Bioconversion of Cellulosic Materials by the action of Microbial Cellulases

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***Abstract-** Lignocellulose comprises more than 60 % of plant biomass produced on earth, which mainly consists of three components: cellulose, hemicellulose and lignin. On worldwide basis, land plants produce about 25 tones of cellulose per person per year. Lignocellulose constitutes a large proportion of agricultural, industrial, municipal and forest wastes, the disposal of which is a growing problem. In India, the output of agricultural lignocellulosic waste alone i.e. wheat straw, rice straw, biogasse and cotton stalks is estimated over 100 million tons per year. Recycling of these wastes would not only aid pollution abatement but would also serve as vital source of bio energy and food for future. Moreover, oil and gas resources are depleting every day at alarming rate. Thus, it is important to overcome these problems. Utilization of cellulose is possible through its hydrolysis to glucose and other soluble sugars that in turn can be used as substrates for production of single cell protein, energy materials or other fermentation materials. A variety of microbial species e. g. bacteria, actinomycetes and fungi are known to produce enzyme systems for efficient biodegradation of cellulose and will ultimately providing means to a 'greener technology'.*

Key words: Cellulose, Cellulase enzyme, bioconversion, biomass.

1. Introduction

Current status of energy and environment are large obstacles to the development of human civilization. The world's energy demand is increasing steadily with the increase in human population, industrialisation and economic development. This energy demand is fulfilled mainly with fossil fuel, which is in limited supply. In addition, use of fossil fuel accelerated release of CO_2, the global greenhouse effect becomes more and more serious. Therefore, continuous efforts have to be emphasized towards the solution of the energy supply depletion problem and the environmental impacts caused due to fossil fuel. It has generated worldwide interest in alternative sources of energy (Aristidou and Penttila, 2000; Jeffries and Jin, 2000; Zaldivar et al., 2001), such as agricultural biomass. The use of biomass for energy can complement solar, wind, and other intermittent energy resources in the renewable energy mix of the future, reduce fossil fuel green house gas emissions, and contribute to ecological sustainability.

One of the most immediate and important applications of biomass energy systems could be in the fermentation of ethanol from biomass (Lin and Tanaka, 2006). Each year, photosynthetic fixation of CO2 yields more than 1011 tons of dry plant material worldwide and almost half of this material consists of cellulose (Leschine, 1995). In addition, agronomic residues arised from human activities, such as corn Stover (corn cobs and stalks), sugarcane waste, wheat or rice straw, forestry, paper mill discards and the municipal wastes which have plentiful cellulose, can be converted into fuel ethanol. Now-a-days, nearly all fuel ethanol is produced by fermentation of starchy grains such as corn, wheat, barley and rice (Lin and Tanaka, 2006). But this technology consumes a lot of food materials and is costly, also under the pressure of world's food crisis at present; there is even a serious competition between human food and fuel ethanol. Thus, there is need to find an alternative sustainable way to solve this problem and make this approach sustainable. These days, research for fuel is mainly concentrated in the field of cellulase enzymes, which can hydrolyze cellulosic biomass present in industrial and agricultural wastes economically with high efficiency.
This review presents a general account of cellulose and cellulase enzyme as well as microorganisms producing cellulases, and focuses on their current status in research and gives insight into some promising prospects for its future.

Cellulose and Cellulase

Lignocellulose comprises more than 60 % of plant biomass produced on earth, which includes various agricultural residues (straws, hulls, stems, and stalks), deciduous and coniferous woods, municipal solid wastes, waste from the pulp and paper industry, and herbaceous energy crops, which mainly consists of three major components of the plant cell wall: cellulose, hemicellulose and lignin. The compositions of these materials vary. The major component is cellulose (35–50%), followed by hemicellulose (20–35%) and lignin (10–25%). Table1 gives the composition of some lignocellulosics. On worldwide basis, land plants produce about 25 tones of cellulose per person per year. Proteins, oils, and ash make up the remaining fraction of lignocellulosic biomass . The structure of these materials is very complex, and native biomass is generally resistant to enzymatic hydrolysis due to the composition and function of the plant cell wall, mainly conferring resistance to biotic and abiotic stress factors. In such situation, only complex microbial consortia have been shown to be able to completely degrade plant biomass. Cellulose is a fibrous, insoluble, crystalline polysaccharide. It is a major polysaccharide constituent of plant cell walls, composed of repeating D-glucose.

Table 1 Composition (%, dry basis) of some agricultural lignocellulosic biomass

Agricultural Residue	Cellulose	Hemicellulose	Lignin
Corn fiber	15	35	08
Corn cob	45	35	15
Corn Stover	40	25	17
Rice straw	35	25	12
Wheat straw	30	50	20
Sugarcane bagasse	40	24	25
Switch grass	45	30	12
Coastal Bermuda grass	25	35	06

* Badal C. Saha

units linked by-1,4-glucosidic bonds (Jagtap and Rao, 2005) and is the most abundant carbohydrate polymer on earth (Guo et al., 2008). Cellulose has attracted worldwide attention as renewable resource that can be converted into value added products and bioenergy. But now-a-days, enormous amounts of agricultural, industrial and municipal cellulose wastes have been accumulating or used inefficiently due to the high cost of their utilization processes (Kim et al., 2003). Therefore, it is of considerable economic interest to develop processes for the effective treatment and utilization of cellulosic wastes as cheap carbon sources. Cellulose is used as substrate by a wide variety of organisms including fungi, bacteria and plants as well as a wide range of invertebrate animals, such as insects, crustaceans, annelids, molluscs and nematodes (Watanabe and Tokuda, 2001; Davison and Blaxter, 2005). These organisms possess cellulases. The complete enzymatic system of cellulase includes three different types exo-β-1,4-glucanases (EC 3.2.1.91), endo-β-1,4-glucanases (EC3.2.1.4), and β-1,4-glucosidase (EC 3.2.1.21) (Wilson and Irwin, 1999). These enzymatic components act sequentially in a synergistic system to facilitate the breakdown of cellulose and the subsequent biological conversion to a utilizable energy source i.e. glucose (Beguin and Aubert, 1994). The endo-β-1,4-glucanases randomly hydrolyze the β -1,4 bonds in the cellulose molecule, and the exo-β-1,4-glucanases in most cases release a cellobiose unit showing a recurrent reaction from chain extremity. Lastly, the cellobiose is converted to glucose by β-1,4-glucosidase (Bhat and Bhat, 1997). This whole enzymatic process to hydrolyze cellulosic materials could be accomplished through a complex synergistic reaction of these enzymatic components in an optimum proportion (Tomme et al., 1995). Cellulases provide a key opportunity for achieving tremendous benefits of biomass utilization (Wen et al.,2005). The overall convenience of this enzyme-based bioconversion technology depends on two significant factors, reaction conditions and production cost of the enzyme system. Therefore, there has been much research aimed at obtaining new microorganisms producing cellulase enzymes with higher specific activities and greater efficiency (Subramaniyan and Prema, 2000).

Microorganisms producing cellulases: Cellulolytic microorganisms degrade primarily carbohydrates and are generally unable to use proteins or lipids as energy sources for growth. Cellulolytic microbes notably the bacteria Cellulomonas and Cytophaga and most fungi can utilize a variety of other carbohydrates in additions to cellulose, whereas the anaerobic cellulolytic species have a restricted carbohydrate range, limited to cellulose and or its hydrolytic products. The ability to secrete large amounts of extra cellular enzymes is characteristic of certain fungi and such strains are most suited for production of higher levels of extra cellular cellulases. One of the most extensively studied fungi is Trichoderma reesei, which converts native as well as derived cellulose to glucose. Most commonly studied cellulolytic organisms include fungi (Trichoderma, Humicola, Penicillium, Aspergillus), bacteria (Bacillus, Pseudomonas) and actinomycetes (Streptomyces, Actinomucor, Streptomyces).

Several fungi can metabolize cellulose as an energy source but only few strains are capable of secreting a cellulase enzyme complex, which could have practical application in the enzymatic hydrolysis of cellulose. Besides T. reesei, other fungi like Humicola, Penicillium and Aspergillus have the ability to yield high levels of extra cellular cellulases. Aerobic bacteria such as Cellulomonas, Cellovibrio and Cytophaga are capable of cellulose degradation in pure cultures. However, the microbes commercially exploited for cellulase preparation are mostly limited to T. reesei, H. insolens, A. niger, Thermospora fusca, Bacillus sp, and a few other organisms (Table 2).

Table 2: Major microorganisms employed for cellulase production

Major group	Genus	Species
Fungi*	Aspergillus	A. niger
		A. nidulans
		A. oryzae (recombinant)
	Fusarium	F. solani
		F. oxysporum
	Humicola	H. insolens
		H. grisea
	Melanocarpus	M. albomyces
	Penicillium	P. brasilianum
		P. occitanis
		P. decumbans
	Trichoderma	T. reesei
		T. longibrachiatum
		T. harzianum
Bacteria*	Acidothermus	A. cellulolyticus
	Bacillus	Bacillus sp
		B. subtilis
	Clostridium	C. acetobutylicum
		C. athermocellum
	Pseudomonas	P. cellulose
	Rhodothermus	R.marinus
Actinomycetes*	Cellulomonas	C. fimi
		C. bioazotea
		C. uda
	Streptomyces	S. drozdowiczii
		S. sp
		S. lividans
	Thermononospora	T. fusca
		T. curvata

*Rajeev K Sukumaran, 2005

Research status of cellulase enzyme: Cellulase is an important enzyme for carrying out the depolymerization of cellulose into fermentable sugars. Cellulose is the major source for energy and raw materials and cellulase is widely used in the bioconversion of renewable cellulosic biomass. Glucose generated from depolymerization of cellulosic biomass, also with the help of biotechnology, can be used in production of value added products such as fuel ethanol, single cell protein, feed stock and important chemicals for food and chemical industry (Gawande and Kamat, 1999; Fujita et al., 2002; Lynd et al., 2002). The importance of this enzyme in so many products has raised considerable interest and research efforts are being currently focused on understanding the reaction mechanism and industrial application of cellulase, which began in the early 1950s. Nature has evolved a number of cellulases for the hydrolysis of cellulose, including exoglucanase enzymes that depolymerize cellulose from the ends progressively, and endoglucanase enzymes that cleave along the cellulose chains randomly (Warren, 1996; Coughlan, 1985; Wilson and Irwin, 1999). Prior studies for natural cellulose hydrolysis have revealed many cellulolytic microorganisms and their complex cellulases (Lowe et al., 1987; Lynd et al., 2005). A number of fungi and bacteria capable of utilizing cellulose as a carbon source have been identified (Kim et al., 2003).

Among the cellulolytic fungi, Trichoderma reesei has the strongest cellulose-degrading activity, and its cellulase has been widely investigated (Penttila et al.,1986; Tomme et al., 1988). Cellulases produced by other fungi such as the Aspergillus and Rhizopus species have also been extensively studied by several researchers (Murashima et al., 2002; Saito et al., 2003). Interestingly, recent works confirmed the production of cellulases from insect for themselves (Watanabe et al., 1998; Girard and Jouanin, 1999; Tokuda et al., 1999) along with reports on the production from symbiotic organisms harboring in the insect gut and both (Ohtoko et al., 2000; Scharf et al., 2003). These new findings challenged the traditional view of cellulase activity that cellulose digestion in insects was mediated by microbial cellulase activity in their gut. Current understanding of the modes of catalysis and the role of various structural domains has been gained from protein engineering, X-ray crystallography and fluorescence studies, which evaluate cellulase–cellulose interactions in bulk (Pilz et al., 1990; Chapon et al., 2001; Violot et al., 2005; Pinto et al., 2007). To date, a battery of cellulase genes have been found and their gene structures and functions have also been studied (Mae et al., 1995; Murray et al., 2003; Lee et al., 2005). The purification and properties of cellulases have been described in many papers (Whitaker, 1951; Kanda et al., 1976; Churilova et al., 1980; Anzai et al., 1984; Mori, 1992; Yeet al., 2001; Kim et al., 2005; Li et al., 2005; Ogura et al., 2006; Lee et al., 2008; Thongekkaew et al., 2008). These studies have paved the way scientists to further understand the molecular mechanisms of cellulase at the fundamental scale of cellulose, expand their understanding of micro scale heterogeneous kinetics and identify essential amino acids in the catalytic site of various enzymes (Wilson and Irwin, 1999; Tomme et al., 1995; Henrissat et al., 1998; Jung et al., 2002; Jung et al., 2003; Zhang and Lynd, 2004). Although there have been many reports dealing with more efficient cellulose degrading enzyme from various organisms such as Trichoderma reesei, Trichoderma viride, Trichoderma lignorum, Chrysosporium lignorum, Chrysosporium pruinosum and Fusarium solani (Selby and Maitland, 1967; Toyama and Ogawa, 1975; Tong et al., 1980), only limited research has identified the yeast as cellulase producer (Oikawa et al., 1998; Hong et al., 2007). However in the last decade, the high production cost and low yields of this enzyme have been the major problems for industrial application. Therefore, investigations on ability of microbial strains to utilize inexpensive substrates (Griffin, 1973; Hurst et al., 1978; Liaw and Penner, 1990; Ju and Afolabi, 1999; Stenberg et al., 2000) and improvement of enzyme productivity (Kumakura et al., 1984; Chadha and Garcha, 1992; Hayward et al., 2000; Bailey and Tahtiharju, 2003;Villena and Gutierrez-Correa, 2006) are being done. Though the cellulase enzyme cost has dropped due to improvements in expression vectors and on-site production (Barros and Thomson, 1987; Din et al., 1990; Sahasrabudhe and Ranjekar, 1990; Harkki et al., 1991; Okamoto et al., 1994; Kobayashi et al., 2003; Kashima and Udaka, 2004), there is still a necessity of engineering a new generation of cellulase cocktails that would further reduce cellulase cost.

2. Applications of Cellulases

Cellulases were initially investigated several decades back for the bioconversion of biomass which gave innovative way to research in industrial applications of the enzyme. It has become the third largest group of enzymes used in the industry since their introduction (Xia et al, 1999). They are used in the biostoning of denim garments for producing softness and the faded look of denim garments (Olson, 1990), in food industry for extraction and clarification of fruit and vegetable juices (Galante et al, 1998), in pulp and paper industry for biomechanical pulping for modification of the coarse mechanical pulp and hand sheet strength properties (Akhtar,1994), deinking of recycled fibres (Pere et al,1990) and for improving drainage and runnability of paper mills (Prasad et al, 1992). Perhaps the most important application currently being investigated actively is in the utilization of lignocellulosic wastes for the production of biofuels.

Apart from these common applications, cellulases are also employed in formulations for removal of industrial slime (van Zessen et al, 2003), in research for generation of protoplast (Wiatr, 1990), and for generation of antibacterial chitooligosaccharides, which could be used in food preservation (Liu et al, 2000), immuno-modulation (Tsai et al, 2000) and as a potent antitumor agent (Wuet et al, 2004).

3. Future prospects

Lignocellulose is the potential source of biofuels, biofertilizers, animal feed and chemicals, besides being the raw material for paper industry. Exploitation of this renewable resource needs either chemical or biological treatment of the material, and in the latter context cellulases have gained wide popularity over the past several decades. Research has shed light into the mechanisms of microbial cellulase production and has led to the development of technologies for production and applications of cellulose degrading enzymes. However, there is no single process, which is cost effective and efficient in the conversion of the natural lignocellulosic material for production of useful metabolites or biofuels. Use of the current commercial preparations of cellulase for bioconversion of lignocellulosic waste is economically not feasible.

The major goals for future cellulase research would be: (1) Reduction in the cost of cellulase production; and (2) Improving the performance of cellulases to make them more effective, so that less enzyme is needed (Qin et al, 2004). The former task may include such measures as optimizing growth conditions or processes, whereas the latter require directed efforts in protein engineering and microbial genetics to improve the properties of the enzymes.

Optimization of growth conditions and process has been attempted to a large extent in improving cellulase production. Many of the current commercial production technologies utilize submerged fermentation technology and employ hyper producing mutants (NREL research review). In spite of several efforts directed at generating hyper producers by directed evolution, the cost of enzymes has remained high (Philippidis, 1994). Alternative strategies thought of in cellulase production include mainly solid substrate fermentation on lignocellulosic biomass particularly by using host/substrate specific microorganisms. There are several reports on such use of filamentous fungi in production of optimal enzyme complex for the degradation of host lignocellulose (Pandey et al, 2001; Tengerdy et al, 2003; Awafo et al, 1996; Chahal et al, 1996).

Performance of enzyme complex on lignocellulosic material is best when these complexes are prepared with the same lignocellulosic material as the host/substrate in fermentation (Chahal et al, 1996; Szakacs et al, 1996). Another strategy is to use mixed culture in the production of enzyme. Several reports have shown that mixed culture gives improved production and enzyme complexes with better hydrolytic activity (Duenas et al, 1995; Krishna, 1999). Thus, SSF may be considered as a cost effective means for large scale production of cellulases which probably would de several fold cheaper compared to the current commercial preparations.

Cellulases are subjected to regulations by various factors and some of the cis-acting promoter elements have been characterized (Aro et al, 2005). Active research in this field has led to genetic improvement of cellulase production by various methods including over expressing cellulases from the cbh1 promoter of T. reesei (Durand et al, 1988) and generation of desired variation in the cellulase production profile of organism (Harkki et al, 1991).

4. Conclusions

More and more research is oriented in genetic manipulations of cellulase process design. Medium formulations have come to an age and the futures definitely requires controlled genetic interventions into the physiology of cellulase producers to improve production and thereby make the cellulase production process more cost effective.

Improvement in cellulase activities or imparting of desired features to enzymes by protein engineering are probably other areas where cellulase research has to advance. Active site modifications can be important through site directed mutagenesis and the mutant proteins can be used for understanding the mechanisms of action as well as for altering the substrate specificities or improving the activities. There are several reports of development in this direction. Meinke et al (Stockton et al, 1991) has generated a mutant enzyme with endoglucanase like features and improved activity by deleting C-terminal loop of Clostridium fimi CELB. Protein engineering has been successfully employed to improve the stability of Humicola cellulose in presence of detergents (Meinke et al, 1995), to improve the thermo stability of an alkaline, mesophilic endo-1,4-β-glucanase from alkaliphilic Bacillus sp (Otzen et al, 1999) and for altering pH profile of cellobiohydrolase(Ozawa et al, 2001) and more recently endoglucanase (Becker et al, 2001) from T. reesei. Such modifications affecting the enzyme properties may be beneficial in improving the overall performance of cellulases and a better understanding of their mode of action, which will enable better utilization of enzymes in biomass conversion. More basic research is needed to make designer enzymes suited for specific applications.

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