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RESEARCH ARTICLE

Cellulases in conversion of lignocellulosic waste into second-generation biofuel

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Abstract

Cellulases are enzymes involved in degradation of lignocellulosic biomass. Lignocellulosic wastes are present in the form of agricultural waste like Rice Straw, Wheat Straw, Corn Straw and Bagasse. These cellulose wastes have potential to be utilized for the production and recovery of several useful products like bioethanol.The first generation bio-fuel are produced from sugar and starch based materials like sugarcane and grains. Rising population and diminishing land resources create a controversy, that whether land used for production of food and feed should be diverted for production of bio-fuel, considering the starving human society. Therefore second generation biofuel derived from lignocellulosic biomass is the most feasable choice for generation of bio-ethanol. A vital objective of second millennium biotechnology is the enzymatic conversion of renewable cellulosic biomass to inexpensive fermentable sugars and fermentation of these sugars into ethanol.

INTRODUCTION

Life is associated with waste production and exploitation and management of these materials as a renewable resource for development of useful product could be a major challenge.Lignocellulosiccellulose, an important cell wall polysaccharide, which is replenished constantly in nature by photosynthesis, goes waste in a lion's share in the form of pre-harvest and post-harvest agricultural losses and wastes of food processing industry. These cellulose wastes have potential to be utilized for the production and recovery of several useful products. The demand for energy is growing globally due to expanding human population and increase in industrial prosperity. The conventional fossil such as oil, coal and natural gasarestillconstituting the major source of energy. The continuous utilization of fossil fuels over the years has severelyaugmented the concentration of greenhouse gases in the atmosphere of earth (Ballesteros et al., 2006). These factors along with continuous decrease in the world's energy supply, and unstable oil market have forced us to look for alternative source of fuels. Ethanol has been considered as an alternative fossil fuel having capacity to be used singly in specially designed engines or can be blended in petrol without any engine modification, if added upto 30%. Today it is believed that bio-ethanol has potential to give energy security to the world, so its global production is exhibiting an upward trend over the last 25 years. The global production ability in 2005 and 2006 were about 45 and 49 billion liters per year, respectively and total amount to be produced in 2015 is predicted to reach over 115 billion liters (Licht, 2006). The first generation bio-fuel are produced from sugar and starch based materials like sugarcane and grains. Rising population and diminishing land resources create a controversy, that whether land used for production of food and feed should be diverted for production of bio-fuel, considering the starving human society. Therefore second generation biofuel derived from lignocellulosic materials is the most viable option for production of bio-fuel. Lignocellulosic waste materials obtained from energy crops, wood and agricultural residues, represent the most abundant global source of renewable biomass (Lin & Tanaka, 2006).The cost of production of ethanol from lignocellulose is the major hindrance in

commercializing the ethanol production from this feedstock. It has been found that enzymatic hydrolysis step is the most expensive step.During the last few years, effort has been put to reduce the cost of ethanol production from lignocellulosic biomass by improving the enzymatic hydrolysis step. Modern tools of biotechnology have been applied to improve thecellulolytic enzymes and reduce the cost and improve the conversion of biomass. The conversion of the cellulosic biomass into sugar is still a major barrier in the production of fuels and other high-value commodity products. The present manuscript describes the status of lignocellulosic waste generated in Asia and World, Nature of lignocellulosic material, overall conversion process of lignocellulosic waste into bio-ethanol, significant role played bycellulases in making of bioethanol and economics and environment involved in bio-ethanol production from lignocellulosic waste.

Status of lignocellulosic waste generated in Asia and World

The four major agro-wastes like Rice Straw, Wheat Straw, Corn Straw and Bagasse are the most suitable feedstocks for production of bio-ethanol due to their abundance and availability throughout the year.The global and continent wise production of these agro-wastes is given in Table 1. Asia is the largest producer of Rice straw and Wheat straw whereas America is the major producer of Corn straw and Bagasse (Table 1). These agricultural wastes vary in their chemical composition (Table 2), cellulose is the largest component in all. A very small part of Wheat straw, Rice straw and Corn straw is utilizable and it also varies geographically .A large fraction of agricultural residues is disposed of as waste. A very good example of this is Rice staw, 600-900 million tons of rice straw is produced world wide, but very small portion is used as animal feed and remaining is removed from the farm by burning. This practice is prevalent world- wide, causing air pollution and reducing the fertility of soil (Béguin & Aubert, 1994). Most of the countries have banned practice of open field burning. In case of Corn straw less than 1% is collected for industrial processing and about 5% is used as fodder. Around 90 % of Corn straw in United states is left in the fields. The bagasse of sugarcane is used as fuel for boilers and generation of electricity (Chandel et al., 2013; Kumar et al., 2008). Considering the enormity and abundance of agricultural waste, bioethanol production from these wastes is a promising option for energy security.

Lignocellulosic biomass composition

The lignocellulosic material is mainly composed of cellulose (30-50%), hemicelluloses (15-35%) and lignin (10-20%) (Chandel et al., 2013). Cellulose and hemicellulose make upto 70% of total biomass. Lignin is the third most abundant component firmly linked to the cellulose and hemicelluloses by covalent and hydrogen bonds making the structure robust and resistant to enzymes (Buaban et al., 2010). The chemical composition of most prevalent agricultural residues are given in Table 2.

Role of cellulase in hydrolysis of cellulose

Cellulose is a fibrous, insoluble and crystalline polysaccharide. It is a major constituent of plant cell walls and composed of D-glucose units linked together by β-1,4-glucosidic bonds (Shahzadi et al., 2014).Cellulose is the most abundant carbohydrate polymer and most sought after renewable resource that can be converted into bio-ethanol and bio-energy. Since cost of production of bio-ethanol and othervalue-added products from cellulosic waste is high, so huge amount of agricultural, industrial and municipal cellulosic waste is accumulating (Wu et al., 2010). Therefore it has become an ecological and economic interest to develop cost-effective utilization and treatment processes for cheap cellulosic sources. A wide variety of organisms including fungi, bacteria, and protists as well as invertebrate animals like insects, crustaceans, annelids, mollusks and nemadodes utilize cellulose as their source of energy.(Kuhad et al., 2011) Cellulase enzyme is present in these organisms and complete enzyme system of cellulase includeexo-β-1, 4-glucanases (EC 3.2.1.91), endo-β-1,4-glucanases (EC 3.2.1.4), and β-1,4-glucosidase (EC 3.2.1.21) (Lynd et al., 2005). The synergistic actionof all threecomponents act sequentially and lead to breakdown of cellulose into fermentable sugar.

The three general components of cellulase enzyme systems are endoglucanases, exoglucanases, and β- glucosidases. Endoglucanasesattack the crystalline region of cellulose randomly. Each break produces two new end in cellulose chain. The exoglucanases attach to free end of the chain and acting along the chain, releases cellobiose and glucose. Cellobiose, consisting of two glucose molecules connected together is then split by β-glucosidase into glucose monomers (Pandey et al., 2013).

Overview of the conversion process

Although there are so many rewards in production of bio-ethanol from lignocellulosic biomass, still technology for this conversion is in its infancy stage. Sugarcane and starch-based materials are mostly used for production of bio-ethanol. The basic technique of producing ethanol from starch or lignocellulosicbiomass includes hydrolysis of lignocelluloses or starch into sugar and finally fermentation of sugar into ethanol. The techno-economic challenges in conversion of lignocellulosic biomass to ethanol is very high considering the following facts (1) Efficient conversion of cellulose and hemicelluloses into fermentable sugars (2) Efficient fermentation of hexoses and

pentoses into ethanol as well as removal of inhibitory compounds. (3) Minimizing the cost of production. (4) Cost-efficient use of lignin.

Pretreatment is the first step in conversion of biomass into ethanol.It includes alkali swelling, acid hydrolysis and steam explosion techniques (Venkata Mohan et al., 2008). All these methods act by different mechanisms to make the lignocellulosic biomass more accessible to enzymes.The sacchrification of lignocellulosic biomass is the second step, where cellulose and hemicelluloses portion is converted into fermentable sugar. Finally sugars are fermented into ethanol or used for preparation of other value-added products. Among all three steps, saccharification of lignocellulosic biomass is the most critical step for bio-ethanol production. Although there are different chemical methods used for hydrolysis,butenzymatic hydrolysis is better as it requires less energy and mild conditions in comparision to chemical method (Ferreira et al., 2009). The optimum temperature and pH for cellulase and xylanase have been reported to be 40-50 °C and 4-5 respectively (Park et al., 2002). The enzymatic hydrolysis is better due to its low toxicity, low utility cost and low corrosion in comparision to chemical methods (Sun & Cheng, 2002; Talebnia et al., 2010). Above all no inhibitory by-product is formed in case of enzymatic hydrolysis (Ferreira et al., 2009). However enzymatic hydrolysis is limited by the factors like substrate concentration, enzyme loading, time and temperature of sacchrification (Tucker et al., 2003).

Microbial cellulases

Cellulases are produced by a variety of microorganisms. These organisms can be separated into two classes on the basis of organization of enzyme in the cell. (1)Microorganisms having cellulases organized into multienzyme complex called cellulosomes, e.g*Clostridiumthermocellulum* and *Cellulomonas.* (2) Microorganisms producing free cellulase that are not attached to each other, e.g *Trichoderma*sp, *Humicolagrisea*, *Aspergillus*sp, *Streptomyces*, *Bacillus* sp etc (Kuhad et al., 2011; Rabinovich et al., 2002). The fungi of class *T. reesei* and *Humicola grisea* and of bacteria *Streptomyces lividans* and *Cellulomonas* produce extracellular cellulase in large amount(Sukumaran et al., 2005). Among all cellulase producers, *Trichoderma* spp. (e.g. *T. reesei*, *T. viride*, *T. longibrachiatum*, *T. pseudokoningii*etc) are the most suitable cellulose degraders, as they produce copious amount of extracellular cellulase. In case of cellulase system of *T. reesei*two CBHs (Cel6A and Cel7A), and atleast five EGs (Cel5A, Cel7B, Cel12A, Cel45A and Cel61A), have been identified. These enzymes belong to six different GH families i.e 5, 6, 7, 12, 45 and 61. The other important cellulase producing fungus are *Penicillium* spp. (e.g *P. citrinum, P. occiantalis, P. italicum*etc) and *Aspergillus* spp. (*A. niger, A. nidulans, A. niger* and *A.oryzae*) (Saini et al.). The important feature of carbohydrate degraders are that, they are generally unable to use proteins or lipids as energy source (Lynd et al., 2002). But they can utilize a wide range of carbohydrates in addition to cellulose (Lynd et al., 2002). The important examples are bacteria *Cellulomonas* and *Cytophaga* and most other fungi , While the anaerobic cellulase producers utilize a limited carbohydrate range including cellulose or its hydrolytic products. Although there are several fungi having ability to utilize cellulose as energy source, but only few secrete cellulase enzymes, having practical applications in hydrolysis of cellulose.In addition to *T. reesie* there are some other fungi like *Humicola*, *Penicillium* and *Aspergillus* secrete extracellular cellulase in large amount(Persson et al., 1991) Microbes commercially exploited for cellulase productions are restricted to *T. reesie*, *H.insolens*, *A.niger*, *Thermomonosporafusca*, *Bacillus*sp and few more (Persson et al., 1991).

Hydrolysis of Lignocellulosic biomass

The hydrolysis of cellulose by enzymes is carried out by highly specific enzyme cellulase. The hydrolysed products are generally reducing sugars. The enzyme hydrolysis is generally carried out at mild conditions (pH 4.8 and temperature 50ºC), so its utility cost is low compared to acid or alkaline hydrolysis. Another advantage is its low corrosion efficiency (Alvira et al., 2010).

There are so many factors, influencing the enzymatic hydrolysis of cellulose e.g pretreatment of cellulosic material, temperature and pH of hydrolyzing condition. Cellulase dosage of 10-30 (FPU/g cellulose) is generally used for efficient hydrolysis with high glucose yield in reasonable time (48-72 h). It is also reported that enzyme loading may vary depending on the pretreatment, type and concentration of raw materials(Talebnia et al., 2010). Today, most of the commercial cellulase is produced from fungi because majority of cellulase producing bacteria are slow growing anaerobes and very few produces extracellular cellulases (Lynd et al., 2002).

The cellulase enzyme act by three steps i.e adsorption, biodegradation and desorption. During processof hydrolysis activity of cellulase decreases due to irreversible adsorption of enzyme on cellulose. The addition of surfactants during enzymatic hydrolysis improve the conversion of cellulose into monomeric sugars (Eriksson et al., 2002). There are various hypothesis supporting the improvement of enzymatic cellulose hydrolysis. The surfactant could change or modify the nature of cellulose surface, reduce irreversible binding of cellulase on cellulose, prevent enzyme denaturation as well as unnecessary binding of cellulase to lignin. Tween 20 was reported to be the most effective for improving enzyme hydrolysis (Taherzadeh & Karimi, 2007). The cocktail of enzyme containing cellulase and other enzyme like hemicellulase results in higher sugar production.

Chemicals and other high-value bio-products

The conversion of lignocellulosic biomass into sugars can contribute significantly to the production of organic chemicals. The sugars derived from biomass can be fermented to ethanol and other commodity chemicals by various microbes. It has been reported that *B. coagulans*can ferment hexoses and pentoses to lactic acid (Ou et al., 2011). More than 75% of organic chemicals are produced from primary base-chemicals: ethylene, propylene, benzene, toluene and xylene which are used to synthesize other organic compounds (Coombs et al., 1987). The aromatic compounds can be produced from lignin, whereas low molecular weight compound can be synthesized from ethanol produced by fermentation of sugars derived from lignocellulosic biomass. Vanillin and gallic acid are one of the two most important compound (Walton et al., 2003).Vanillin is used for various purposes including being an intermediate in the chemical and pharmaceutical for the production of herbicides, anti-foaming agents or drugs such as papaverine, L-dopa and the antimicrobialagent, trimethoprim. It is also used in household products such as air-fresheners and door polishes (Walton et al., 2003). Hemicelluloses are of particular industrial interest because these are a readily available bulk source of xylose from which xylitol and furfural can be derived. Xylose produced from palm waste can be used for the production of xylitol (Rahman et al., 2007).

Fermentation

The saccharified biomass is used for fermentation by several microorganisms. However, the industrial utilization of lignocelluloses for bioethanol production is hindered due to the lack of pentose and hexose sugar fermenting microorganisms (Kim et al., 2010). For a microrganisim to behave as commercially sustainable ethanol producer it should follow certain criteria such as it should have broad substrate utilization, high ethanol yield and productivity, have the ability to withstand high concentrations of ethanol and high temperature, should be tolerant to inhibitors present in hydrolysate and have cellulolytic activity. Genetically modified or engineered microorganisms are thus used to achieve complete utilization of the sugars in the hydrolysate and better production benefits (Fernandes & Murray, 2010). The most commonly used methods for fermentation of lignocellulosichydrolysate are simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF). Of them SSF is superior over SHF for ethanol production because it can improve ethanol yields by removing end product inhibition, eliminate the need for separate reactors and cost effective (Taherzadeh & Karimi, 2007). The higher ethanol yield coefficient from SSF would be partially due to more conversion of xylose to xylitol under the SSF conditions (Sarkar et al., 2012). The reason for slow xylose consumption during fermentation in SHF may be the presence of toxic compounds which obstruct the growth and fermentation activity of the microorganism (Sarkar et al., 2012). However the use of thermo-tolerant microorganism overcomes the drawback of SSF s such as *Kluyveromycesmarxianus* which resist the higher temperatures needed for enzymatic hydrolysis (Ballesteros et al., 2004). Besides SSF or SHF, there are few alternatives available such as consolidated bioprocessing (CBP) and simultaneous saccharification and co-fermentation (SSCF) (Lynd et al., 2005). In CBP also known as direct microbial conversion (DMC), cellulase production, biomass hydrolysis and ethanol fermentation are all together carried out in a single reactor. In this process the fermentation of cellulose to ethanol is carried out by mono- or co-culture of microorganisms. This method involves no capital investment for purchasing and production of enzyme (Hamelinck et al., 2005). Bacteria such as *Clostridium thermocellum* and few fungi including *Neurosporacrassa*, *Fusariumoxysporum* and *Paecilomyces*sp have shown this type of activity. However, the major drawback of CBP is poor ethanol yield and long fermentation periods (3-12 days) therefore it is not efficiently used (Szczodrak & Fiedurek, 1996). Sequential fermentation using *S. cerevisiae*in the first phase for hexose utilization and *C. shehatae* in the second phase for pentose utilization has also been employed for better utilization of sugar but ethanol yields achieved are not high(Sánchez & Cardona, 2008). Few examples of microorganisms used in ethanol fermentation are *S. cerevisiae, Escherichia coli, Zymomonasmobilis, Pachysolentannophilus, C. shehatae, Pichiastipitis, Candida brassicae, Mucorindicus*etc. (Sánchez & Cardona, 2008). Among all the reported yeast and bacteria employed in ethanol production from hexoses, *S. cerevisiae* and *Z. mobilis*, respectively were found to be best (Talebnia et al., 2010). However, *S. cerevisiae* cannot utilize the main C-5 sugar xylose of the hydrolysate(Xu et al., 1998). The innate organisms such as *Pichia* and *Candida* species can utilize xylose and can be a better alternate for *S. cerevisiae* but their ethanol production rate is five times lower than later (Xu et al., 1998). Different microorganisms have shown different yields of ethanol depending on their monomer utilization. A number of genetically modified microorganisms have been developed to improve higher yield of ethanol and wide substrate utilization to increased recovery rates such as *P. stipitis* BCC15191(Buaban et al., 2010), *P. stipitis* NRRLY-7124 (Moniruzzaman, 1995), recombinant *E. coli* KO11 (Takahashi et al., 2000), *C. shehatae* NCL-3501 (Abbi et al., 1996), *S. cerevisiae* ATCC 26603 (Moniruzzaman, 1995). Strict anaerobic hemophilic bacteria such as *Clostridium* sp. and *Thermoanaerobacter* sp. (Sánchez & Cardona, 2008), and Some other thermo-tolerant microorganisms such as *K.*

marxianus, *Candida lusitanieae* and *Z. mobilis* (Bjerre et al., 1996) have been proposed to explore the benefits of fermentation at elevated temperatures.

Cost involved in biofuel production

The major hurdle in bioethanol production from lignocellulosic biomass is cost management. The major cost involved is due to low yield and inefficient hydrolysis. It has been estimated that feedstock can account for more than 40% among all costs involved, so conversion of biomass to bioethanol is critically dependent on rapid and efficient conversion of all hexoses and pentoses present in celluloses and hemicelluloses respectively.

Economic considerations for cellulosic ethanol production

The bioethanol production cost should be lower than the current price of gasoline to make the bioethanol more competitive and acceptable on larger scale (Subramanian et al., 2005). The researchers are putting lot of efforts to make bioconversion technology cheap and efficient. However, there is still much to be done to bring down the price of bioethanol. The two most important factors contributing dominantly in bioethanol price are feedstock and cellulase enzyme. Since cost of biomass feedstock is around 40%, rest is mostly spent on cellulolytic enzyme.

An analysis of the potential of bioethanol in short and long term (2030) in terms of performance, key technologies and economic aspects such as cost per kilometer driven has been conducted recently by Hamelinck et al.(2005). The integrated approach of process optimization, fermentation and enzyme technology could significantly improve the economics of bioethanol production. The cost of cellulosic ethanol will be brought down from more than Rs 50 to Rs 25/l, with an estimated cost of less than Rs 12.5 per litre in near future (Aristidou & Penttilä, 2000). Wooley et al (1999) have described further the economic analysis of bioethanol ($ 0.78/gallon) and further suggested a projected cost Rs 10/l by 2015 if enzymatic hydrolysis step and biomass improvement targets are achieved. The estimated cost of ethanol production from cellulosic biomass as per earlier estimates (Rs 231.5) has been reduced four times over last 20 years. It has been found that distillation cost per unit volume is very high, if concentration of ethanol is substantially low, so it is better to concentrate the sugar solutions before fermentation. The lowest operational cost has been reported with membrane distillation process. Apart from this it is simple, user-friendly, efficient and cost effective distillation process (Camacho et al., 2013).

Conclusion

Interest in lignocellulosic biomass utilization has increased dramatically over the last few years as a renewable resource alternative for fossil fuels as well as input into other industrial processes. The environmental impacts of lignocellulosic biomass utilization for energy and other commodity products are quite significant and are arguably greater in scale and scope than any other class of energy resources. The majority of biomass is found in rural areas resulting from agriculture processes. Therefore, the bio-economy has the potential to provide much needed diversification of the rural economy. The biomass has been viewed as an alternative to energy; while it has potential to be used as input into various biotechnological processes for production of various consumer products. Production of value-added by-products serves to expand a bio-based economy or sustainable development. A greater reliance on bio-based resources and biological processes is an envitable part of an overall sustainability transtition, and thus main questions of technical innovation and policy development. In many developing countries, biomass is currently a significant source of energy and materials only for local and traditional uses. The biomass is generally used inefficiently with very few higher-value-added product markets. Bio-based renewable resources can provide raw materials for many new and growing biotechnological industries, while also stimulating rural development, job creation, and GHG reduction.Lignocellulosic biomass has been projected to be one of the main resources for economically attractive bioethanol production. Though theoretical ethanol yields from sugar and starch (g ethanol/ g substrate) are higher than from lignocellulose, these conventional sources are insufficient for worldwide bioethanol production. In that aspect agricultural wastes are renewable, less costly and abundantly available in nature. Agricultural wastes do not demand

separate land, water, and energy requirements. They do not have food value as well. For economically feasible bioethanol production, several hindrances are to be overcome. These refer to the four major aspects which are feedstock, conversion technology, hydrolysis process, and fermentation configuration. With regard to feedstock major obstacles are cost, supply, harvesting, and handling. As regards conversion technology the hindrances are biomass processing, proper and cost effective pretreatment technology to liberate cellulose and hemicellulose from their complex with lignin.

Fig.1 **Overview of the conversion process of lignocellulosic biomass**

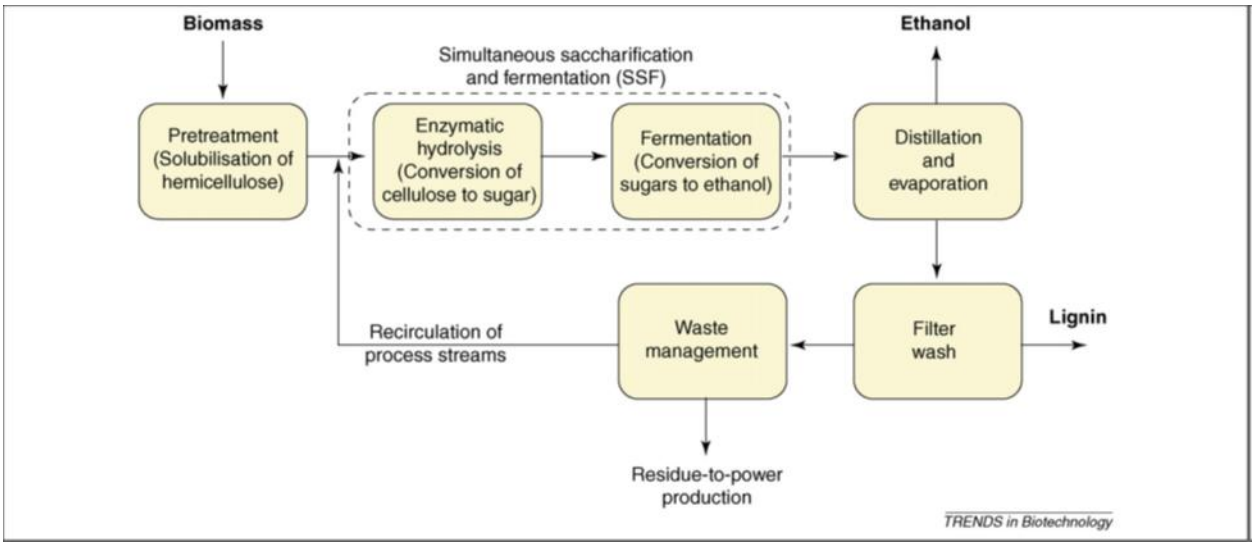

Table 1 Quantities of agricultural waste (million tons) reportedly available for bioethanol production

Agrowaste	Africa	Asia	Europe	America
Rice straw	20.9	667.6	3.9	37.2
Wheat straw	5.34	145.20	132.59	62.64
Corn straw	0.00	33.90	28.61	140.86
Bagasse	11.73	74.88	0.01	87.62

Table no 2. Composition of most common agricultural wastes

Substrate	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Ash (%)
Rice Straw	32-47	19-27	5-24	12.4
Wheat Straw	35-45	20-30	8-15	10.1
Maize straw	42.6	21.3	8.25	4.3
Bagasse	65 (Total carbohydrate)	-	18.4	2.4
Sorghum straw	27	25	11	-
Oat straw	31-48	-	16-19	4-6.5
Barley straw	31–45	27–38	14-19	2-7

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