioenergy are given in Table 1.

Chemically, biomass is composed of cellulose, hemicellulose and lignin, but the proportion of these components may vary from plant to plant, but collectively they constitute 90% of the plant dry weight (19). Table 2, shows the percentage composition of different cell wall components in different biomass

Table 2. Percentage amount of cellulose, hemicelluloses, and lignin in various biomasses

Biomass	Cellulose%	Hemicellulose %	Lignin%	REFERENCES
Corn stover	37.5.	22.4.	17.6.	(99, 100)
Rice straw	32	24	13	(99, 101)
Barley straw	37.5.	25.3.	26.1.	(102)
Rye straw	38	36.9.	17.6.	(102)
Wheat straw	38.2.	21.2.	23.4.	(99, 103)
Napier grass	45.7.	33.7.	20.6.	(102, 104)
Eucalyptus	38-45	12-13	25-37	(99)
Giant reed stalk	33.1.	18.5.	24.5.	(102)
Giant reed leaves	20.9.	17.7.	25.4.	(102)
Sugarcane bagasse	21.1.0	27	45.5.	(105)
Reed	39.5.	29.8.	24	(106)
Rapeseed stover	27.6.	20.2.	18.3.	(106)
Bermuda grass	47.8.	13.3.	19.4.	(106)
Reed canary straw	42.6.	29.7.	7.6.	(107)
Sugarcane leaves	18	25	45	(108)
Bamboo	28.1.	24.6.	46.7.	(109)
Switch grass	31	22	18	(110)
Monterey pine (*Pinus radiate*)	41.7.	20.5.	25.9.	(110)
Hybrid poplar	40	22	24	(110)

feedstocks. Cellulose is the most abundant renewable polysaccharide on earth and major component of lignocellulosic materials. Structurally, it is a linear homopolymer of β-1,4-linked D glucose molecules, with the dimer cellobiose as the repeating unit. Hemicellulose, is the second major constituent of lignocellulose, and acts as a linking material between cellulose and lignin. Lignin is a non-linear, branched heteropolymer with lower degree of polymerization (<200) than cellulose. It is mainly consists of hexose sugars such as D-glucose, D-galactose and D-mannose, and of pentoses such as D-xylose and L-arabinose, linked together by β-1,4- and sometimes by β-1,3- glycosidic bonds (20, 21). Barakat *et al*. (22) reported that the lignocellulosic content affect the specific energy requirement (SER), and they also mentioned that arabinose/xylose ratio and accessible surface area lead to the increased of SER. On the contrary, the content of cellulose, lignin, crystallinity and p-coumaric acids links were found to have a positive effect on the reduction of the SER.

4. KEY STEPS IN CONVERSION OF LIGNOCELLULOSE TO BIOETHANOL

The plant biomass stored large amount of sugars that can be fermented into ethanol and other liquid fuels. The process of biofuel production involves assortment of biomass, unfolding the cell wall into pentose and hexose sugars (pretreatment and saccharification), and conversion of these sugars into bioethanol (fermentation) (23). The lignocellulosic utilization processes involve five essential steps, namely; (i) Biomass pretreatment, (ii) saccharification (iii) fermentation of monosaccharides (iv) separation and (v) effluent treatment (24). There are numerous reasons for supporting biofuels production as pertinent technologies because it can provide energy security, savings of foreign exchange, environmental protection, and employment in rural sector (25). The whole process of biomass conversion to bioethanol production is shown in Figure 2.

4.1. Pretreatment technology: removing barriers for hydrolysis of lignocelluloses

The pretreatment of lignocellulosic substrate is a fundamental process for successful breakdown of biomass over enzymatic hydrolysis as it releases only less than 20% glucose from the cellulose fraction (21). The prime goals of the biomass pretreatment are; (a) to increase the enzyme accessibility to cellulose and promote cellulose decrystallization, (b) unwinding of cellulose and hemicelluloses (c) solublization of hemicelluloses & lignin, (d) structural modification of lignin (e) enzymatic digestibility of the pretreated biomass, (f) minimization of loss of sugars, and (g) minimize investment and processing costs. Among the all pretreatment methods, the best pretreatment method must protect the hemicellulose portion, and

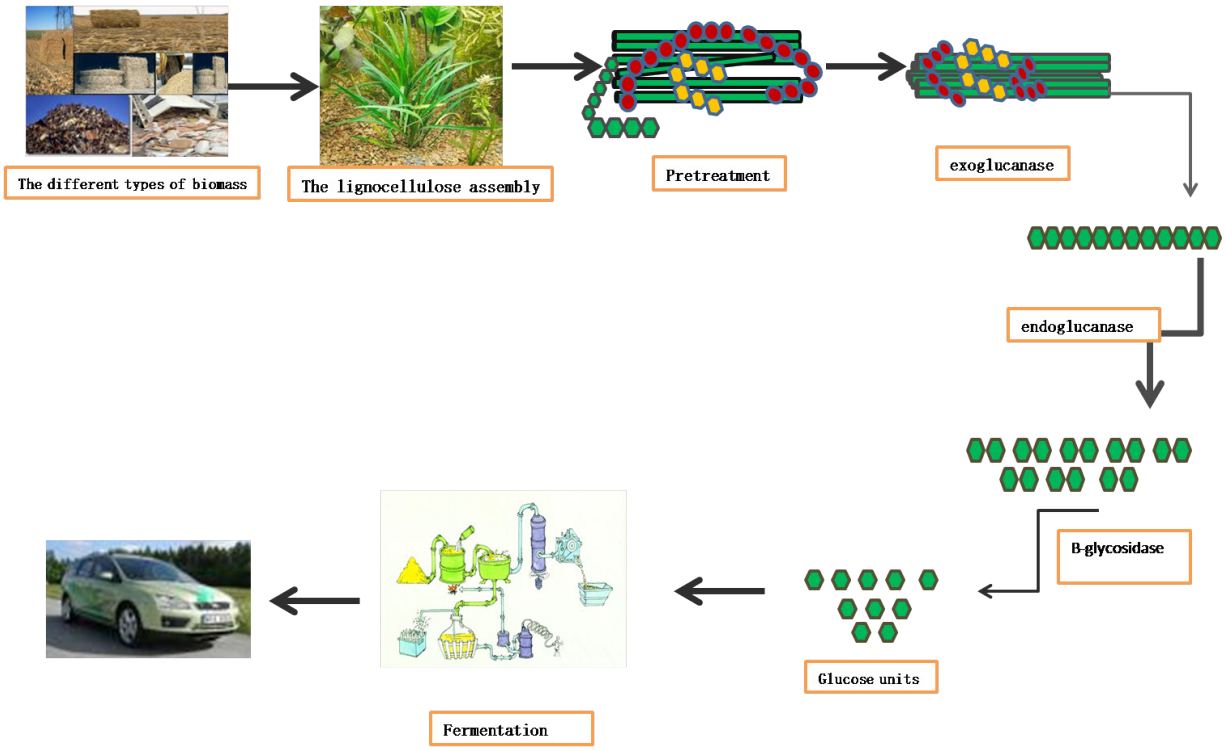

Figure 2. Whole process of biomass conversion to bioethanol production

circumvent the requirements for reducing the particle size of biomass, and reduces the production of toxic components (21). Different types of pretreatment are effective in dealing with the different types of biomass, but specific pretreatment can increase the porosity of specific substrate. Some of these effects are (a) the elimination of some or all of the lignin from the cell wall, it will increase the porosity of the substrate (b) the unfolding of lignin (c) removal of hemicelluloses from the cell wall (d) increase the disturbance in the cellulose and hemicelluloses (e) increase the disturbance in the crystallinity of the cellulose (f) increase the degree of depolymerization of cellulosic microfibrils and, (g) decrease the particle sizes of plant biomass.

Presence of lignin in biomass feedstock's is one of the major constraints that limits hydrolysis of the biomass by cellulolytic and hemicellulolytic enzymes (26). Lignin reduces the hydrolysis of biomass may be by providing a physical barrier between cellulose and hemicellulose (26), cellulase enzymes adsorbed to the lignin non-specifically, which decreases hydrolysis of the substrate (27), inhibition of the hydrolytic enzymes by lignin (28), and the blockage of cellulase activity by lignin (29).

The removal of lignin from cell wall may be achieved through physical, chemical or biological means. Use of grinding or milling in substrate size diminution also makes the pretreatment more effective (30). Varnai *et al*. (26), in their work mentioned about the efficacy of pretreatment methods in changing the structure of lignin. They have reported that total delignification and the changes in the position of lignin in biomass can increase the hydrolysis of biomass without the removal of lignin from the biomass. Pretreatment methods must be devised in such a way that save the sugar products and lignin degradation products, which can be further fermented. Pretreatment also helps in enhancement of enzymatic hydrolysis with a need of low enzyme loadings. An ideal pretreatment process must improve the following parameters; sugar yield after enzymatic saccharification, minimal effluent generation, reduction of the degradation of carbohydrates, low energy demand and low capital and operational cost requirement (11).

Therefore, more research is required to develop better cellulase preparations which are best suited for use in bio refineries, such as high catalytic efficiency, increased thermostability, and wide pH range and greater tolerance to end-product inhibition. Till date, enzymatic saccharification is the most expensive step in bioconversion of lignocellulosic biomass to ethanol. Kristensen *et al*. (31) reported the role of hydrothermal and steam explosion in unfolding of hemicellulose structure, lignin re-localization and also for the removal of wax, but this method was unable to degrade the fibrillar structure of cellulose. Ferro *et al*. (32) reported the steam explosion pretreatment

which disrupted inter fiber surface of rockrose with preferential solubilization of the water soluble fraction of hemicelluloses that partially degrades the lignin. This lignin could be removed to significant extent by alkali extraction of R-SE and enhance the yield of ethanol production higher in SSF than to SHF up to 22.1. ± 0.2. g of ethanol per 100 g of dry R-SE-OH. Chan *et al*. (33) reported that dilute acid and ferrous ion co-catalyst pretreatment is effective in increasing the amount of solubilized sugars and reducing sugars in the lignocellulosic residue, whereas, Ji *et al*. (34) suggested that dilute acid pretreatment is an attractive method because it induced selective solubilization of hemicelluloses and lignin migration within tissues that together facilitated the loosening of cell wall structure. This treatment not only opened up the cell wall structure but also had an impact on the cleavage of lignin–carbohydrate linkages that were resulting from hydroxycinnamic acids removal. These alterations further enhanced the accessibility of enzymes to cellulose, as cellulose surfaces became more exposed. Meng *et al*. (35) concluded that, acid pretreatment method is superior in comparison to water and alkaline pretreatment in terms of increasing the cellulose accessibility, and also by increasing the nanopore space between the coated microfibrils. After dilute acid pretreatment, about 500 mg of glucose/ gram of dry pretreated biomass could be released after 60 min at 160 °C. Corbin *et al*. (36) reported that 0.5. M sulphuric acid increase the yield of glucose by 10%, and bioethanol from grape marc up to 400L/t and 270 L/t bioethanol from soluble carbohydrate by enzymatic saccharification. Kim *et al*. (37) reported the use of nitric acid pretreatment in corn stover for the production of bioethanol and biodiesel up to 22.4.g/L & 1.0.4g/L. Negro *et al*. (38) in their study, concluded that one step extrusion is effective in olive tree purning and increase the yield of glucose near to 69% from raw material. Saratale and Oh (39) reported the alkaline pretreatment (2% NaOH, 121°C, 30 min) of rice paddy straw (PS) resulted in a maximum yield of 703 mg of reducing sugar per gram of PS with 84.1.9% hydrolysis yield after a two-step enzymatic hydrolysis process. Klein *et al*. (40) reported that the use of polyoxometalate (HSIW)/Graphene as a catalyst in the process of degradation increase the rate of glucose production from biomass.

Ionic liquids are a good alternative for chemical treatment and are the cheapest and environmental friendly method for unfloding of biomass. Khare *et al*. (24) reported that ionic liquid 1-ethyl-3-methylimidazolium acetate is effective, and increases the rate of saccharification up to 90% & they also reported that *an Alicyclobacillus acidocaldarius bacterium is* a good source of endoglucanase. In their work, they synthesized ionic liquids by combining cation and anion by considering the requirement of feedstock, cost, stability and their degrading potential, and reported highest saccharification yield (75%) from synthesized ionic liquid (1-ethyl-3-methylimidazolium acetate) (41). Zhang *et al*. (42) reported that metal salts with mechanical treatment have positive effect on cellulose structure, the presence of $Al(NO_3)_3$ significantly improved the enzymatic breakdown of cellulose. They concluded that mechanical activation and metal salts (MAMS) pretreatment technology is simple, efficient and eco-friendly and can offer an extensive range of potential applications for the cellulose degradation. Liu *et al*. (43) reported that high dose of irradiation (>1000 kGy) could evidently decompose the crystalline structure of MCC (microcrystalline cellulose). SEM, FT-IR analysis of degraded MCC cellulose into reducing sugar and bioethnol showed that efficiency of pretreatment in following order; ionic liquids = irradiation pretreatment > AA-ILs pretreatment > 1% HCl pretreatment > 1% H_2SO_4 pretreatment. Steam explosion pretreatment of cardoon allowed the disruption of interfibrillar surface with preferential solubilization of the hemicellulosic water-soluble fraction, thus producing solid residues richer in cellulose and in lignin after that SSF process allowed the highest maximum ethanol concentration of 66.6.% (44). Travaini *et al*. (45) reported that ozonolysis has proved its efficiency as pretreatment for diverse lignocellulosic biomass and providing high delignification (~80%) and total sugar release (~75%) with very low carbohydrate losses. The less generation of inhibitory compounds enables subsequent enzymatic hydrolysis and fermentation steps for biofuels production. Jia *et al*. (46) reported that synergism between cellulase and xylanase in the hydrolysis of bagasse was affected by structural and compositional differences between the substrates resulting from the different pretreatments. PAA (peracetic acid) pretreatment removed part of hemicellulose but left more crystalline cellulose, resulting in a high degree of synergy for glucan conversion. In contrast, (Emim) (OAc) pretreatment likely disrupted less hemicellulose-cellulose associations but generated more amorphous cellulose, resulting in a high degree of synergy for xylan conversion. The molecular structure of enzymes also affected the synergism. Owing to the cross linking of hemicellulose and cellulose (46).

5. BREAKING THE PLANT DEFENSE: THE ROLE OF MICROBIAL ENZYMES

Structurally plants are diverse in composition, and are made up of at least 35 different cell types and created a strong defense to prevent the entry of pathogens. Plant cell wall is organized into three layers, the middle lamella, primary cell wall, and secondary cell wall (S1, outer; S2, middle; and S3, inner). The primary constituents of cell walls are cellulose (20–50% on a dw basis), hemicellulose (15–35%), and lignin (10–30%), while proteins (3–10%), lipids (1–5%), soluble sugars (1–10%), and minerals (5–10%)

are minor components (47, 48). The complexity and heterogeneity of plant biomass are reflected in the microbial diversity and variety of their enzymes that are produced naturally to degrade plant biomass (49).

Microorganisms play a vital role in the production of enzymes for biomass saccharification (Table 3, Figure 3 & 4). Therefore, different strategies are used for the prospection of novel and/or more efficient enzymes that hydrolyze lignocellulose. One example consists of bioprospecting of microorganisms in specific environmental niches with posterior investigation of their ability to hydrolyze crude substrates, followed by a screening of the best candidates that possess interesting enzymes (50, 51). Another strategy is the metagenomic tool, which is extensively used for the genetic composition analysis of microorganism mixtures (52). It is already known that enzyme extracts obtained from a single microorganism are not so efficient in biomass hydrolysis, mainly because of the imbalance of enzymes. Usually enzymes cocktails having different enzymes in an adequate proportion so they are specific to individual pretreated biomass compositions. During enzymatic treatment of biomass, polysaccharides of the cell wall exposed to degradation by an array of enzymes. Though, the most important problem with this process is biomass recalcitrance and less cellulases production in microbes. Improving enzymatic bioconversion of lignocelluloses to bioethanol, enzymes must have high adsorption ability, with improved catalytic efficiencies, high stability to variable temperature, and low end-product inhibition (53). For effective degradation of lignocellulose those microbial strains are required, which produce applicable levels of endoglucanase, exoglucanase and β-glucosidase, Different types of inhibitions were experienced in attaining greater saccharification yields using these enzymes. Furthermore, enzymes should not get affected by temperature and pH ranges, show resistance to product inhibition, synergism in actuation and high catalytic activity. Blending of individual enzymes and complementing crude enzyme extracts shows promise, since it can result in synergistic effects to improve biomass saccharification efficiency (54). Co-cultivation has often been performed to obtain improved lignocellulose hydrolysis. Several studies strongly justify the use of these microbial enzymes in biomass degradation. Anand *et al*. (55) reported that *Serratia liquefaciens* is able to employ three polysaccharides including CMcellulose, xylan and pectin. *Bacillus circulans* is able to utilize all four polysaccharides with different efficacy. Dantur *et al*. (1) in their study showed that bacteria which are isolated from *Diatrea saccralis* larvae have a high cellulolytic, endo and exoglucanase activity. These isolates are *Klebsiella, Pneumonia, Klebsiella variicola, Stenotrophomonas maltophilia, Stenotrophomonas rhizophila,* and *Bacillus pumilus.* For example, the new strain of *Pichia anomala* GS2-3 (after DNA shuffling) is a good producer of ethanol it can produce 47.1. g/L total sugar alcohols from 100 g/L glucose, which was 32.3.% higher than the original strain (56).

Lignocellulose can be broken down into simple sugars either enzymatically or chemically. However, enzymatic hydrolysis is a better choice because it needs less energy input and mild environment conditions, while fewer fermentation inhibitor products are generated. Though, plant cell wall convolution and heterogeneity requires a mixture of exo- and endo-enzymatic actions (57). Microbial conversion of lignocellulosics into fermentable sugars provides the condition, which needs slight or no pretreatment to produce biofuel or by-product as discussed earlier. Currently researchers are looking for lowering the costs of bioconversion and particularly focusing on technological development for effective biomass pretreatment and improvement of fermentation yield. Researchers all over the world, have adopted several strategies for decreasing the cost of cellulase production by screening of hyper cellulase producing strains, increasing cellulase titer and productivity by optimization of fermentation process parameters, adopting cheaper bioprocess technology such as solid-state fermentation (SSF), improving cellulase properties for efficient saccharification by protein engineering or blending of different cellulase, etc. Onsite cellulase production could further play an important role for decreasing the cost of overall bioethanol production (58).

Numerous lignocelluloses degrading enzymes may be classified previously in different ways on the basis of specificity of catalyzed reaction, structural/evolutionary relation and also on added aspects (59). Based on their sequence and structural homology enzymes are carbohydrate-active enzymes (http://www.cazy.org), fungal oxidative lignin enzymes (FOLy), lignocellulose-degrading enzymes belong to the category of glycoside hydrolases (GH), polysaccharide lyases (PL), carbohydrate esterases (CE), lignin oxidases (LO), and lignin degrading auxiliary enzymes (LDA) families. Modifying the lignin, using genetic engineering, may have potential in improving saccharification and thus improves the yield of biofuels (60). The cellulosome is a multiprotein complex, produced by anaerobic microbes, whose main function is to degrade lignocellulosic materials (61). Several lignocellulose-decomposing enzymes utilize hydrolytic reactions (chiefly acting on hemicellulose), whereas others uses oxidoreductive ones (mainly acting on lignin), to convert lignocellulose into bioethanol. In nature, cellulolytic microbes produce three main types of cellulases that work synergistically: endoglucanases, exoglucanases, and β-glucosidases. Endoglucanases enzymes break internal β-1,4-glycosidic bonds in the polymer, which creates reducing and non-reducing ends that further hydrolyzed by exoglucanases. By

Table 3. List of bacterial isolates degrading lignocellulosic biomass

Bacteria reported to degrade lignin under aerobic conditions
• *Pseudomonas spp.*
• *Acinetobacter spp.*
• *Pseudomonas spp.*
• *Xanthomonas spp.*
• *Streptomyces badius*
• *Streptomyces viridosporous*
• *Streptomyces cyaneus*
• *Thermomonospora mesophila*
• *Pandorea norimbergensis LD001*
• *Psuedomonas sp. LD002*
• *Bacillus sp. LD003*
Bacteria reported to hydrolyse cellulose under anaerobic conditions
Anarocellum thermophilum
• *Ruminococcus albus*
• *Clostridium thermocellum*
• *Caldicellulosiruptor saccharolyticus*
• *Ruminococcus flavefaciens*
• *Clostridium cellulolyticum*
• *Clostridium thermocellum*
• *Caldicellulosiruptor bescii*
• *Bacteroides succinogenes+Selenomonas ruminantium*
• *Clostridium thermocellum+Clostridium thermohydrosulfuricum*
• *Clostridium thermocellum + Clostridium thermohydrosulfuricum*
• *Fibrobacter succinogenes*
Bacteria reported to hydrolyse cellulose under aerobic conditions
• *Clostridium thermocellum*
• *Cellulomonas fermentans*
• *Fibrobacter succinogenes*
• *Ruminococcus flavefaciens*
• *Clostridium thermocellum*
• *Clostridium cellulolyticum*
• *Cellulomonas udaJC3*
• *Klebsiella oxytoca*
• *Klebsiella pneumonie*
• *Klebsiella variicola*
• *Bacillus pumilus*
• *Enterococcus casseliflavus*
• *Stenotrophomonas maltophilia*
• *Microbacterium testeceum*
Lignocellulose degrading microbes
• *Proteus vulgaris*
• *Bacillus circulans*
• *Klebsiella pneumonia*
• *Pseudomonas fluorescens*
• *Enterobacter* sp.,
• *P. aeruginosa,*
• *Aeromonas* sp.,
• *Citrobacter freundii*
• *Serratia liquefaciens*
• *Escherichia coli*
• *Erwinia* sp

Adapted from (111).

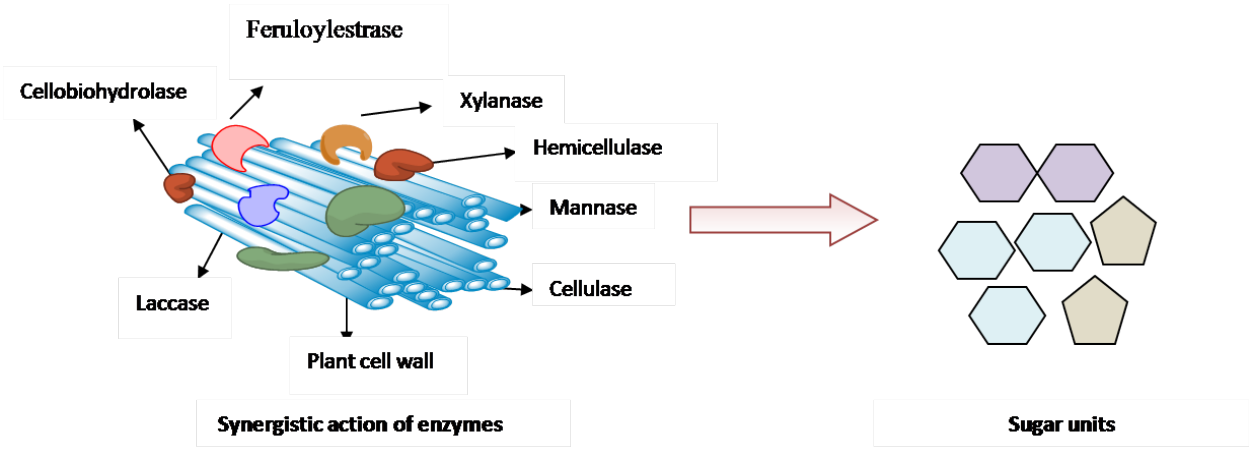

Figure 3. Synergistic action of microbial enzymes for biomass conversion into sugar units

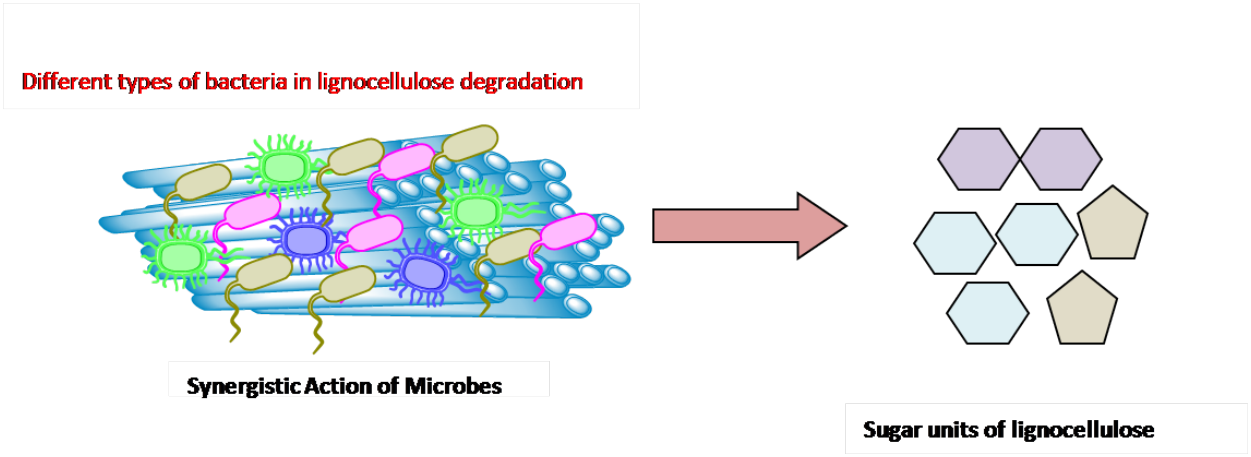

Figure 4. Synergistic action of microbes for biomass conversion into sugar units

working in coordination, the enzymes create shorter cellodextrins, with the disaccharide cellobiose, which is further degraded by β-glucosidases into its component sugars. The hemicellulose constituent of lignocellulose is made up of pentose and hexose sugars. To release sugars, microbes take up a variety of hemicellulases that have distinctive substrate specificities, with exoxylanases, endoxylanases, arabinases, and mannanases, among others. Lignin degradation by bacterial isolates remained less understood till date, while literature showed the potential role of white-rot fungi, in degradation which involves a combination of extracellular peroxidases and laccases enzymes (62, 63). Lu *et al*. (64) reported that *Clostridium thermocellum* CTL-6 has high capacity to degrade cellulose upto 80.9.%. Van and Pletschke (65) in their article reviewed the lignocellulose bioconversion by using enzymatic hydrolysis and described about the enzymes synergy and the role of different factors which affect enzymes, conversion, and synergy. Kumar and Murthy (66) reported stochastic molecular model of enzymatic breakdown of cellulose in ethanol production. Hu *et al*, (67) reported the role of synergistic action of accessory enzymes that improves the breakdown capacity of a “cellulase mixture” but it is highly substrate specific. Pérez-Rangel *et al*. (68) reported that the epiphytic microorganism are novel source of enzyme because they de-lignified the lignocellulose and convert hemicelluloses sugars into hydrogen efficiently. Lima *et al*. (69), in their study evaluated the Brazilian biomass composition and its processing potential as a novel source for sustainable bio renewable production. Robl *et al*. (70) reported *Annulohypoxylon stygium* DR47 fungus is a good producer of group of cell wall degrading enzymes which are b-glucosidase, pectinase, and glycohydrolase families, such as GH3, GH18, GH35, GH54 and GH92. Kim *et al*. (37) studied role of synergistic proteins for the enhanced cellulase production for hydrolysis of cellulose. In this work, they focus on action of non-GH proteins, which enhance lignocellulosic biomass degradation. To make proper use of these microbial

Table 4. Enzymes involved in lignocellulose degradation

Lignocellulose monomers	Enzymes
Lignin	Laccase, Manganese peroxidase, & lignin peroxidase
Pectin	Pectin methyl esterase, pectatelyase, polygalacturonase, rhamno galacturonanlyase, protopectinases, polygalacturonase, lyases, Swollenin, and GH 61
Hemicellulose	Endo-xylanase, β-xylosidase, endomannanase, acetyl xylan esterase, β-mannosidase, α-L-arabinofuranosidase, ferulic acid esterase, α- glucuronidase, α-galactosidase, p-coumaric acid esterase,xyloglucanases, and glucuronidases
Cellulose	Cellobiohydrolase, endoglucanase, and β-glucosidase

isolates and their enzymes, more research is required to understand the interactions among the enzymes, enzyme–cell, enzyme–substrate, and cell–substrate. So there is an urgent need of some new tools and techniques which can provide the information near to the nano level. Different types of microbial enzymes required to convert lignocellulose into sugar monomers are presented in Table 4.

6. FERMENTING THE SUGARS RELEASED FROM BIOMASS DEGRADATION

Several microorganisms are involved in converting biomass into ethanol after pretreatment. But there are several hurdles in conversion of lignocelluloses to bioethanol, the most important one is availability of ideal microbes that can well ferment both pentose and hexose sugars (71). Genetic modification of microbes is required that can efficiently utilize sugars into ethanol. Bioconversion of plant biomass into fuels and industrially relevant chemicals involves one of three strategies: separate hydrolysis and (co-) fermentation (SH(c)F), simultaneous saccharification and (co-) fermentation (SS(c)F), and consolidated bio-processing (CBP) (49). CBP involves straight conversion of lignocellulosic biomass to ethanol in a single processing step. CBP is a promising strategy for reducing the cost as it simplifies the operating requirements and avoid exogenous enzyme supplementation (63). The processes frequently engaged in the lignocellulosic hydrolysate fermentation are known as simultaneous saccharification fermentation (SSF), separate hydrolysis, and fermentation (SHF). SSF process is superior to SHF for ethanol production as it can advance ethanol yields by eliminating product inhibitors and also reduce the need for separate reactors. This process is economical but difference in temperature optima of enzyme for hydrolysis and fermentation pose some restrictions (72). In case of SSF, ethanol yield is higher, which may be partially due to efficient conversion of xylose to xylitol under the SSF environment (73). Overall, SSF is a better option in comparison to SHF (74). Klinke *et al*. (75) reported that *Thermoanaerobacter mathranii* A3M3 can grow on pentoses sugar and produce ethanol in hydrolysate without any need for detoxification. A co-fermentation process involving *Saccharomyces cerevisiae* and *Fusarium oxysporum* culture increased ethanol production by 19%, leading to a final ethanol concentration of 58 g L^{-1}, but could also lead to lower overall cost of the process by incorporating in-situ enzyme production (76). Asada *et al*. (77) reported that *Ureibacillus thermosphaericus* A1 has the capacity to increase fermentation, and resulted in increased of 74% ethanol production in the presence of inhibitory materials (such as formic acid, furfural, and 5-hydroxymethylfurfural). Saha *et al*. (78) reported that recombinant *E. coli* strain FBR5 can convert all these sugars (pentose and hexose) into ethanol. Huang *et al*. (79) used thermo-tolerant *Saccharomyces cerevisiae* ZM1-5 in simultaneous saccharification and fermentation of SB pulp to ethanol, which was performed in multiple parallel fermentation tanks (500 mL working volume). A considerable amount of ethanol (18.7.9 g/L at 0.4.2 g ethanol/g cellulose) was produced when the solid loading was 60 g/L. Their results indicated that SB pulp could be employed as an alternative material for bioethanol production (79).

7. INSTRUMENTS AND METHODS USED IN BIOMASS ASSESSMENT

In last decades, new methods are being developed to assess the rapid conversion of biomass to bioethanol and byproduct generation. Previously, many standard methods are available but are labor intensive, costly, and harmful for the environment. Very old method based on use of a two-stage sulfuric acid for release of sugars from lignin dates to the early 19th century. However, in 1920 to 1940, methods based on wood lignin isolation were developed by different scientists. Analytical methods developed by NREL, USA generate valuable data on various biomass feedstocks (80). Sluiter *et al*. (80) in their article discussed various methods used for the biomass assessment and analysis. A traditional method for biomass analysis includes two-stage sulfuric acid hydrolysis, gravimetric & instrumental analysis for compositional analysis. These methods were frequently used for studies of woody biomass, bioenergy production, and areas related to biomaterials (81). Wet chemical analysis holds potential for biomass analysis. These methods include thioacidolysis, acidolysis, transesterification, acetyl bromide method, nitrobenzene oxidation, orcinol method, and vansoest method, etc. Long-established fuel investigation of biomass includes

ultimate analysis, proximate analysis, and thermo-gravimetric analysis. Numerous standard methods for analysis of biomass are tedious and slow and use toxic chemicals. Saldarriaga *et al*. (82) reported a methods for biomass characterization, that depends upon thermo-gravimetric analysis, deconvolution of the DTG signal .

The application of spectroscopic methods is invaluable in analysis of biomass as they are simpler and quicker. Analytical and nondestructive methods are based on spectroscopy, such as Fourier transform infrared spectroscopy (FTIR), Near infra-red (NIR), Raman spectroscopy and Nuclear magnetic resonance (NMR), and are extensively used to measure functional groups and chemical bonds in biomass. Data obtained by these methods for biomass characterization and fuel analysis are more suitable in comparison to traditional chemical methods.

Standard spectroscopic methods for cellulose analysis are FTIR, NMR and for measuring the cellulose crystallinity NMR and FT-Raman instrument are used. Methods used for structural analyses of polysaccharides are FTIR, FT-Raman, Dispersive Raman, NMR and Fluorescence spectrophotometer. For lignin estimation FTIR, UV-VIS, UV-Raman and NMR method can be used effectively (83). Techniques are available to characterize lignocellulose during degradation process after or during enzyme treatment are categorized into: (1) primarily imaging techniques, (2) physicochemical techniques, and (3) spectro-microscopy techniques (84). Recently for characterization of biomass, high-throughput analytical techniques, such as NIR and Py-mbms have been proved significant in unraveling the chemical nature of diverse biomass samples and it requires minimal sample preparation. These high-throughput methods (HT) coupled with multivariate analysis have been established to be capable of identifying outliers, comparing samples (using principal component analysis), and building of prediction models (using partial least square).

Among the microscopic methods, atomic force microscopy (AFM) technique is used for imaging the surface structure of untreated and treated biomass, as well as to see the binding and assembly of the cellulosome complex. These microscopic investigations have revolutionized our understanding about the molecular structure of plant cell walls. AFM is the best tool to interpret cellulosome functions by correlative imaging using a combination of spectroscopy and other optical microscopy techniques (61). Recently the use of single-molecule spectroscopy has been explored for studying issues in biomass degradation. Ding *et al*. (61) have used the technique in which they have fluorescently tagged CBMs probe on the surface of carbohydrate-containing materials to map the distribution of cell wall polymers at the molecular level of resolution. By using this approach the distance between the cellulose and hemicelluloses may be correlated and calculated (61). The conversion and allocation of crystalline cellulose and lignin have been characterized by AFM, SEM and FTIR. These techniques are useful for molecular characterization of biomass (31). Tetard *et al*. (85) concluded that the cell wall structural information and cellulose globular structure is determined by atomic force microscopic techniques. Cao *et al*. (86) reported that the distribution of lignin is more in the xylem, while cellulose distribution is uniform, and hemicellulose content is high in the pith, in contrast to lignin in crop stalks, while in corn stalk lignin is more in the mechanical tissues, and again, cellulose is relatively uniformly distributed, while hemicelluloses is more in the parenchyma, in contrast to lignin. The results show that FTIR micro-spectroscopic imaging is an ideal technique for analyzing the chemical structures linked to tissue structure in crop stalk transverse section. Karimi and Taherzadeh (87) reported the application of thermogravimetric analysis (TGA) to study lignin, hemicelluloses and α-cellulose contents in biomass. TGA method proved to produce better and reliable results than the common methods used for the determination of the a-cellulose content. Fu *et al*. (88) reported the application of 13C cross-polarization, magic-angle spinning, and solid-state NMR for the direct quantification of lignin in biomass. By constructing a standard curve from pristine lignin and cellulose, the lignin content is accurately determined through direct measurement without the need of chemical or enzymatic pre-treatment (88).

2D-NMR is one of the most prevalent techniques developed during the last decade for probing lignin structure, linkages to carbohydrates, and quantifying specific functionalities and linkages present in lignin (89). Chan *et al*. (33) reported that furfuryl alcohol oligomers, C9–C14, were identified by combined spectroscopic technique and analytical methods, i.e., UV/Visible Raman and Infrared spectroscopy, gas chromatography and mass spectrometer. Szymanska-Chargot *et al*. (90) concluded that FT-IR spectroscopy combined with chemometric methods has potential for fast and reliable determination of the main constituents of fruit cell walls i.e, galacturonic acid, hemicellulose and cellulose and this method is used for both qualitative and quantitative analysis of cell wall. Lupoi *et al*. (91) reported the power of using Raman spectroscopy to supplement tedious, destructive methods for the evaluation of the lignin S/G ratio of diverse plant biomass materials.

Thomas *et al*. (92) concluded that X-ray powder diffraction (XRPD) and laser micro-Raman techniques are useful to measure the functional group transformations and the consequent crystalline sample changes during the process of cellulose degradation

Table 5. Instruments used in the analysis of lignocellulose degradation

S. No.	Instruments	Use	Reference
1.	Scanning electron microscopy (SEM)	Analysis of lignocellulosic biomass degradation and modification after the pretreatment and enzymatic action and generation of excellent 3-D images of bacteria on different surfaces	(112)
2.	Colorimetric method	It used for the analysis of lignocellulose concentration after and before degradation.	(113)
3.	Infrared	It is used for qualitative and quantitative study of biomass with both near-infrared and mid-infrared spectroscopy. And also for the wet chemical methods for composition analysis	(114)
4.	TGAQ500IF of TA Instrument	It is used to investigate the reactivity of the carbonaceous materials. TGA curve generally used for moisture loss, cellulose decomposition and CO/CO2formation and catalytic cellulose conversion is also determined	(115)
5.	Fluorescence	It is used for tracking the activity of polymer-immobilized enzymes. Capable of localization of lignin in plant cell wall, More selective than absorbance and Non-destructive	(89, 116)
6.	X-ray diffraction atomic force microscopy and small angle neutron scattering	Used for further characterizing enzyme-polymer systems	(117)
7.	X-ray crystallography/NMR	For the characterization of protein and enzyme structure and action	(9)
8.	HPLC	The contents of cellulose and other sugars in the enzymatic hydrolyzed samples were determined by HPLC system, Separation of non-volatile or thermally unstable molecules	(80) (91)
9.	IR and Raman Spectroscopy	Comprehensive investigation of the biomass derived furfuryl alcohol oligomer formation over tungsten oxide catalysts	(33)
10.	GC	Better spectral resolution than HPLC, Selectively analyze only volatile species, Headspace sampling limits clean-up requirements	(91)
11.	MS	It is a destructive technique and this can be used for the mass weight determination	(91)
12.	MIR Spectroscopy	It is a non destructive technique and may require specific sample preparation (i.e., KBr pellet). Spectral sensitivity to water; may require extensive sample drying.	(91)
13.	NMR	Solid state NMR methods are used to analyze biomass as a function of its chemical or biological treatment for biofuels, chemicals, or biochar production. Whole cell wall NMR by the direct-dissolution of biomass	(118, 119)
14.	Raman	Non-destructive method for the assessment of biomass degradation	(120)
15.	FT-NIR	It is more robust and nondestructive and useful in analysis of these components such as glucan, xylan, mannan, arabinan, galactan, lignin, and ash content in lignocellulosic biomass	(121)
16.	HR-TGA	This instrument can measure lignin, cellulose, and hemicellulose content with precision and identifying compositional differences. HR-TGA can be coupled with 1H-NMR	(122)
17.	Transmission electron microscopy	High-resolution images of the bacterial cells and the surrounding extracellular matrix and clearly visualize the structural differentiation in lignocellulosic biomass	(123)
18.	Atomic force microscopy	Bacterial structure, membrane components and cell to cell interactions can be seen with high-resolution, without the need of any fixation or dehydration	(123)
19.	Environmental scanning electron microscopy	Bacteria can be visualized in their native state without the need of any fixation or dehydration.	(123)

and glucose production. Ji *et al*. (34) reported that Raman mapping technique is useful for visualization of lignin, cellulose distribution and redistribution after pretreatment within specific tissues in different bands. CRM imaging and TEM measurements provided more complete information on the removal, migration and re-localization of lignin resulting from dilute acid pretreatment. Traoré *et al*. (93) suggested that FTIR is a good tool for the wood lignin, cellulose, hemicelluloses and carbohydrates; they also confirmed the relationship between carbohydrate and lignin content of soft wood and hard wood plant. Agarwal *et al*. (94) concluded that, Raman and NMR is a good tool for the estimation of syringyl-to-guaiacyl (S/G) ratio in woods. The SFG and XRD analysis revealed the changes in crystal size might be due to the aggregation of cellulose crystals, along with the increase in crystalline cellulose amount when the process of delignification approached. Application of heat treatment, without employing reactive agents, drastically increased the amount of crystalline cellulose and the XRD crystallite size, though the lignin content was not changed as much (95). Table 5, is showing instruments used frequently in analysis of biomass degradation.

8. CHALLENGES OF LIGNOCELLULOSICS CONVERSION INTO BIOETHANOL

There are several bottlenecks or key challenges in lignocelluloses conversion into bioethanol, which have to be resolved before the commercialization of biofuel production technology.

a. The biofuel production industry is currently trying to overcome recalcitrance of lignocellulose biomass as it remains a major economic and technical barrier for lignocellulose-based biofuel formation.
b. The appropriate choice of pretreatment used and the availability of lignocellulosic feedstock are closely related to the success and low cost conversion of biomass into bioethanol.
c. In depth understanding of the pretreatment process is a necessary prerequisite to know how pretreatments affect the physicochemical nature of heterogeneous cell walls; and how enzymes deconstruct the cell wall effectively.
d. The designing of superior biocatalysts; and co-optimization of the pretreatment process, enzymatic hydrolysis, and fermentation are vital for making the whole conversion cost effective.
e. During pretreatment and hydrolysis, characterization of the molecular structure of the cellulose microfibril is essentials (61).
f. The plants cell wall possesses more carbohydrate and aromatic polymers that have high oxygen contents over crude oil; thus, reduction to higher energy density molecules is imperative for producing biofuels that are attuned with the existing transportation infrastructure.
g. Effective release of sugars from lignocellulose is among the greatest technical and economic barriers because leading lignocellulose pretreatment technologies experiencing low sugar yields, and severe reaction conditions, and high cellulase use, narrow substrate applicability, and high cost, etc (96).

9. CONCLUSIONS

Lignocellulose present in biomass can be used for the energy production and byproduct generation for industry, but its recalcitrance to biological hydrolysis requires pretreatment before going for the fermentation. Application of proper pretreatments according to biomass types needs extensive research. Exploring novel microbes, within the immense biodiversity and adverse environments, for better adaptive characters in terms of temperature, pH and wide adaptability to low-cost substrates may be a viable strategy for biomass deconstruction. Furthermore, the use of non-destructive methods for biomass assessment and rapid screening of microbes for lignocellulolytic enzymes would need more focused research. Recent progress in this area is gaining a deeper understanding and building a momentum for rapid biomass conversion. Though, there are several key challenges which limit lignocellulose conversion into bioethanol, that are inadequate feedstock availability, rudimentary supply-chain logistics, biocatalytic inefficiency of rapid conversion of insoluble biomass to sugars, high oxygen-to-carbon content, and short of robust microbial catalysts. To resolve these issues, future research must be directed towards the development of cost effective, better pretreatment methods, optimization of saccharification process, and exploration of lignin degrading microbes, lignin engineering and lignin degradation.

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