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Key Technologies for the Production of Lignocellulolytic Enzymes

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Abstract

Enzymatic hydrolysis of lignocellulosic materials, most abundant renewable resources on the earth, is a key approach to convert them into bioethanol. Lignocellulolytic enzymes of various microorganisms, particularly bacteria and fungi, play dandiest role for this bioconversion from the complex cellulose into monomeric sugars which are converted into bioethanol with the help of traditional fermentation tehchnology by the yeast, *Saccharomyces cerevisae*. But the high cost of celluloytic enzymes is the major hinderance for commercial applications of this technology. To acquaint ourselves with the latest trends for reducing the cost of lignocellulolytic enzymes, this paper reviews various possible approaches based on relevant researches.

Keywords: Lignocellulosic; Biomass; Bioethanol; Lignin-degrading enzyme

Introduction

For the last several years, constant increase in the global population, industrialisation and depletion of fossil fuels together with the growing concerns over greenhouse gas emissions have shifted the worldwide focus for sustainable and environment-friendly resources as a potential alternative for the traditional fuels. In this context, biofuels, particularly bioethanol, are receiving increasing attention as alternative renewable energy sources. Production of bioethanol is increasing every year because of its use not only as biofuel but also in medicines, cosmetics and industrial materials (Sanchez and Cardona, 2008). In the United states, ethanol is already mixed with gasoline at a level of 10% and support from vehicle manufacturers has resulted in vehicles that can use upto 85% ethanol-15% gasoline mixture (Redding *et al.,* 2011). In India,the Ethanol Blended Program (EBP) was initiated in 2003, mandated blending of 5% ethanol in four union territories (out of seven) and in nine states (out of 28), the target was later extended to 20 states from November 2006 (Sorda *et al.,* 2010). However, in 2008, Indian

government has mandated 10% blending of ethanol with petrol which amounted to about 1100 million liters of ethanol use (Ravindranath *et al.,* 2011).Owing to these facts, bioethanol has become the most produced biofuel worldwide with almost 74 billion litres in 2009, European Union (EU) ranking third behind United States (54%) and Brazil (34%) with a production of 3.7 billion litres (Soccol *et al.,* 2011). Indian distilleries use molasses as the feedstock for ethanol production and the annual supply of molasses is suffi-cient only for producing approximately 2.7 billion litres of ethanol (Sukumaran *et al.,* 2010).

Although the current industrial production of bioethanol mainly relies on sucrose- and starch- containing raw materials, which may not be desirable due to their significant impact on food prices and food security (Naik *et al.,* 2010). These limitations can be addressed by producing ethanol from lignocellulosic materials (2nd generation biofuels) such as agricultural and forest waste residues (McIntosh and Vancov, 2011) which serve as a low cost feedstock for the production of bioethanol and other value added products (Sindhu *et al.,* 2014). It has been estimated that 442 billion liters of bioethanol can be produced from lignocellulosic biomass and that total crop residues and wasted crops can produce 491 billion liters of bioethanol per year, about 16 times higher than the actual world bioethanol production (Kim and Dale, 2004). In addition, conversion of agricultural wastes reduces their environmental ill-impacts by efficiently using them to produce secondary energy (Liu *et al.,* 2012).

Lignocellulose consists primarily of plant cell wall materials; it is a complicated natural composite with three major constituents, *i.e.*, cellulose, hemicellulose and lignin (Wu *et al.,* 2011). The key step in the utilization of cellulose for bioethanol production is its enzymatic hydrolysis into monomeric sugars and subsequent conversion into valuable chemicals and energy (Gupta *et al.,* 2011). Because of the recalcitrant crystalline nature of cellulosic fibers, significantly larger amounts of enzymes are needed during cellulose hydrolysis than in the process of starch hydrolysis where amylases were utilized in the production of first-generation bioethanol (Merino and Cherry, 2007). The cellulose utilizing population includes aerobic and anaerobic mesophilic bacteria, filamentous fungi, thermophilic and alkaliphilic bacteria, actinomycetes and certain protozoans (Alexander, 1961). All or some of these microorganisms need to be optimised for the efficient production of cellulases so that bioethanol could be produced cost effectively. This critique also deals with the concurrent research in this field.

Meterial and Methods

Related material, reports and research papers were searched from the Sciencedirect and Google by modifying the search topics.

Lignocellulose Degrading Enzymes

Cellulases

The complete enzymatic degradation of cellulosic substrates depends upon the production of a complex enzyme system. Following three groups of enzymes may be

distinguished in this respect: consisting of endoglucanases (EG) (1,4- -D-glucano-hydrolase; EC 3.2.1.4), exocellobiohydrolase (CBH) (1,4- -D-glucan glucohydrolase; EC 3.2.1.74) and -glucosidase (BGL) (-D-glucoside glucohydrolase; EC 3.2.1.21) (Esterbauer *et al.,* 1991; Bhatt, 2000; Krogh *et al.,* 2009; Elyas *et al.,* 2010; Swiatek *et al.,* 2014). Cellulases are highly specific enzymes. The entire process of cellulosic hydrolysis proceeds in three successive stages: adsorption of cellulases on celluloseøs surface, hydrolysis of cellulose to glucose, and desorption of cellulases on substrateøs surface (El-Zawawy *et al.,* 2011). Endoglucanases produce nicks in the cellulose polymer exposing reducing and non-reducing ends, cellobiohydrolases acts upon these reducing and non-reducing ends to liberate cello-oligosaccharides and cellobiose units, and -glucosidases cleaves the cellobiose to liberate glucose, thereby completing the hydrolysis (Sukumaran *et al.,* 2005). Accumulation of cellobiose inhibits both endoglucanases and exoglucanases thus limiting the saccharification of cellulose (Yue *et al.,* 2009).

Filamentous fungi are the major sources of commercial cellulases. Although *Trichoderma, Aspergillus*, *Penicillium* and *Fusarium* species are commonly used for cellulases production (Iqbal *et al.,* 2011) but cellulolytic fungi belonging to the genera *Trichoderma* (*T. viride, T. longibrachiatum, T. Reesei, T. atroviridae*) have long been considered the most productive and powerful destroyers of crystalline cellulose (Margeot *et al.,* 2009; Fang *et al*., 2010). However, *Aspergillus* is also known to be a good producer of cellulases (Sherief *et al*., 2010a) and are the most efficient producer of -glucosidase compared with *Trichoderma* spp. (Wen *et al*., 2005). Selections of desired fungal strains depend on several factors including selection of substrate for optimizing the cellulase producing conditions (Shazia *et al.,* 2007).

Xylanases

Xylan is the principal hemicellulose, consisting of 1,4- -linked D-xylose units substituted with different side groups such as l-arabinose, D-galactose, acetyl, feruloyl, p-coumaroyl and glucuronic acid residue (Kuhad *et al.,* 1997), which is the major component of plant cell wall, second plentiful polysaccharide after cellulose (Coughlan and Hazlewood, 1993), in both hard woods and annual plants (Qie *et al.,* 2010; Polizeli *et al.,* 2005) and accounts for 20ó35% of the total dry weight in tropical plant biomass. Xylanases is a group of enzymes that having properties such as tolerance to high pH and temperature, catalyze the hydrolysis of xylan (Coughlan and Hazelwood,1993). Endoxylanase [EC 3.2.1.8, endo- -(1,4)-xylanase] and endomannanase [EC 3.2.1.78, endo- -(1,4)-D-mannanase] represent enzymes degrading the main polysaccharide chain of xylan and glucomannan, respectively. The degradation of hemicellulose to short oligomeric compounds proceeds upon the action of such enzymes as: -xylosidase (EC 3.2.1.37), -mannosidase (EC 3.1.1.25), and -glucosidase (EC 3.2.1.21). Oligomers possess lateral groups that are successively removed by -glucuronisidase (EC 3.2.1.139), -arabinosidase (EC 3.2.1.55), and -D-glucosidase (EC 3.2.1.22) (Collins *et al.,* 2005). Acetyl substituents are successively removed by esterases (Palonen, 2004).

However, fungi are the most common sources of xylanases which can produce thermophilic enzymes ranging from 40^0C to 60^0C (Latif *et al.,* 2006) but filamentous fungi, mainly *Aspergillus* and *Trichoderma* spp., are gaining importance as producers of xylanase than yeast and bacteria from the industrial point of view due to non-pathogenic nature, ease in cultivation under fermentation conditions and capable of producing high levels of extracellular enzymes (Kar *et al.,* 2006). Also, wheat straw, rice straw and corn stover are good inducers of acidophilic xylanolytic enzymes (Liao *et al.,* 2012). Xylanase production by a newly isolated *Streptomyces sp.* RCK-2010 was optimized for varying culture conditions following one factor at a time (OFAT) and response surface methodology (RSM) approaches. Among various carbon sources tested, the actinomycete secreted higher level of xylanase on wheat bran and the enzyme was found to be most active at 60^0C and pH 6.0 and almost 40% stable after 4h at optimum temperature (Kumar *et al.,* 2012). Wheat bran holds the greatest promise for cost-effective production of the xylanase enzyme production by *Penicillium chrysogenum* PCL 501 (Okafor *et al.,* 2007). It was also found that crude xylanase produced by *Aspergillus carneus* M34 had a higher pH tolerance over a broad range of pH values and showed specificity for various agricultural wastes, especially that resulting from the use of coba husk (Fang *et al.,* 2007).

Ligninases

The enzymes commonly found in a lignolytic enzyme complex consist mainly of enzymes with lignin-degrading peroxidase activity (LiP; E.C.1.11.1.14), manganese peroxidase activity (MnP; E.C.1.11.1.13) and the lignin-degrading enzyme laccase activity (E.C.1.10.3.2). Some or all of these enzymes and their isozymes are produced by a number of wood-rotting fungi (Singh and Chen, 2008). White-rot fungi employ an array of extracellular hydrolases, that attack cellulose and hemicelluloses and secrete one or more of three extracellular enzymes, which are essential for lignin degradation, namely, lignin peroxidase, manganese-dependent peroxidase and laccase, and can fully mineralize all cell wall polymers in hardwood decay (Martinez *et al.,* 2005; Lundell *et al.,* 2010).

The white-rot basidiomycete, *Phanerochaete chrysosporium* is reported to have high lignolytic activity. This strain is considered to be a model strain for the development and understanding of the lignolytic enzyme production system because it can produce a more complete lignolytic enzyme complex than most other strains (Orth *et al.,* 1993).

Lignocellulolytic Enzyme Production Techniques

The fermentation process for enzyme production can be conducted either in a liquid medium, known as submerged fermentation (SmF), or a solid medium, the solid-state fermentation (SSF) (Table 1) (Farinas *et al.,* 2011). Submerged medium is usually selected for analytical purpose where rapid growth in liquid medium takes place due to equal distribution of nutrients for maximum fungal growth (Bisaria and Ghose, 1981). Approximately 90% of all industrial enzymes are produced by SmF, often using genetically modified microorganisms (Holker *et al.,* 2004). Lignocellulolytic fungi can be cultured upon different kinds of fermentative media,

both submerged and solid state, for the production of different lignocellulolytic enzymes (Gautam *et al.,* 2011).

Table 1: Fermentaion process/es for the production of various lignocellulolytic enzymes by different microorganisms on various lignocellulosic biomasses

Microorganism	**Substrate (s)**	**Method**	**Enzyme (s)**	**Reference**
Aspergillus niger and *Trichoderma viride*	Wheat bran	Submerged fermentation	Cellulolytic and Hemicellulolytic enzymes	Haq *et al.* (2006)
Aspergillus ellipticus	Wheat straw	Solid state fermen-tation	Cellulase	Acharya *et al.* (2010)
Aspergillus fumigatus	Rice straw and wheat bran	Solid state fermentation	Cellulase	Sherief *et al.* (2010b)
Aspergillus niger	Guar gum	Shake flask culture	-mannanase	Mohamad *et al*. (2011)
Aspergillus niger	Paddy straw	Submerged fermentation	Cellulase	Sakthi *et al.* (2011)
Aspergillus niger	Wheat bran	Solid state fermentation	Cellulase and Xylanase	Farinas *et al.* (2010)
Aspergillus niger	Saw dust	Submerged fermentation	Cellulase	Acharya *et al.* (2008)
Aspergillus niger KA-06 and *Chaetomium* sp. KC-06	Orange fruit Waste (peel and pulp)	Solid state fermentation	Cellulolytic and Pectinolytic enzymes	Yalemtesfa *et al.* (2010)
Aspergillus sp., *Penicillum* sp., *and Rhizopus* sp.	Lignocellulosic wastes	Submerged fermentation	Xylanase	Sridevi and Charya (2011)
Aspergillus terreus	Bagasse	Submerged fermentation	Endoglucanase	Youssef and Berekaa (2009)
Bacillus circulans D1	Sugarcane bagasse and grass	Submerged fermentation	Xylanase	Bocchini *et al.* (2005)
Fusarium solani F7	Wheat straw, rice straw and bran and wood husk	Solid state fermentation	Xylanase	Gupta *et al.* (2009)
Penicillium oxalicum GZ-2	Wheat straw and bran, rice straw and corn stover	Submerged fermentation	Xylanase	Liao *et al.* (2012)
Termitomyces clypeatus	Mustard stalk and straw	Submerged fermentation	Lignocellulolytic enzymes	Pal *et al.* (2013)
Thermoascus aurantiacus RBB1	Wheat bran	Solid state fermen-tation	Cellulolytic enzymes	Dave *et al.* (2013)
Trichoderma harzianum	Wheat straw	Solid state fermentation	Carboxymethyl Cellulase	Iqbal *et al.* (2010)

Solid state fermentation (SSF) has been defined as the bioprocess carried out in the absence, or near-absence of free water; however, the substrate must possess enough moisture to support the growth and metabolic activity of the microorganism (Chahal, 1985; Thomas *et al.,* 2013). On industrial scale, solid state medium is preferred where the bulk quantities of enzymes are required with low analytical grade quality (Lynd *et al.,* 2002). Mostly solid state fermentation was employed for enzymes production due to its numerous advantages such as high volumetric productivity, relatively higher concentration of the products, less effluent generation, requirement for simple fermentation equipment, lower capital investment and lower operating cost (Holker and Jurgen, 2005). In addition, SSF processes are more efficient than submerged fermentation in the utilization of agro-industrial residues, and therefore are a low-cost alternative for the production of various microbial products, including cellulolytic enzymes (Krishna, 2005; Mohana *et al.,* 2008), solid waste management and reduced energy requirements (Shah and Madamwar, 2005).

The filamentous fungi are considered to be strong cellulolytic enzymes secreting strains in a solid state fermentation (Bhargav *et al.,* 2008) since SSF resembles the natural habitat of the fungi (Hamidi-Esfahani *et al.,* 2004). This may allow them to produce certain enzymes and metabolites, which usually would not be produced or would only be produced at a low yield in SmF (Irbe *et al.,* 2014). In SSF, the fugal mycelia can spread more easily while in SmF they always aggregate (Li *et al.,* 2013).The remarkable differences of protein contents and enzyme activities between SSF and SmF might result from the different protein secretion abilities of the same strain cultivated in SSF and SmF. Cellulases produced in SSF show remarkable stability towards temperature, pH and metal ions (Singhania *et al.,* 2010), and even some enzymes not secreted to the medium in SmF are secreted in SSF (Barrios-González, 2012).

However, removal of metabolic heat during SSF is a challenge that could seriously hamper the processø productivity and potential for large-scale commercial operation (Rathbun and Shuler, 1983; Ghildyal *et al.,* 1994 and Pirota *et al*., 2013). Several bioreactor designs, including trays, packed beds, rotary drums and fluidized beds have been proposed to increase the oxygen transfer rate while limiting the deleterious effects of shear stresses on filamentous microorganisms, minimizing power consumption and circumvent the heat dissipation problem (Khanahmadi *et al.,* 2004).

Forced aeration improves growth and productivity of fungal cultures in SSF and also acts as a heat transfer fluid for temperature control and heat removal (Smits *et al.,* 1996). A 35L draft-tube airlift bioreactor equipped with a mechanical impeller was developed and validated to grow *Trichoderma reesei* RUT-C30 in a cellulose culture medium with lactose and lactobionic acid as fed batch. Cultures carried out without mechanical agitation resulted in higher volumetric enzyme productivity (200UL ^{1}h ^{1}), filter paper activity (17UmL ^{1}), carboxymethyl cellulase activity (11.8UmL ^{1}) and soluble proteins (3.2mgmL ^{1}) when compared to those with agitation (Ahamed and Vermette, 2010) as airlift bioreactors provide rapid mixing and also retaining narrower shear stress distribution.

Further Research and Development in Enzyme Production

High cost of the enzymes, low hydrolysis rate caused by product (sugar) inhibition and low productivity of the microorganisms (Ruan *et al.,* 2012) are some limiting factors in commercialization of cellulolytic enzymes production. In many bioconversion strategies, the cellulase required for biomass conversion may account for as much as 40% of the total process cost (Ahamed and Vermette, 2008). Efforts towards cost reduction have been directed at increasing enzyme production by finding/ engineering hyperactive microbial strains (Narasimha *et al.,* 2006), efficient fermentation techniques and recovery systems (Xu *et al.,* 2005).

As the proportions of cellulase enzyme components and associated enzymes are largely varying and are a function of the substrate used for growth (Pandey *et al.,* 2000), the cost of the culture media, especially carbon resource, has been estimated to be the major contribution to cellulase production cost. In this context, lignocellulose is envisaged to be the cheapest raw material and hence several lignocellulosic substrates such as wheat straw, bagasse, waste paper sludge, waste newsprint and aspen wood have been used as main component of media for enzyme production (Table 1). A newly isolated oleaginous fungus *Aspergillus tubingensis* TSIP9 exhibited satisfactory proportion of enzymes and lipid production in a solid state fermentation of palm pressed fiber and palm empty fruit bunch. The results also showed that the pretreatment of the substrate enhanced enzymes production by the fungi (Kitcha and Cheirsilp, 2014) as due to the close connection of cellulose and hemicellulose with lignin in the plant cell wall, pretreatment was found necessary to make these carbohydrates available for enzymatic hydrolysis and fermentation (Radeva *et al.,* 2012). A proficient pretreatment strategy includes: (1) disrupting and removing the cross-linked matrix of lignin and hemicelluloses that embeds the cellulose Łbers (2) disrupting hydrogen bonds in crystalline cellulose, and (3) increasing the porosity and surface area of cellulose for subsequent enzymatic hydrolysis (Li *et al*., 2010). Several physical (milling and grinding), physico-chemical (steam explosion/autohydrolysis, hydrothermolysis and wet oxidation), chemical (alkali, dilute acid, oxidizing agents and organic solvents) and biological processes (Balat, 2011) are currently employed to overcome the recalcitrance of lignocellulose so that the enzymatic hydrolytic efficiency could be enhanced leading to the improved yields of monomeric sugars.

A number of microorganisms particularly fungi possessing cellulose degrading enzymes have been isolated and studied extensively. But their cellulase system is, in general, deficient in -glucosidases, particularly in brown-rot fungi (Arantes *et al.,* 2012), causing accumulation of cellobiose, which results in repression and end product inhibition of the enzymes endoglucanase and exoglucanase (Ju and Afolabi, 1999; Adsul *et al.,* 2007). *Trichoderma* is one of the most important microorganisms used in industry which allows a relatively higher enzyme production of cellulases consisting mainly of cellobiohydrolases, endoglucanases and -glucosidases (Fang *et al.,* 2010). Corroboratively, Rosgaard *et al.* (2006) found that the commercial cellulase preparations from *Trichoderma reesei* were very popular because they contained high activities of both exoglucanase and endoglucanase, but low levels of -glucosidases.

Hence, the attention has been diverted to other organisms, which possess all the three essential components of cellulase system. A mixture of cellulolytic, hemicellulolytic, amylolytic and pectinolytic enzymes could be produced by *Aspergillus niger* NS-2 in solid state fermentation (SSF) and surface culture fermentation (SCF) using wheat bran as the substrate (Bansal *et al.,* 2011). A newly isolated strain with high level production of cellulase under SSF was identified as *A. fumigatus*, the optimal cultivation condition for cellulase production was 50^0C, 80% initial moisture, initial pH 4.0 and 7% initial inoculum. Cellulases secreted by the strain showed maximal activity at 50^0C. The optimal pH for the CMCase activity was 7.0 and pH stability was over a broad pH range. The crude enzymes secreted *by A. fumigatus Z5* were capable of hydrolyzing cellulose and produced considerable reducing sugars suggesting that the fingus *A. fumigatus Z5* had a great potential capacity in bioethanol production (Liu *et al.,* 2011).

Stimulation of enzyme activities by incorporation of synthetic and/or natural chemical compounds into the fermentation media is another key research direction, where some research groups have succeeded in enhancing activities by using Tween 80, Tween 20, Triton X-100 and polyethylene glycol (Alkasrawi *et al.,* 2003;). Such surfactants were found crucial in enhancing microbial growth in SSF by promoting the penetration of water into the solid-substrate matrix, leading to an increase of surface area (Singh *et al*., 1991; Asgher *et al*., 2006). The cellulase production by *Aspergillus terreus* was also enhanced by two-fold with the addition of calcium chloride (3mM) and magnesium sulfate (5mM) in the medium alongwith Tween 80 (2 mlL^{-1}) as surfactant (Shahriarinour *et al.,* 2011).

During the past four decades, a lot of efforts have also been devoted to obtain either mutagenetically modified (Xu *et al.,* 2011; Vu *et al.,* 2011) or genetically engineered strains capable of producing high levels of cellulase (Qin *et al.,* 2008). A mutant fungal strain, *Chaetomium cellulolyticum* NRRL 18756 was created by mutagenesis with Co 60 -rays radiation (Fawzi and Hamdy, 2011); under the deduced optimized medium and SSF conditions, the CMCase production of the mutant fungus was 80.0 U/g representing a 4.0-fold increase in CMCase production than that produced in wheat bran basal medium by the wild type strain (20.1 U/g). A mutant strain of *Trichoderma asperellum* RCK2011 was developed through UV-irradiation for enhanced cellulase production and lower catabolite repression (Raghuwanshi *et al.,* 2014). The mutant strain produced FPase (2.2 IU/gds), CMCase (13.2 IU/gds), and -glucosidase (9.2 IU/gds) under optimized conditions, which was 1.4, 1.3, 1.5-fold higher than the wild type. In another study, fungal strain was subjected to chemical mutations with Ethyl Methane Sulfonate (EMS) and the resultant mutant fungal culture, *Aspergillus niger* GNEM7 depicted significant enhancement in cellulase production using pea seed husk as substrate (Pradeep and Narasimha, 2011). Such improved strains could be able to reduce the cost of the processes with increased productivity and may also possess some specialized desirable characteristics (Karanam and Medicherla, 2008). A -mannanase gene (CsMan5A) from the fungus, *Chaetomium* sp. was cloned and expressed in *Pichia pastoris* under the control of AOX1 promoter for the first time. The enzyme expression levels were by far the highest obtained, thus making it highly cost-effective for commercial production. The enzymes exhibited high specific

activity, good thermal and pH stability as well as resistance to many metal ions (Katrolia *et al.,* 2012). The results of Zhong *et al.* (2009) and Silva *et al.* (2013) suggested that co-culture could be a potential technique for producing recombinant hydrolase cocktails at lower cost than those associated with the production of a single culture. In addition to that, combining both cellulase and -glucosidase in the same organism is a good strategy and has been used to improve enzymatic hydrolysis efficiency of lignocellulosic biomass for ethanol production in engineered microbes co-expressing endoglucanase and -glucosidase (Methew *et al.,* 2008)

Conclusion

Solid state fermentation is as attractive process to produce cellulases and hemicellulases economically. It is always affected by the nature of the used substrate. Therefore, choice of an appropriate substrate is important and use of cheap and easily available substrate as lignocellulasic waste will help to reduce the cost of lignocellulolytic enzymes production. Also, implications in understanding genetics of microorganisms producing cellulase enzymes will enable improved production processes which are critical to reduce the cost of biomass conversion.

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