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Review Article

Lignocelluloses: An Economical and Ecological Resource for Bio-Ethanol Production – A Review

Sokan-Adeaga Adewale Allen[1, *], Ana Godson R. E. E.[1], Sokan-Adeaga Micheal Ayodeji[2], Sokan-Adeaga Eniola Deborah[3]

[1]Department of Environmental Health Sciences, Faculty of Public Health, University of Ibadan, Ibadan, Nigeria

[2]Department of Microbiology, Faculty of Sciences, Obafemi Awolowo University, Ile-Ife, Nigeria

[3]Department of Physiology, Faculty of Basic Medical Sciences, Ladoke Akintola University of Technology, Ogbomosho, Nigeria

Email address:

sokanadeaga.adewaleallen@yahoo.com (Sokan-Adeaga A. A.), agree2000@yahoo.com (Ana G. R. E. E.), sokanmicheal@yahoo.com (Sokan-Adeaga M. A.), sokaneniola@yahoo.com (Sokan-Adeaga E. D.)

[*]**Corresponding author**

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Abstract: At present, the world is confronted with the twin crises of fossil fuel depletion and environmental degradation. This has made the search for alternative and renewable sources of energy inevitable. Today, examples of such efforts are seen in the production of biofuels from wastes of organic origin, often known as Lignocellulosic Biomass. Lignocellulosic wastes are generated during the industrial processing of agricultural products. These wastes are generated in large amounts throughout the year, and are the most abundant renewable resources on earth. Due to their large availability and composition rich in compounds they could be used in other processes, there is a great interest on the reuse of these wastes, both from economical and environmental viewpoints. This paper present a concise overview of lignocelluloses, their chemical composition, economical and biotechnological potentials in bio-ethanol production with special emphasis on the choice of lignocellulosic substrates, pretreatment methods and types of microorganisms that have been used for optimal, ecological and economic production of ethanol. Also reviewed are the different methods used to improve microbiological lignocellulolytic enzymatic systems including the current status of the technology for bio-conversion of lignocellulose residues by microorganisms (particularly yeasts and fungi), with focus on the most economical and eco-friendly method for ethanol production. Although the production of bioethanol offers many benefits, more research is needed in the aspects like feedstock preparation, fermentation technology modification, etc., to make bioethanol more economically viable. This paper opined that lignocellulosic waste will become the main feedstock for ethanol production in the near future. Scaling up the production of lignocellulosic ethanol, however, requires further reduction of the production cost. Conclusively, the review suggested that in order to improve the technology and reduce the production cost, two major issues have to be addressed: i) improving technologies to overcome the recalcitrance of cellulosic biomass conversion (pretreatment, hydrolysis and fermentation) and ii) sustainable production of biomass in very large amounts.

Keywords: Lignocellulosic Wastes, Biomass, Bio-Ethanol, Economical, Environmental, Biotechnological

1. Introduction

The enormous growth in the world populations, during the last few decades has led to a difficult situation in the field of energy supply and demand. At present, the world is confronted with the twin crises of fossil fuel depletion and environmental degradation. Indiscriminate extraction and consumption of fossil fuels have led to a reduction in the underground carbon sources. The global reserves of primary energy and raw materials are obviously limited. According to an estimate, the reserves will last for 218 years for coal, 41

years for oil, and 63 years for natural gas under a business-as-usual scenario coupled with their inherent environmental impact [1]. This has made the search for alternative and renewable sources of energy inevitable. Many industrialized countries are pursuing the development of expanded or new biofuels industries for the transport sector, and there is growing interest in many developing countries similarly "modernizing" the use of biomass in their countries and developing greater access to clean liquid fuels while helping to address energy costs, energy security and global warming concerns associated with fossil fuels [2].

Currently, bioethanol is being commercially produced only from edible feedstock such as corn-starch and sugarcane juice. The European Union (EU) had established a goal of 5.75% biomass-derived transportation fuels by December, 2010. The use of fuel ethanol has been quite successful in Brazil, where it is being produced at a very low cost by fermentation of sugarcane. In the US, corn is the dominant biomass feedstock for production of ethanol, and in the EU, straw and other agricultural wastes are the preferred types of biomass for ethanol production [3]. These bio-ethanol production systems pose a concern about competition with food and feed supplies. To avoid this competition, bioethanol production from non-edible lignocellulosic biomass such as wheat straw, rice straw, bagasse, corn stover, wood, peels of fruits and vegetables is attracting keen interest.

Lignocelllosic biomass can be utilized to produce ethanol, a promising alternative energy for the limited crude oil [4]. There are mainly two process involved in the conversion hydrolysis of cellulose in the lignocellulosic biomass to produce reducing sugars and fermentation of the sugars to ethanol [5-8]. The hydrolysis of cellulose is usually catalysed by cellulase enzymes, and fermentation is carried by yeasts or bacteria. During the enzymatic hydrolysis, cellulose is degraded by the cellulase to reducing sugars that can be fermented by yeast or bacteria to ethanol [4] the optimization of cellulose enzymes and enzymes loading can also improve the hydrolysis. Simultaneous saccharification and fermentation effectively removes glucose, which is an inhibitory to cellulose activity, thus increasing the yield and rate of cellulose hydrolysis. Lignocellulosic feed stocks such as agricultural wastes have favourable utilization potential for bio-ethanol production because of their quantity and competitive price. The main contributive parameter of bio-ethanol is the cost of the raw material and in order to reduce the overall cost of production corn cob which is abundant and do not interfere with food security was used for this experiment [6, 9]. Apart from the solvent nature of ethanol, it could also serve as a basic raw material for the synthesis of other products. It is also a safer alternative to methyl tertiary butyl ether (MTBE), which is usually added to gasoline in order to achieve a better and healthier combustion [10]. The United States Environmental Protection Agency announced its intentions to regulate MTBE addition to gasoline because of its toxic nature and its possible role in the contamination of community water sources [11]. In view of this, the demand for ethanol could further increase [12].

This paper present a concise overview of lignocelluloses, their chemical composition, abundance and biotechnological potentials in bio-ethanol production with special emphasis on the choice of lignocellulosic substrates, pretreatment methods and types of microorganisms that have been used for optimal, ecological and economic production of ethanol. Also reviewed are the different methods used to improve microbiological lignocellulolytic enzymatic systems including the current status of the technology for bio-conversion of lignocellulose residues by microorganisms (particularly yeasts and fungi), with focus on the most economical and eco-friendly method for ethanol production.

2. Overview of Lignocellulose

2.1. Lignocellulose Biomass

The term "biomass" generally refers to renewable organic matter generated by plants through photosynthesis in which solar energy combines with CO_2 (carbon dioxide) and moisture to form carbohydrates and oxygen materials having combustible organic matter are referred to as biomass. Biomass contains carbon, Hydrogen and Oxygen (oxygenated hydrocarbon, with high level of moisture and volatile matter, low bulk density and calorific value [13]. Lignocellulose biomass refer to the major structural component of woody and non-woody plants such as grass and represents a major source of renewable organic matter. A lignocellulosic biomass composed primary of plant fibres that are inedible by humans and have cellulose as a prominent component. Lignocellulose biomass as shown in Fig. 1consists of Lignin, hemicelluloses and cellulose. The composition is in the following proportion: cellulose (30 – 50%), hemicelluloses (20 – 35%) and lignin (5 – 30%) of plant dry matter. Lignocellulose biomass is a renewable resource that is virtually inexhaustible and is a potential feedstock for alternate fuel production. It may be available as either (a) residues corn stalks or other non-edible parts of plants used to produce food, municipal solid waste, pulp and paper industry wastes; (b) dedicated crops grown for the primary purpose of energy production [14].

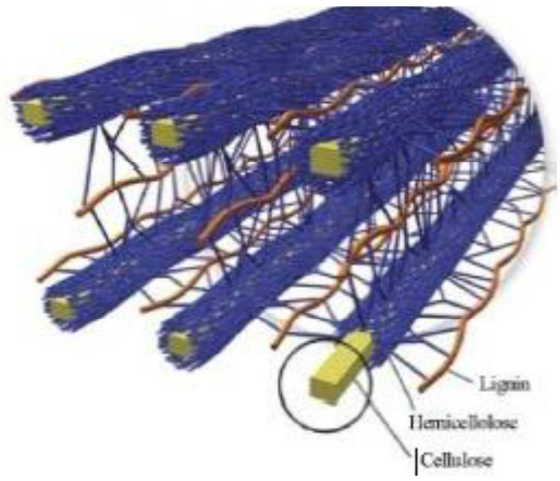

Figure 1. *Typical Lignocellulose biomass.*

2.2. Components of Lignocellulose

Lignocellulose is a renewable organic material and is the major structural component of all plants. Lignocellulose is a loose compound of lignin and cellulose. Lignin is not a single chemical compound. The name represents a class of closely resembling chemical compounds. Lignocellulose consists of lignin, hemicellulose and cellulose and Table 1 shows the typical compositions of lignocellulosic materials.

Table 1. *Lignocellulose contents of common agricultural residues and wastes.*

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood stems	40-55	24-40	18-25
Softwood stems	45-50	25-30	25-35
Nut shells	25-30	25-30	30-40
Paper	85-99	0	15
Wheat straw	30	50	15
Rice straw	32.1	24	18
Sorted refuse	60	20	20
Leaves	15-20	80-85	0
Cotton seeds hairs	80-95	5-20	20
Newspaper	40-55	25-40	18-30
Waste paper from chemical pulps	60-70	10-20	5-10
Primary wastewater solids	8-15	NA	24-29
Fresh bagasse	33.4	30	18.9
Swine waste	6	28	NA
Solid cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
Coastal Bermuda grass	25	35.7	6.4
Switch grass	45	31.4	12.0
S32 rye grass (early leaf)	21.3	15.8	2.7
S32 rye grass (seed setting)	26.7	25.7	7.3
Orchard grass (medium maturity)	32	40	4.7
Grasses (average values for grasses)	25-40	25-50	10-30
Bagasse	41.4	21.9	25.5
Forest residue	51	13	26.5

Source: Compiled from Betts *et al*. [15]; Sun and Cheng [4].

2.2.1. Cellulose

Cellulose $(C_6H_{10}O_5)n$ is a carbohydrate. It forms the primary structural component of green plants. For the plants, the primary cell wall is made of cellulose and the second cell wall is made of cellulose with a varying amount of lignin. Cellulose is also the most abundant form living terrestrial biomass in the world, which in combination with lignin and hemicellulose can be found in all the plants [16]. It is also the major constituent of paper and for the synthesis of the plastic celluloid. Cellulose, the major constituent of all plant material and the most abundant organic molecule on earth, is a linear biopolymer of anhydroglucopyranose-molecules, connected by β-1, 4-glycosidic bonds [Fig. 2]. Cellulose or β-1-4-glucan is a linear polysaccharide polymer of glucose made of cellobiose units. The cellulose chains are packed by hydrogen bonds in so-called 'elementary microfibrils'. These fibrils are attached to each other by hemicelluloses, amorphous polymers of different sugars as well as other polymers such as pectin, and covered by lignin. The microfibrils are often associated in the form of bundles or macrofibrils. This special and complicated structure makes cellulose resistant to both biological and chemical treatments. [17, 16].

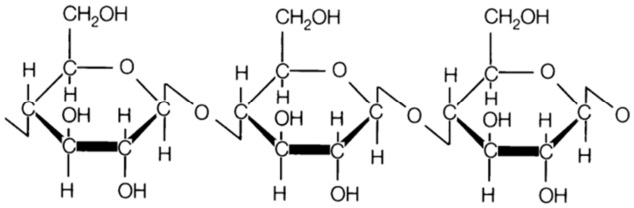

Figure 2. *Structure of Cellulose.*

In most lignocellulosic materials cellulose forms the major part of the three components. Cellulose is composed of insoluble, linear chains of β-(1→4)-linked glucose units with an average degree of polymerisation of about 10,000 units but could be as low as 15 units. It is composed of highly crystalline regions and amorphous (non-crystalline) regions forming a structure with high tensile strength that is generally resistant to enzymatic hydrolysis, especially the crystalline regions [18].

Cellulases, responsible for the hydrolysis of cellulose, are composed of a complex mixture of enzyme proteins with different specificities to hydrolyse glycosidic bonds. Cellulases can be divided into three major enzyme activity classes [19, 20]. These are endoglucanases or endo-1,4-β-glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). Endoglucanases, often called carboxymethylcellulose (CM)-cellulases, are proposed to initiate attack randomly at multiple internal sites in the amorphous regions of the cellulose fibre opening- up sites for subsequent attack by the cellobiohydrolases. Cellobiohydrolase, often called an exoglucanase, is the major component of the fungal cellulase system accounting for 40-70% of the total cellulase proteins and can hydrolyse highly crystalline cellulose [21].

2.2.2. Hemicellulose

Hemicellulose, the second most abundant component of lignocellulosic biomass, is a heterogeneous polymer of pentoses (including xylose and arabinose), hexoses (mainly mannose, less glucose and galactose) and sugar acids. Hemicellulose is less complex, its concentration in lignocellulosic biomass is 25 to 35% and it is easily hydrolysable to fermentable sugars [22]. The dominant sugars in hemicelluloses are mannose in softwoods and xylose in hardwoods and agriculture residues [23, 24].

Hemicellulose is similar to cellulose but is less complex. Hemicelluloses bind with pectin to cellulose to form a network of cross-linked fibers in plants. The hemicellulose has its main component xylan between that of the hardwood and softwood. Hemicellulose [Fig. 3] is a collective term referring to those polysaccharides soluble in alkali, associated with cellulose of the plant cell wall, and these would include non-celluloseβ – D – g lucans, pectic substances (polygalacturonans), and several heteropolysaccharides such as those mainly consisting of galactose (arabinogalactans), mannose (galactogluco-and

glucomannans) and xylose (arabinoglucuro- and glucuronoxylans). However, only the heteropolysaccharides, those with a much lower degree of polymerisation (100-200 units) as compared to that of cellulose (10000-14000 units) are referred to as hemicelluloses. The principal sugar components of these hemicellulose heteropolysaccharides are: D-xylose, D-mannose, D-glucose, D-galactose, L-arabinose, D-glucuronic acid, 4-O-methyl-D-glucuronic acid, D-galacturonic acid, and to a lesser extent, L-rhamnose, L-fucose, and various O-methylated sugar [24].

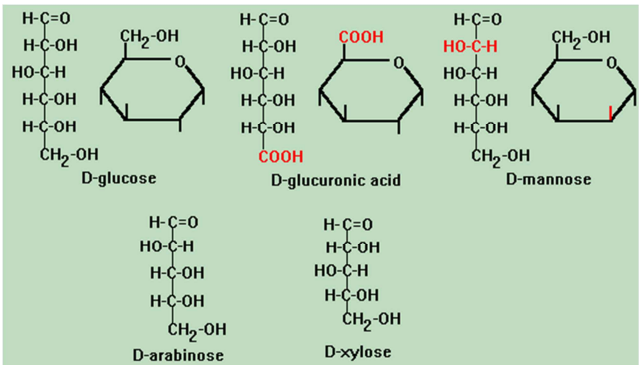

Figure 3. *Structure of Hemicellulose.*

Rabinovich et al., [19] and Shallom and Shoham [25] present recent reviews covering the types, structure, function, classification of microbial hemicellulases. The details of catalytic mechanism and structure of glycoside hydrolases, are also reported in [26, 20]. Hemicellulases like most other enzymes which hydrolyse plant cell polysaccharides are multi-domain proteins [27, 28]. These proteins generally contain structurally discrete catalytic and non-catalytic modules. The most important non-catalytic modules consist of carbonhydrate binding domains (CBD) which facilitate the targeting of the enzyme to the polysaccharide, interdomain linkers, and dockerin modules that mediate the binding of the catalytic domain via cohesion-dockerin interactions, either to the microbial cell surface or to enzymatic complexes such as the cellulosome [25, 28]. Based on the amino acid or nucleic acid sequence of their catalytic modules hemicellulases are either glycoside hydrolases (GHs) which hydrolyse glycosidic bonds, or carbonhydrate esterases (CEs), which hydrolyse ester linkages of acetate or ferulic acid side groups and according to their primary sequence homology they have been grouped into various families [27, 19, 20].

2.2.3. Lignin

Lignin [Fig. 4], the third main heterogeneous polymer in lignocellulosic residues, generally contains three aromatic alcohols including coniferyl alcohol, sinapyl and p-coumaryl. Lignin serves as a sort of 'glue' giving the biomass fibers its structural strength. Lignin acts as a barrier for any solutions or enzymes by linking to both hemicelluloses and cellulose and prevents penetration of lignocellulolytic enzymes to the interior lignocellulosic structure. Not surprisingly, lignin is the most recalcitrant component of lignocellulosic material to degrade [29, 30].

In general lignin contains three aromatic alcohols (coniferyl alcohol, s-inapyl and p-coumaryl). In addition, grass and dicot lignin contain large amounts of phenolic acids such as p-coumaric and ferulic acid, which are esterified to alcohol groups of each other and to other alcohols such as s-inapyl and p-coumaryl alcohols. Because of the difficulty in dissolving lignin without destroying it and some of its subunits, its exact chemical structure is difficult to ascertain [31].

Lignin is further linked to both hemicellulose and cellulose forming a physical seal around the latter two components that is an impenetrable barrier preventing penetration of solutions and enzymes. Of the three components, lignin is the most recalcitrant to degradation whereas cellulose, because of its highly ordered crystalline structure, is more resistant to hydrolysis than hemicellulose [32].

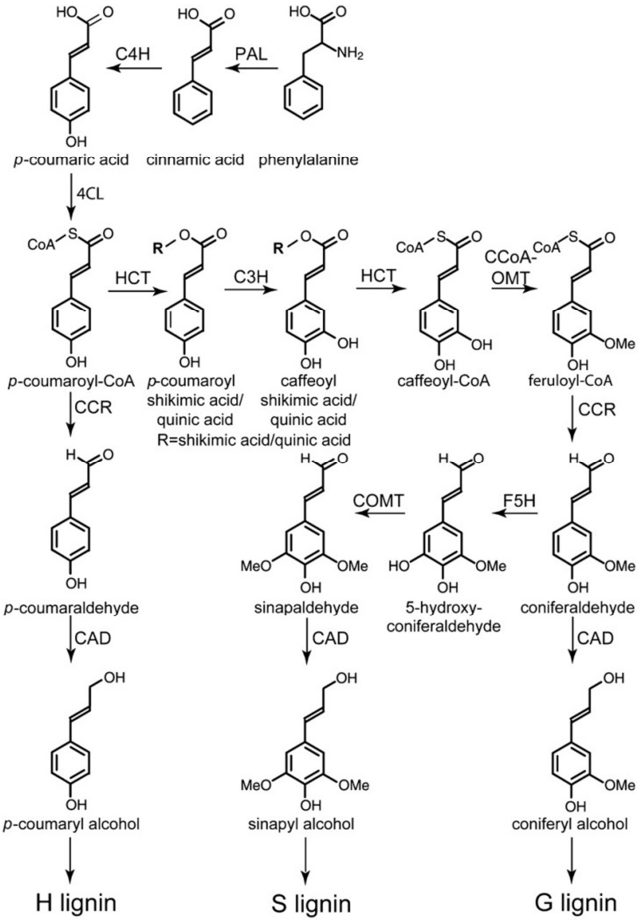

Figure 4. *Structure of Lignin.*

2.3. Potential Sources of Agro-based Wastes (Residues)

Billions of tons of agricultural waste are generated each year in the developing and developed countries. Agricultural residues includes all leaves, straw and husks left in the field after harvest hulls and shells removed during processing of crop at the mills, as well as animal dung. The types of crop residue which play a significant role as biomass fuel are relatively few [33]. The quantity of agricultural residues produced differs from crop to crop and is affected by seasons, soil types, and irrigation conditions. Production of

agricultural residues is directly related to the corresponding crop production and ratio between the main crop produce and the residues, which varies from crop to crop and, at times, with the variety of the seeds in one crop itself. Thus, for known amounts of crop production, it may be possible to estimate the amounts of agricultural residues produced using the residue to crop ratio [34].

Iyer et al., [35], reported, that agro-residue does suffer two major constraints: high moisture content and relatively low bulk density. These constraints inhibit their economical transportation over long distances, thereby necessitating their utilization near the sources of production. Unlike fossil fuels, which are concentrated sources of energy and chemicals the management strategy for agro-residues utilization has to be different. These are, therefore, most appropriate for decentralized technological applications in rural environments. The processing of the agricultural produce and utilization of agro-residues, therefore, can contribute their maximum share to rural development.

2.4. Microorganisms and Their Lignocellulolytic Enzymes

A diverse spectrum of lignocellulolytic microorganisms, mainly fungi [36, 37] and bacteria [38] have been isolated and identified over the years and this list still continues to grow rapidly. Already by 1976 an impressive collection of more than 14,000 fungi which were active against cellulose and other and other insoluble fibres were collected [39].

Despite the impressive collection of lignocellulolytic microorganisms only a few have been studied extensively and mostly *Trichoderma reesei* and its mutants are widely employed for the commercial production of hemicellulases and cellulases [40]. This is so, partly because T. reesei was one of the first cellulolytic organisms isolated in 1950's and because extensive strain improvement and screening programs, and cellulase industrial production processes, which are extremely costly, have been developed over the years in several countries.

T. reesei might be a good producer of hemi-and cellulolytic enzymes but is unable to degrade lignin. The white-rot fungi belonging to the *basidiomycetes* are the most efficient and extensive lignin degraders [41] with *P. chrysosporium* being the best-studied lignin-degrading fungus producing copious amounts of a unique set of lignocellulytic enzymes. *P. chrysosporium* has drawn considerable attention as an appropriate host for the production of lignin-degrading enzymes or direct application in lignocellulose bioconversion processes [42]. Less known, white-rot fungi such as *Daedalea flavida*, *Phlebia fascicularia*, *P. floridensis* and *P. radiate* have been found to selectively degrade lignin in wheat straw and hold out prospects for bioconversion biotechnology where the aim is just to remove the lignin leaving the other components almost intact [43]. Less prolific lignin-degraders among bacteria such as those belonging to the genera *Cellulomonas, Pseudomonas*, *Actinomycetes, Thermomonospora* and Microbispora and bacteria with surface-bound cellulase-complexes such as *Clostridium thermocellum* and *Ruminococcus* are beginning to receive attention as representing a gene pool with possible unique lignocellulolytic genes that could be used in lignocellulase engineering [44, 45].

It is conventional to consider lignocellulose-degrading enzymes according to the three component of lignocellulose (lignin, cellulose and hemicellulose) which they attack but bearing in mind such divisions are convenient classifications since some cross activity for these enzymes have been reported [46]. The exact mechanism by which lignocellulose is degraded enzymatically is still not fully understood but significant advances have been made to gain insight into the microorganisms, their lignocellulolytic genes and various enzymes involved in the process.

2.5. Overview of Lignocellulosic Biomass Conversion into Bio-Ethanol

Schematic picture for the conversion of lignocellulosic biomass to bio-ethanol, including the major steps can been seen in Figure 5. Pretreatment of the lignocellulosic residues is necessary because hydrolysis of non-pretreated materials is slow, and results in low product yield. Some pretreatment methods increase the pore size and reduce the crystallinity of cellulose [47]. Pretreatment also makes cellulose more accessible to the cellulolytic enzymes, which in return reduces enzyme requirements and, thus, the cost of ethanol production. The pretreatment not only enhance the biodigestibility of the wastes for ethanol production, but also results in enrichment of the difficult biodegradable materials, and improves the yield of ethanol from the wastes.

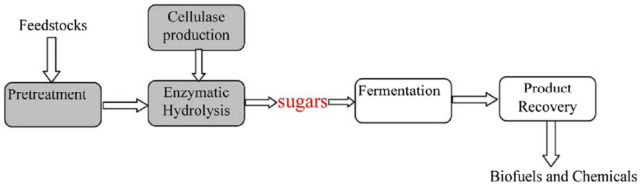

Figure 5. *Major steps involved in the conversion of lignocellulosic biomass to ethanol (Dashtban et al., [48]).*

2.6. Pre-treatment of Lignocellulose

Numerous pretreatment strategies have been developed to enhance the reactivity of cellulose and to increase the yield of fermentable sugars. Typical goals of pretreatment include:

i. Production of highly digestible solids that enhances sugar yields during enzyme hydrolysis, avoidance of degradation of sugars (mainly pentoses) including those derived from hemicelluloses.
ii. Minimization of formation of inhibitors for subsequent fermentation steps.
iii. Recovery of lignin for conversion into valuable co-products.
iv. Cost effectiveness by operating in reactors of moderate size and by minimizing heat and power requirements [49-51]. Figure 6 depicts schematic of goals of pretreatment on lignocellulosic material.

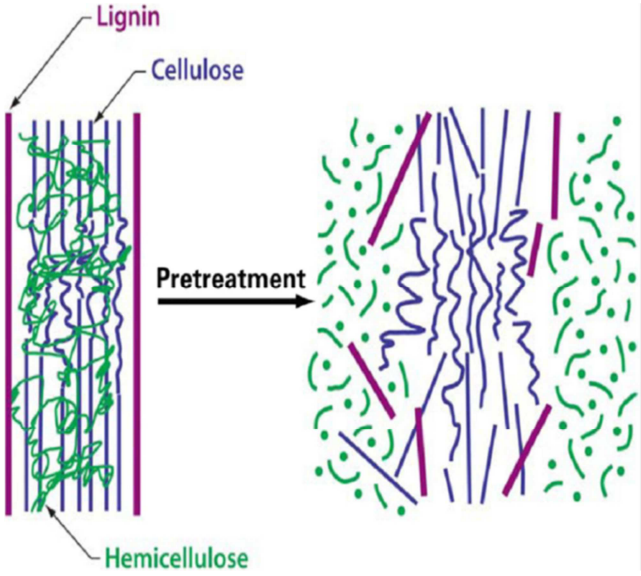

Figure 6. *Schematic of goals of pretreatment on lignocellulosic material.*

2.6.1. Physical Pretreatments Methods

Physical pretreatments methods such as ball milling and grinding have been used for degradation of lignocelluloses with limited success. This method of pretreatment being cost effective and ecofriendly, and one on which relatively little work has been done and reported, so far, would form one of the thrust areas of future research.

Waste materials can be comminuted by a combination of chipping, grinding and milling to reduce cellulose crystallinity. The size of the materials is usually 10–30 mm after chipping and 0.2–2 mm after milling or grinding. Vibratory ball milling has been found to be more effective in breaking down the cellulose crystallinity of spruce and aspen chips and improving the digestibility of the biomass than ordinary ball milling [52]. The power requirement of mechanical comminution of agricultural materials depends on the final particle size and the waste biomass characteristics [53]. Pyrolysis has also been used for pretreatment of lignocellulosic materials. When the materials are treated at temperatures greater than 300°C, cellulose rapidly decomposes to produce gaseous products and residual char [54]. The decomposition is much slower and less volatile products are formed at lower temperatures.

The efficiency of ultrasound in the processing of vegetal materials has been already proved [55]. The known ultrasounds benefits, such as swelling of vegetal cells and fragmentation due to the cavitational effect associated with the ultrasonic treatment, act by increasing the yield and by shortening of the extraction time. The effect of ultrasound on lignocellulosic biomass has been employed in order to improve the extractability of hemicelluloses [56], cellulose [57], lignin [58] or to get clean cellulosic fiber from used paper [59] but only few attempts to improve the susceptibility of lignocellulosic materials to biodegradation by using ultrasound have been described. It was found out that ultrasound has a beneficial effect on saccharification processes. Sonication has been reported to decrease cellulase requirements by 1/3 to ½ and to increase ethanol production from mixed waste office paper by approximately 20% [60]. It was notice that the effect of ultrasound fragmentation of Avicel (microcrystalline cellulose formed by acid treatment) is similar to that of the enzymes for short incubation intervals. The time needed for ultrasonic treatment could be reduced when increasing the irradiation power [61].

2.6.2. Chemical Pretreatment Methods

i. Alkaline Pretreatment

Alkaline pretreatment involves the use of bases, such as sodium, potassium, calcium, and ammonium hydroxide, for the pretreatment of lignocellulosic biomass. The use of an alkali causes the degradation of ester and glycosidic side chains resulting in structural alteration of lignin, cellulose swelling, partial decrystallization of cellulose [62, 63] and partial solvation of hemicelluloses [64, 65]. Sodium hydroxide (NaOH) has been extensively studied for many years, and it has been shown to disrupt the lignin structure of the biomass, increasing the accessibility of enzymes to cellulose and hemicellulose [66]. Another alkali that has been used for the pretreatment of biomass is lime [$Ca(OH)_2$]. Lignocellulosic feedstocks that have been shown to benefit from this method of pretreatment are corn stover, switch grass, bagasse, wheat, and rice straw.

ii. Acid Pretreatment Methods

Acid pretreatment involves the use of concentrated and diluted acids to break the rigid structure of the lignocellulosic material. The most commonly used acid is dilute sulphuric acid (H_2SO_4), which has been commercially used to pre-treat a wide variety of biomass types-switchgrass, corn stover, spruce (softwood), and poplar. Acid pretreatment (removal of hemicellulose) followed by alkali pretreatment (removal of lignin) has shown to yield relatively pure cellulose [67]. Strong acid allows complete breakdown of the components in the biomass to sugars, but also requires large volumes of concentrated sulfuric acid and can result in the production of furfural, an inhibitory byproduct [68]. Dilute acid allows reduced acid concentrations, but requires higher temperatures, and again gives furfural.

A key advantage of acid pretreatment is that a subsequent enzymatic hydrolysis step is sometimes not required, as the acid itself hydrolyses the biomass to yield fermentable sugars [69]. A mixture of H_2SO_4 and acetic acid resulted in 90% Saccharification [70]. Hemicellulose and lignin are solubilized with minimal degradation, and the hemicellulose is converted to sugars with acid pretreatment. The major drawback to these acid processes is the cost of acid and the requirement to neutralize the acid after treatment.

iii. Wet Oxidation

Wet oxidation utilizes oxygen as an oxidizer for compounds dissolved in water. Typically, the procedure for wet oxidation consists of drying and milling lignocellulosic biomass to obtain particles that are 2 mm in length, to which water is added at a ratio of 1 L to 6 g biomass. Wet oxidation has been used to fractionate lignocellulosic material by solubilizing hemicellulose and removing lignin [71]. It has

been shown to be effective in pretreating a variety of biomass such as wheat straw, corn stover, sugarcane bagasse, cassava, peanuts, rye, canola, faba beans, and reed to obtain glucose and xylose after enzymatic hydrolysis [72, 70, 73]. During wet oxidation, lignin is decomposed to carbon dioxide, water and carboxylic acids. Biomass such as straw, reed and other cereal crop residues have a dense wax coating containing silica and protein which is removed by wet oxidation [74].

iv. Green Solvents

Processing of lignocellulosic biomass with ionic liquids (IL) and other solvents has gained importance in the last decade due to the tenability of the solvent chemistry and hence the ability to dissolve a wide variety of biomass types. Ionic liquid (IL) was found to possess a great potential in dissolving cellulose [75]. Ionic liquids are salts, typically composed of a small anion and a large organic cation, which exist as liquids at room temperature and have very low vapor pressure. The chemistry of the anion and cation has been tuned to generate a wide variety of liquids which can dissolve a number of biomass types-corn stover [76], cotton [77], bagasse [78], switch grass, wheat straw [79]. Dadi and coworkers [80] have studied the enzymatic hydrolysis of Avicel regenerated from two different ILs, 1-n-butyl-3-methylimidazolium chloride and 1-allyl-3-methylimidazoliumchloride. Hydrolysis kinetics of the IL-treated cellulose was significantly enhanced. A limitation in using ionic liquids is the fact they tend to inactivate cellulose.

A solvent which has been effective in dissolution of cellulose and has a low vapor pressure similar to that of the ionic liquids is N-methyl morpholine N-oxide (NMMO). NMMO retains all the advantages of the ionic liquids ability to dissolve a variety of lignocellulosic substrates [81] without the need to chemically modify them and >99% of the solvent can be recovered due to its low vapor pressure. It is also nontoxic and biodegradable as proven by the work of Lenzing and other researchers [82]. Further research is needed to evaluate and improve the economics of usage of ILs and NMMO for pretreatment of biomass. Pretreatment of lignocellulosic materials with acidified organic solvents (mixture of 80% ethylene glycol, 19.5% water and 0.5% HCl at 178°C for 90 min) has also been successfully used [83]. The advantages of these methods include recovery and recycling of organic solvents as they can be easily distilled out. The disadvantages are that the process requires expensive high pressure equipment. Their performances could be improved by heating, microwave, or sonication [84].

2.6.3. Physicochemical Pretreatment Methods

i. Steam-explosion

Steam-Explosion pretreatment is one of the most commonly used pretreatment options, as it uses both chemical and physical techniques in order to break the structure of the lignocellulosic material [85]. This hydrothermal pretreatment method subjects the material to high pressures and temperatures for a short duration of time after which it rapidly depressurizes the system, disrupting the structure of cellulose microfibrils. The disruption of the fibrils increases the accessibility of the cellulose to the enzymes during hydrolysis.

Steam explosion is typically initiated at a temperature of 160–260°C (corresponding pressure 0.69–4.83 MPa) for several seconds to a few minutes before the material is exposed to atmospheric pressure. The process causes hemicellulose degradation and lignin transformation due to high temperature, thus increasing the potential of cellulose hydrolysis [86].

However, some disadvantages have been seen when using this process. Dilute acids are required to be added during softwood pretreatment or even when increased yields are warranted for lower acetylated feedstock. The factors that affect steam explosion pretreatment are residence time, temperature, chip size and moisture content. Recent studies indicate that lower temperature and longer residence time are more favorable [8].

ii. Liquid Hot Water (LHW)

Much like the steam-explosion process, liquid hot water (LHW) pretreatment uses water at elevated temperatures and high pressures to maintain its liquid form in order to promote disintegration and separation of the lignocellulosic matrix. Temperatures can range from 160°C to 240°C over lengths of time ranging from a few minutes up to an hour with temperatures dominating the types of sugar formation and time dominating the amount of sugar formation [87].

This process has been found to be advantageous from a cost standpoint in that no additives such as acid catalysts are required. Furthermore, expensive reactor systems have not been necessary to use due to the low corrosive nature of this pretreatment technique. Neutralization of degradation products is not needed due to their fractionation and utilization in the liquid fraction. In the same sense, inhibitory products have not been reported to form overwhelmingly in the respective fractions allowing higher yields under specific conditions.

iii. Ammonia Fiber Explosion (AFEX)

The ammonia fiber/freeze explosion (AFEX) process is another physicochemical process, much like steam explosion pretreatment, in which the biomass material is subjected to liquid anhydrous ammonia under high pressures and moderate temperatures and is then rapidly depressurized. The moderate temperatures (60°C to 100°C) are significantly less than that of the steam explosion process, thus allowing less energy input and overall cost reduction associated with the process [88].

There have been extensive literature reviews on this type of pretreatment over the last decade, focusing on the advantages and disadvantages of the AFEX process used for different feedstocks [89]. An overview of some of the advantages include lower moisture content, lower formation of sugar degradation products due to moderate conditions, 100% recovery of solid material, and the ability for ammonia to lessen lignin's effect on enzymatic hydrolysis. A smaller number of disadvantages can be seen in the form of higher

costs due to recycle and treatment of chemicals that are being used.

iv. Ammonia Recycle Percolation (ARP)

Ammonia recycle percolation (ARP) has been paired with the AFEX pretreatment process by many authors, but it can have some different characteristics that need to be taken into consideration when looking at different pretreatment options (Kim and Lee, 2005)[90]. In this process, aqueous ammonia of concentration between 5-15% (wt %) is sent through a packed bed reactor containing the biomass feedstock at a rate of about 5 ml/min. The advantage with this process over AFEX is its ability to remove a majority of the lignin (75–85%) and solubilize more than half of the hemicellulose (50–60%) while maintaining high cellulose content [90]. Primarily, herbaceous biomass has been most treated with this process: 60-80% delignification has been achieved for corn stover and 65–85% delignification for switchgrass [91].

v. Supercritical Fluid (SCF) Pretreatment

A supercritical fluid is a material which can be either liquid or gas, used in a state above the critical temperature and critical pressure where gases and liquids can coexist. It shows unique properties that are different from those of either gases or liquids under standard conditions-it possesses a liquid like density and exhibits gas-like transport properties of diffusivity and viscosity [92]. Thus, SCF has the ability to penetrate the crystalline structure of lignocellulosic biomass overcoming the mass transfer limitations encountered in other pretreatments. The lower temperatures used in the process aids in the stability of the sugars and prevents degradation observed in other pretreatments. Kim and Hong [93] investigated supercritical CO_2 pretreatment of hardwood (Aspen) and southern yellow pine with varying moisture contents followed by enzymatic hydrolysis. SCF pretreatment showed significant enhancements in sugar yields when compared to thermal pretreatments without supercritical CO_2. Alinia and coworkers [94] investigated the effect of pretreatment of dry and wet wheat straw by supercritical CO_2 alone and by a combination of CO_2 and steam under different operating conditions (temperature and residence time in the reactors). It was found that a combination of supercritical CO_2 and steam gave the best overall yield of sugars.

2.6.4. Biological Pretreatment Methods

Biological pretreatment uses microorganisms and their enzymes selectively for delignification of lignocellulosic residues and has the advantages of a low-energy demand, minimal waste production and a lack of environmental effects. In biological pretreatment processes, microorganisms such as brown-, white- and soft-rot fungi are used to degrade lignin and hemicellulose in waste materials [95]. White-rot basidiomycetes possess the capabilities to attack lignin. *Penicillium chrysosporium*, for example, has been shown to non-selectively attack lignin and carbohydrate [96]. *P. chrysosporium* has been successfully used for biological pretreatment of cotton stalks by solid state cultivation (SSC) and results have shown that the fungus facilitates the conversion into ethanol [97].

Brown rots mainly attack cellulose, while white and soft rots attack both cellulose and lignin. White-rot fungi are the most effective *basidiomycetes* for biological pretreatment oflignocellulosic materials [98]. Other *basidiomycetes* such as *Phlebia radiata, P. floridensis* and *Daedalea flavida*, selectively degrade lignin in wheat straw and are good choices for delignification of lignocellulosic residues [99]. *Ceriporiopsis subvermispora*, however, lacks cellulases (cellobiohydrolase activity) but produces manganese peroxide and laccase, and selectively delignifies several different wood species [100]. The advantages of biological pretreatment include low energy requirement and mild environmental conditions. However, the rate of hydrolysis in most biological pretreatment processes is very low.

2.7. Hydrolysis of Pretreated Biomass

After pretreatment, the released cellulose and hemicelluloses are hydrolyzed to soluble monomeric sugars (hexoses and pentoses) using cellulases and hemicellulases, respectively. The initial conversion of biomass into sugars is a key bottleneck in the process of biofuel production and new biotechnological solutions are needed to improve their efficiency, which would lower the overall cost of bioethanol production. Enzymatic hydrolysis has been considered key to cost-effective bioethanol in the long run, and the reaction is carried out with mainly cellulase and hemicellulase for cellulose and hemicellulose, respectively. The advantages of using enzyme (cellulase) over acid are to eliminate corrosion problems and lower maintenance costs with mild processing conditions to give high yields.

Despite the fact that some fungal strains have the advantages of being thermostable and producing cellulases, most of these fungal strains do not produce sufficient amounts of one or more lignocellulolytic enzymes required for efficient bioconversion of lignocellulosic residues to fermentable sugars. In addition, plant cell walls are naturally resistant to microbial and enzymatic (fungal and bacterial) deconstruction, collectively known as 'biomass recalcitrance' [101]. These rate-limiting steps in the bioconversion of lignocellulosic residues to ethanol remain one of the most significant hurdles to producing economically feasible cellulosic ethanol. Improving fungal hydrolytic activity and finding stable enzymes capable of tolerating extreme conditions has become a priority in many recent studies.

2.7.1. Fungal Extracellular Cellulases

Enzymatic saccharification of lignocellulosic materials such as sugarcane bagasse, corncob, rice straw, Prosopis juliflora, Lantana camara, switch grass, saw dust, and forest residues by cellulases for biofuel production is perhaps the most popular application currently being investigated [102, 103]. Both bacteria and fungi can produce glucanases (cellulases) that hydrolyze of lignocellulosic materials. These microorganisms can be aerobic or anaerobic and mesophilic or thermophilic. Bacteria belonging to genera of *Clostridium, Cellulomonas, Bacillus, Thermomonospora, Ruminococcus,*

Bacteriodes, Erwinia, Acetovibrio, Microbispora, and *Streptomyces* are known to produce cellulase [104]. Anaerobic bacterial species such as *Clostridium phytofermentans, Clostridium thermocellum, Clostridium hungatei,* and *Clostridium papyrosolvens* produces cellulases with high specific activity [8]. Most commercial glucanases (cellulases) are produced by *Trichoderma ressei* and β-D-glucosidase is produced from Aspergillus niger [105]. Fungi known to produce cellulases include *Sclerotium rolfsii, Phanerochaete chrysosporium* and various species of *Trichoderma, Aspergillus, Schizophyllum* and *Penicillium* [98, 8]. Among the fungi, *Trichoderma* species have been extensively studied for cellulase production.

High temperature and low pH tolerant enzymes are preferred for the hydrolysis due to the fact that most current pretreatment strategies rely on acid and heat [106]. In addition, thermostable enzymes have several advantages including higher specific activity and higher stability which improve the overall hydrolytic performance [107]. Ultimately, improvement in catalytic efficiencies of enzymes will reduce the cost of hydrolysis by enabling lower enzyme dosages. Some fungal strains such as *T. emersoniI* [108], *Chaetomium thermophilum* [109] and *Corynascus thermophiles* [110] can produce thermostable enzymes which are stable and active at elevated temperatures (60°C) well above their optimum growth temperature (30-55°C) [111]. Due to the promising thermostability and acidic tolerance of thermophilic fungal enzymes, they have good potential to be used for hydrolysis of lignocellulosic residues at industrial scales.

2.7.2. Fungal Hemicellulases

Several different enzymes are needed to hydrolyze hemicelluloses, due to their heterogeneity [22]. Xylan is the most abundant component of hemicellulose contributing over 70% of its structure. Xylanases are able to hydrolyze β-1,4 linkages in xylan and produce oligomers which can be further hydrolyzed into xylose by β-xylosidase. Not surprisingly, additional enzymes such as β-mannanases, arabinofuranosidases or α-L-arabinases are needed depending on the hemicellulose composition which can be mannan-based or arabinofuranosyl-containing. Also similarly to cellulases, most of the hemicellulases are glycoside hydrolases (GHs), although some hemicellulases belong to carbohydrate esterases (CEs) which hydrolyze ester linkages of acetate or ferulic acid side groups [25]. A mixture of hemicellulases or pectinases with cellulases exhibited a significant increase in the extent of cellulose conversion. Many fungal species such as *Trichoderma, Penicillium, Aspergillus* and *T. emersonii* have been reported to produce large amounts of extracellular cellulases and hemicellulases.

2.7.3. Fungal ligninases

Fungi degrade lignin by secreting enzymes collectively termed “ligninases”. These include two ligninolytic families; i) phenol oxidase (laccase) and ii) peroxidases [lignin peroxidase (LiP) and manganese peroxidase (MnP)] [112]. White-rot basidiomycetes such as *Coriolus versicolor* [113], *P. chrysosporium* and *T. versicolor* [114] have been found to be the most efficient lignin-degrading microorganisms studied. Interestingly, LiP is able to oxidize the non-phenolic part of lignin, but it was not detected in many lignin degrading fungi. In addition, it has been widely accepted that the oxidative ligninolytic enzymes are not able to penetrate the cell walls due to their size. Thus, it has been suggested that prior to the enzymatic attack, low-molecular weight diffusible reactive oxidative compounds have to initiate changes to the lignin structure and hemicellulose, fungal cellulosomes are much less well characterized compared to bacterial cellulosomes.

2.8. Fermentation

In the fermentation process, the hydrolytic products including monomeric hexoses (glucose, mannose and galactose) and pentoses (xylose and arabinose) will be fermented to valuable products such as ethanol. Among these hydrolytic products, glucose is normally the most abundant, followed by xylose or mannose and other lower concentration sugars.

The last two steps of bioconversion of pretreated lignocellulolytic residues to ethanol (hydrolysis and fermentation) can be performed separately (SHF) or simultaneously (SSF). In the separate hydrolysis and fermentation (SHF), the hydrolysate products will be fermented to ethanol in a separate process. The advantage of this method is that both processes can be optimized individually (e.g. optimal temperature is 45-50°C for hydrolysis, whereas it is 30°C for fermentation). However, its main drawback is the accumulation of enzyme-inhibiting end-products (cellobiose and glucose) during the hydrolysis. This makes the process inefficient, and the costly addition of β-glucosidase is needed to overcome end-product inhibition [115].

Further process integration can be achieved by a process known as consolidated bioprocessing (CBP) which aims to minimize all bioconversion steps into one step in a single reactor using one or more microorganisms. CBP operation featuring cellulase production, cellulose/hemicellulose hydrolysis and fermentation of 5- and 6- carbon sugars in one step have shown the potential to provide the lowest cost for biological conversion of cellulosic biomass to fuels, when processes relying on hydrolysis by enzymes and/or microorganisms are used [116].

The simultaneous saccharification and fermentation (SSF) process was first studied by Takagi et al., [117] for cellulose conversion to ethanol. The SSF process was originally developed for lignocellulosic biomass by researchers at Gulf Oil Company in 1974 [118]. The SSF process eliminates expensive equipment and reduces the probability of contamination by unwanted organisms that are less ethanol tolerant than the microbes selected for fermentation [119].

Over the years, various groups have worked on the SSF process to improve the choice of enzymes, fermentative microbes, biomass pretreatment, and process conditions. Extensive studies on SSF have since been conducted focusing on the production of ethanol from cellulosic

substrates. Phillipidis *et al.,* [120] have studied the enzymic hydrolysis of cellulose in an attempt to optimize SSF performance. Ghose *et al.,* [121] have increased ethanol productivity by employing a vacuum cycling in an SSF process using lignocellulosic substances. Zhu et al., [122] evaluated the suitability of production of ethanol from the microwave-assisted alkali pretreated wheat straw, the simultaneous saccharification and fermentation (SSF) of the microwave-assisted and conventional alkali pretreated wheat straw to ethanol.

Candida brassicae is accepted as the yeast of choice as far as SSF is considered, although both *Saccharomyces cerevisiae* and *S. carlsbergensis* have been found to offer similar rates. Several other yeasts as well as the bacteria *Zymomonas mobilis* have been studied with cellulose from *T. ressei* mutants for SSF processes. Researchers have also examined several combinations of enzymes with *Z. mobilis, S. cerevisiae,* and other ethanol producer, but they have only considered substrate levels lower than necessary to prove economic viability. Wyman *et al.,* [123] evaluated the cellobiose-fermenting yeast *Brettanomyces clausenii* for the SSF of cellulose to ethanol.

There are number of different methods to quantitate ethanol in samples. HPLC has been utilized to monitor the fermentation process This method has the advantage of being able to monitor not only the production of ethanol, but also the reaction substrates and byproducts [124]. Fourier transform infrared spectroscopy [125], gas chromatography [126], and Infrared [127] technologies have also been used to detect and quantitate ethanol in samples. While FTIR requires a large investment in instrumentation, the use or less expensive IR technology has been demonstrated to be just as accurate [127]. Gerchman *et al.,* [128] developed a cheap and rapid approach for ethanol quantification in aqueous media during fermentation steps as part of the conversion of biomass to ethanol. The suggested method requires a sample of a small volume and consists of organic extraction, followed by direct use of gas chromatography with a flame ionization detector (GC-FID). The feasibility of such approach is obvious since there is no need for the head-space system, distillation, expensive reagents and sophisticated equipment. The proposed method was also tested for its 'real-life' applicability for ethanol quantification from fermentation process.

2.9. Methods Used to Improve Fungal Enzyme Production, Activity and/or Stability

In order to produce ethanol industrially, the fermentative microorganism needs to be robust. The utilization of all the sugars generated from lignocellulosic hydrolysate is essential for the economical production of ethanol [22]. The conventional ethanol fermenting yeast (Saccharomyces cerevisiae) or bacterium (*Zymomonas mobilis*) cannot ferment multiple sugar substrates to ethanol [129]. A major technical hurdle to converting lignocellulose to ethanol is developing an appropriate microorganism for the fermentation of a mixture of sugars such as glucose, xylose, arabinose, and galactose [129]. A number of recombinant microorganisms such as *Escherichia coli, Klebsiella oxytoca, Z. mobilis,* and *S. cerevisiae* have been developed over the last 25 years with a goal of fermenting mixed sugars to ethanol [130, 131]. Saha and Cotta's [132] research unit has developed a recombinant *E. coli* (strain FBR5) that can ferment mixed multiple sugars to ethanol [133]. The strain carries the plasmid pLOI297, which contains the genes for pyruvate decarboxylase (pdc) and alcohol dehydrogenase (adh) from *Z. mobilis* necessary for efficiently converting pyruvate into ethanol [134].

Technologies required for bioconversion of lignocelluloses to ethanol and other valuable products are currently available but need to be developed further in order to make biofuels cost competitive compared to other available energy resources such as fossil fuels. The most recent and important improvements in production/activity of fungal enzymes using different techniques such as mutagenesis, co-culturing and heterologous gene expression of cellulases are discussed below.

2.9.1. Mutagenesis

Many fungal strains have been subjected to extensive mutagenesis studies due to their ability to secrete large amounts of cellulose-degrading enzymes. Cellulolytic activity of *T. reesei* QM6a has been improved by using different mutagenesis techniques including UV-light and chemicals, resulting in the mutant QM 9414 with higher filter paper activity (FPA) [135]. *T. reesei* RUT-C30 is one of the best known mutants, producing 4–5 times more cellulase than the wild-type strain (QM6a). A recent study by Kovacs *et al.,* [136] has shown that wild-type *Trichoderma atroviride* (F-1505) produces the most cellulase among 150 wild-type *Trichoderma*. Moreover, *T. atroviride* mutants were created by mutagenesis using N-methyl-N'-nitro-N-nitrosoguanidine (NTG) as well as UV-light. These *T. atroviride* mutants (e.g. *T. atroviride* TUB F-1724) produce high levels of extracellular cellulases as well as β-glucosidase when they are grown on pretreated willow. Cellulase and xylanase activities in *Penicillium verruculosum* 28 K mutants were improved about 3-fold using four cycles of UV mutagenesis. The enzyme production was further improved by 2- to 3-fold in a two-stage fermentation process using wheat bran, yeast extract medium and microcrystalline cellulose as the inducer [137].

2.9.2. Co-culturing

Fungal co-culturing offers a means to improve hydrolysis of lignocellulosic residues, and also enhances product utilization which minimizes the need for additional enzymes in the bioconversion process. In the case of cellulose degradation, for example, all three enzymatic components (EG, CBH and β-glucosidase) have to be present in large amounts. However, none of the fungal strains, including the best mutants, are able to produce high levels of the enzymes at the same time. *T. reesei* for example produces CBH and EG in high quantities whereas its β-glucosidase activity is low [138]. *A. niger*, however, produces large amounts of β-glucosidase, but has limited EG components [139]. In

addition, hemicellulose hydrolysis must also be considered when lignocellulosic residues are subjected to biomass conversion. However, this will be determined by the pretreatment methods. Specifically in an alkali pretreatment method, a part of lignin will be removed and thus hemicellulose has to be degraded by the use of hemicellulases, whereas in acid-catalyzed pretreatment, the hemicellulose layer will be hydrolyzed [140]. Again, some fungal strains have been shown to work more efficiently on cellulosic residues whereas others produce more hemicellulolytic enzymes and efficiently hydrolyze hemicellulosic portions [141]. Conversion of both cellulosic and hemicellulosic hydrolytic products in a single process can be achieved by co culturing two or more compatible microorganisms with the ability to utilize the materials. In fact, in nature, lignocellulosic residues are degraded by multiple co-existing lignocellulolytic microorganisms.

2.9.3. Metabolic Engineering

Metabolic engineering is a powerful method to improve, redirect, or generate new metabolic reactions or whole pathways in microorganisms. This enables one microorganism to complete an entire task from beginning to end. This can be done by altering metabolic flux by blocking undesirable pathway (s) and/or enhancement of desirable pathway (s). For example by application of homologous recombination, the production of *T. reesei* β-glucosidase I was enhanced using xylanase (xyn3) and cellulase (egl3) promoters which improved β-glucosidase activity to 4.0 and 7.5 fold compared to the parent, respectively. This will permit one fungal strain such as *T. reesei* to be more efficient on hydrolysis of cellulose to glucose which improve the yield and therefore lower the cost [142]. Becker and Boles [143] described the engineering of a Saccharomyces cerevisiae strain able to utilize the pentose sugar L-arabinose for growth and to ferment it to ethanol. Expanding the substrate fermentation range of S. cerevisiae to include pentoses is important for the utilization of this yeast in economically feasible biomass-to-ethanol fermentation processes. After overexpression of a bacterial L-arabinose utilization pathway consisting of *Bacillus subtilis* AraA and *Escherichia coli* AraB and AraD and simultaneous overexpression of the L-arabinose-transporting yeast galactose permease, we were able to select an L-arabinose-utilizing yeast strain by sequential transfer in L-arabinose media. High L-arabinose uptake rates and enhanced transaldolase activities favor utilization of L-arabinose.

2.9.4. Heterologous Expression

Heterologous expression is a powerful technique to improve production yield of enzymes, as well as activity. In order to make a robust lignocellulolytic fungal strain, many different fungal cellulases with higher and/or specific activity based on the need for a functional cellulose system in the organism have been cloned and expressed. For example, thermostable β-glucosidase (cel3a) from thermophilic fungus *T. emersonii* was expressed in *T. reesei* RUT-C30 using a strong T. reesei cbh1 promoter. The expressed enzyme has been shown to be highly thermostable (optimum temperature at 71.5°C) with high specific activity [144]. In the study for the improvement of biofinishing of cotton, *T. reesei* cellobiohydrolase (I & II) were overexpressed using additional copy (s) of the genes cloned under T. reesei cbh1 promoter. The results have shown that the expression of CBHI was increased to 1.3- and 1.5-fold with one or two additional copies of the gene, respectively.

2.9.5. Immobilization

Immobilization of microbial cells and enzymes has showed certain technical and economical advantages over free cell system. Using immobilized enzymes not only leads to greater product purity, cleaner processes, and economic operational costs but also makes the use enzyme cost effective and recoverable [145]. The immobilized biocatalysts have been extensively investigated during last few decades. An immobilized cellobiase enzyme system has been used in the enzymatic hydrolysis of biomass for the generation of cellulosic ethanol [146]. Production of alcohol and biodiesel fuel from triglycerides using immobilized lipase has been carried out using porous kaolinite particle as a carrier [147].

The use of an immobilized yeast cell system for alcoholic fermentation is an attractive and rapidly expanding research area because of its additional technical and economical advantages compared with the free cell system. A reduction in the ethanol concentration in the immediate microenvironment of the organism due to the formation of a protective layer or specific adsorption of ethanol by the support may act to minimize end product inhibition. The most significant advantages of immobilized yeast cell systems are the ability to operate with high productivity at dilution rates exceeding the maximum specific growth rate, the increase of ethanol yield and cellular stability and the decrease of process expenses due to the cell recovery and reutilization [148]. Other advantages of immobilized cell system over presently accepted batch or continuous fermentations with free-cells are: greater volumetric productivity as a result of higher cell density; tolerance to higher concentrations of substrate and products; lacking of inhibition; relative easiness of downstream processing etc. in different types of bioreactors, such as packed bed reactor, fluidized bed reactor, gaslift reactor and reactor with magnetic field [149, 150]. Perspective techniques for yeasts immobilization can be divided into four categories: attachment or adsorption to solid surfaces (wood chips, delignified brewer's spent grains, DEAE cellulose, and porous glass), entrapment within a porous matrix (calcium alginate, k-carrageenan, polyvinyl alcohol, agar, gelatine, chitosan, and polyacrilamide), mechanical retention behind a barrier (microporous membrane filters, and microcapsules) and self-aggregation of the cells by flocculation [151].

2.9.6. Process Integration

One of the most important approaches for the design of more intensive and cost-effective process configurations is process integration. Process integration looks for the

integration of all operations involved in the production of fuel ethanol. This can be achieved through the development of integrated bioprocesses that combine different steps into one single unit. Thus, reaction–separation integration by removing ethanol from the zone where the biotransformation takes place, offers several opportunities for increasing product yield and consequently reducing product costs. Other forms of integration may significantly decrease energetic costs of specific flowsheet configurations for ethanol production. Process integration is gaining more and more interest due to the advantages related to its application in the case of ethanol production: reduction of energy costs, decrease in the size and number of process units, intensification of the biological and downstream processes. Integration of fermentation and separation processes for reduction of product inhibition, development of efficient cogeneration technologies using cane bagasse, development of CBP, application of membrane technology (e.g. for ethanol removal or dehydration) are examples of process integration.

3. Conclusion

Lignocellulolytic microorganisms, especially fungi, have attracted a great deal of interest as biomass degraders for large-scale applications due to their ability to produce large amounts of extracellular lignocellulolytic enzymes. Many successful attempts have been made to improve fungal lignocellulolytic activity including recombinant and non-recombinant techniques. Process integration has also been considered for the purpose of decreasing the production cost, which was partly achieved by performing hydrolysis and fermentation in a single reactor (SSF) using one or more microorganisms (co-culturing).

These laboratory improvements should now be verified in pilot and demonstration plants. Scaling up the production of lignocellulosic ethanol, however, requires further reduction of the production cost. Thus, in order to improve the technology and reduce the production cost, two major issues have to be addressed: i) improving technologies to overcome the recalcitrance of cellulosic biomass conversion (pretreatment, hydrolysis and fermentation) and ii) sustainable production of biomass in very large amounts.

Future prospects

It is considered that lignocellulosic waste will become the main feedstock for ethanol production in the near future. In the case of large scale biomass production, additional waste stocks can be tested and used as substrates to meet the needs. On the other hand, biotechnological approaches including systems biology and computational tools are likely good candidates to overcome these issues. Future trends for costs reduction should include more efficient pretreatment of biomass, improvement of specific activity and productivity of cellulases, improvement of recombinant microorganisms for a greater assimilation of all the sugars released during the pretreatment and hydrolysis processes, and further development of co-generation system. Undoubtedly, ongoing research on genetic and metabolic engineering will make possible the development of effective and stable strains of microorganisms for converting cellulosic biomass into ethanol. Process engineering will play a central role for the generation, design, analysis and implementation of technologies improving the indexes of global process, or for the retrofitting of employed bioprocesses.

Undoubtedly, process intensification through integration of different phenomena and unit operations as well as the implementation of consolidated bioprocessing of different feedstocks into ethanol (that requires the development of tailored recombinant microorganisms), will offer the most significant outcomes during the search of the efficiency in fuel ethanol production. This fact will surely imply a qualitative improvement in the industrial production of fuel ethanol in the future.

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