CHAPTER 5

Production of Cellulolytic Enzymes

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Abstract

For the conversion of plant biomass into various bioproducts, a significant bottleneck is enzymatic hydrolysis of lignocelluloses to soluble sugars. These sugars are then metabolized through various natural or engineered pathways toward products of interest. The success of projected biorefinery processes depend to a large extent on the economics of hydrolytic enzyme production. Presently, mesophilic fungal strains

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like *Trichoderma reesei* and *Aspergillus niger* produce cellulases at an industrial scale. Various recombinant cellulases have been successfully expressed in industrial strains which can improve the economics due to their high specificities for targeted bioproducts. In addition, designer cellulosomes and xylanosomes are expected to make the hydrolytic enzymes more effective because of efficient surface binding and direct action on lignocelluloses. This chapter describes various genetic and process approaches which have been used to increase the production of cellulase and xylanase enzymes.

5.1 INTRODUCTION

With the growing human population, the world is facing tremendous pressure to meet its needs of food, feed, chemicals, and energy, and also to balance the demand and supply in keeping with environmental safeguards. The natural gas and oil fields are shrinking fast to meet the demands of our progress. Today, alternate energy sources from environmental wastes and energy crops are high priority research interests worldwide. For production of biocommodities from biomass, one of the major bottlenecks has been the efficient hydrolysis of lignocelluloses to sugars. Primarily, lignocelluloses comprise cellulose (~30 to 50%), hemicellulose (~20% to 35%), and lignin (~15% to 25%). This composition can vary amongst plant species and plant tissues. Even in the same species of corn, there is considerable variability in the composition of corn stover due to genetic background and environmental conditions (Templeton et al., 2009). The plant kingdom produces lignocellulosic biomass to the tune of 10–50 billion tons per annum worldwide (Sticklen, 2006). This renewable biomass can be hydrolyzed to pentose and hexose sugars that serve as building blocks for various industrial products (Chandrakant and Bisaria, 1998; Lynd et al., 2005; Bevan and Franssen, 2006; Bayer et al., 2007; Hatti-Kaul et al., 2007; Madhavan et al., 2012; Seiboth et al., 2012). For this, enzymatic methods to hydrolyze the plant biomass are preferred due to non-inhibitory by-products and nontoxic effluents.

Hydrolytic enzymes like cellulases and xylanases convert lignocelluloses to sugars that can be fermented by various microbes to biofuels and other value-added products. The relatively high cost of these enzymes remains a major barrier to their commercial application in any bioindustry, although significant reduction in the cost of these enzymes has been made in recent years. The focus areas of research have been to improve the efficiency of known enzymes, identify new and more active enzymes, find optimized enzyme mixes for pretreated lignocelluloses and reduce the cost of enzyme production (Merino and Cherry, 2007). Also, for an industrial process to be economically viable, enzymatic breakdown of lignocellulose to fermentable sugars must occur as quickly as possible, preferably in hours. This chapter describes various promising microbial sources of hydrolytic enzymes, and the genetic and process methods employed to produce the enzymes.

5.2 HYDROLYTIC ENZYMES FOR DIGESTION OF LIGNOCELLULOSES

5.2.1 Cellulases

Cellulase is the most prominent group of hydrolytic enzymes that catalyze the hydrolysis of β-1,4 linkages present in cellulose to give glucose. This hexose is fed into the central metabolic pathways of various microorganisms to produce various bioproducts. The cellulases are primarily produced in nature by plants, fungi, bacteria, and even some protozoa, mollusks, and nematodes (Watanabe and Tokuda, 2001). Presently, the common microbial sources of cellulases for industrial-scale production are mesophiles grown in the temperature range of 30–35°C, such as *Trichoderma reesei* (also known as *Hypocrea jecorina*) and *Aspergillus niger*. The enzymes from thermophiles grown in the temperature range of 50–90°C are advantageous for harsh industrial processes, have faster reaction rates and are less prone to contamination. A few examples of such promising microorganisms are *Clostridium thermocellum*, *Thermoanaerobacter* sp, and *Thermotoga maritima* (Gomes and Steiner, 2004). The enzymes from psychrophiles such as *Clostridium* strain PXYL1 (Akila and Chandra, 2003) and *Pseudoalteromonas haloplanktis* (Violot et al., 2003) which grow in the temperature range of 5–20°C have advantages in applications where temperature is detrimental to the product and also reduce the energy consumption. Hence, depending on the suitability of the desired product, an appropriate microbial producer of hydrolytic enzymes can be selected from a wide array available in nature.

Cellulases are multienzyme complexes that consist of three major components: (1) endo-β1-4-glucanases (endo-β1-4-D-glucan 4-glucanohydrolase, EC 3.2.1.4), (2) exo-β1-4-glucanase or cellobiohydrolase (exo-β1-4-D-glucan 4-cellobiohydrolase, EC 3.2.1.91), and (3) β-glucosidase (EC 3.2.1.21); some cellulase systems also contain exo-β1-4-D-glucan 4-glucohydrolase (EC 3.2.1.74) and exo-β1-4-cellobiosidase (EC 3.2.1.176). These three components act synergistically to hydrolyze cellulose to glucose. Endo-glucanase hydrolyzes internal β-1,4 linkages of cellulose chains and creates new reducing and nonreducing ends. Thereupon exoglucanase cleaves disaccharide cellobiose from the nonreducing end (cellobiohydrolase) and in some cases from the reducing end (cellobiosidase) of the cellulose chain. These cellobiose units and short-chain cellodextrins are hydrolyzed by β-glycosidase into individual monomeric units of glucose (Beguin and Aubert, 1994). Various forms of these enzymes are present in different species to digest diverse types of celluloses present in nature. Therefore, the cellulase activities are expressed in terms of the substrates used to quantify them; for example, hydrolysis of carboxymethyl cellulose is measured as carboxymethyl cellulase (CMCase), filter paper hydrolysis as filter paper cellulase units (FPU), Avicel cellulose digestion as Avicelase, cotton fiber hydrolysis as cotton cellulase, etc.

The best known producer of cellulases, *T. reesei*, has two exoglucanases, Cel6A and Cel7A. Cel6A digests cellulose chain from the nonreducing end and Cel7A from the reducing end. It is supported by 8 endoglucanses and 7 β-glycosidases in

the multienzyme cellulase system (Martinez et al., 2008). The genome sequence of hyperthermophile, *Caldicellulosiruptor bescii* and *Caldicellulosiruptor saccharolyticus*, reveals diverse glycoside hydrolase families. It has at least four endo- or exo-acting cellulases and a β-glycosidase with the ability to hydrolyze crystalline cellulose (Blumer-Schuette et al., 2010; Kanafusa-Shinkai et al., 2013). The diversity of cellulases in nature secreted by various microbes makes them versatile and capable of digesting almost all kinds of plant material made of cellulose.

Different mechanisms for digestion of cellulose are employed by various cellulase-producing microorganisms. Most aerobes have cellulases made up of carbohydrate-binding module (CBM), a flexible linker protein and catalytic domain. CBMs are responsible for increasing the interaction between cellulase and cellulose surface. Higher binding affinity of CBM has been shown to be responsible for higher cellulose conversion (Linder et al., 1995; Takashima et al., 2007). Soluble forms such as carboxymethyl cellulose and amorphous forms of cellulose do not require CBM for hydrolysis. The crystalline form of cellulose requires CBM of cellulases for its hydrolysis (Rabinovich, 2002). The cellulosomal cellulases consist of a dockerin domain that binds to cohesion modules of the scaffolding subunit. The dockerin–cohesin complex binds to enzyme by a flexible linker peptide to display catalytic domain. The scaffolding also has a CBM to recognize and bind to cellulosic substrates (Bayer et al., 2004).

5.2.2 Xylanases

For maximizing hydrolysis of lignocellulosic residues by cellulase enzymes, synergistic action of xylanase is required (Ghose and Bisaria, 1979). The cellulose fibers in plant biomass are held together by lignin and xylan. The major enzymes responsible for the hydrolysis of xylan are xylanase (endo-1,4-β-D-xylanohydrolase, EC 3.2.1.8) and β-xylosidase (1,4-β-D-xylohydrolase, EC 3.2.1.37). Xylanase cleaves internal β-1,4 linkages of the xylan backbone. β-Xylosidase hydrolyzes xylobiose and small xylooligosaccharides to xylose and facilitates the hydrolysis of xylan. A few additional debranching enzymes are also required for complete hydrolysis of xylans by removal of the side chain substituents from the xylan backbone. Often xylans are in partially acetylated form, which are cleaved by acetylxylan esterase (EC 3.1.1.72) (Tenkanen and Poutanen, 1992). Arabinosyl substituents are removed by α-L-arabinofuranosidase (EC 3.2.1.55), which results in an increase in the number of potential access points of xylanase to the xylan backbone (Dekker, 1985). 4-*O*-Methyl glucuronic acid side groups, which are among the major substituents of wood xylans, form ester linkages between uronic acid residues and lignin. The debranching enzyme α-glucuronidase acts synergistically with xylanases to digest glucuronoxylan and releases 4-*O*-methyl glucuronic acid (Puls et al., 1987). Thus, xylanase and various debranching enzymes act together to breakdown xylan to primarily pentose sugars while improving accessibility of cellulases to hydrolyze cellulose fibers.

Xylanases are synthesized by fungi, bacteria, marine algae, insects, seeds, etc. but the principal source of commercial xylanases is filamentous fungi. The fungi secrete xylanase into the extracellular medium along with several accessory xylanolytic

enzymes for debranching substituted xylans. There is considerable interest in the enzymes of xylan hydrolysis for their use as a supplement in animal feed, manufacture of bread, food, textiles, pulp bleaching and in the production of ethanol and xylitol (Polizeli et al., 2005). For commercial production of these enzymes, the focus is on utilization of agro-residual wastes along with development of efficient bioprocess strategies to obtain high-enzyme titers.

5.3 DESIRABLE ATTRIBUTES OF CELLULASE FOR HYDROLYSIS OF CELLULOSE

The hydrolytic enzymes which are to be produced should be of desirable characteristics for their application in the hydrolysis of lignocelluloses. Some of the desirable characteristics of the enzymes are (1) catalytic efficiency, (2) thermal stability, (3) adsorption, (4) end-product inhibition resistance, and (5) shear inactivation (Kloysov, 1988; Bisaria, 1998). The catalytic efficiency, for example, of one of the most active endoglucanases from *T. reesei* on crystalline cellulose is on the order of 0.051 per second. Glucoamylase, on the other hand, has catalytic efficiency of 58 per second which is nearly the same as shown by endoglucanase against soluble cellulose, CMC. This indicates that the catalytic efficiency is low for crystalline cellulose owing to the inherent features of cellulose. Therefore, crystalline cellulose is normally subjected to a pretreatment process for efficient cellulose hydrolysis. The desirable attributes of cellulase for efficient hydrolysis of cellulose and the impact of these attributes on cellulose hydrolysis are listed in Table 5.1. A review of cellulase properties produced by *T. reesei* indicates that they also have the following disadvantages under the actual operating conditions of cellulose hydrolysis.

(a) Low thermostability which requires replenishment of lost activity at prolonged usage.
(b) High inhibition which results in cessation of hydrolysis at increased concentration of glucose and cellobiose.
(c) Inactivation by shear due to mixing by impellers in cellulose hydrolysis reactors.
(d) Low adsorption of some cellulase components on crystalline cellulose, which may result in their washing away in a flow-through reactor during continuous hydrolysis.

In view of the projected large-scale usage of cellulase enzymes in bioconversion of lignocellulosic residues, there is a continuous renewed interest in search of novel sources of cellulases which produce these enzymes with desired novel properties that cause rapid hydrolysis of cellulose. Several research groups, including those from National Renewable Energy Laboratory (NREL), Genencor, Novozymes, etc., have reported improvements in one or more of these properties, especially with respect to thermal stability (Mousdale, 2010). It is pertinent to mention here that such an

TABLE 5.1 Desirable Attributes of Cellulase for Efficient Hydrolysis of Cellulose

Attributes	Impact
1. High specific activity	Enzymes with higher specific activity (units/mg protein) will be required in lower quantities to achieve the same degree of hydrolysis.
2. High catalytic efficiency against crystalline cellulose	Cellulases with high catalytic efficiency will reduce the time required to hydrolyze crystalline cellulose.
3. High thermostability	Thermostable enzymes can be used at higher temperatures to accelerate the rates of cellulose hydrolysis.
4. Resistance to end-product inhibition	Most cellulases are inhibited by the products of cellulose hydrolysis (i.e, glucose and cellobiose). End-product inhibition resistant cellulases can produce more concentrated mixtures of sugars, which will obviate the need to concentrate them before bioconversion into bioproducts such as ethanol.
5. Stability against shear forces	Shear-resistant cellulases will be useful where agitation must be provided to suspend the solid cellulose in a hydrolysis reactor.

improvement is desirable in view of the fact that hydrolysis rate doubles with 10°C increase in reaction temperature.

5.4 STRATEGIES USED FOR ENHANCED ENZYME PRODUCTION

5.4.1 Genetic Methods

Strain improvement by random mutagenesis is often limited by the microbe's genetic makeup. To overcome such barriers toward developing a workhorse strain, various genetic tools are used with good success. Thus, a tailored enzyme for a specific need of a bioprocess can be designed through genetic engineering of the strain. Various industrial microbial strains like *T. reesei*, *A. niger*, *Pichia pastoris*, *Saccharomyces cerevisae*, *Escherichia coli,* and *Bacillus subtilis* have been genetically modified to express heterologous genes of commercial significance. A number of genetic approaches have been used over the last two decades to overexpress cellulase and xylanase genes. We have not made an attempt to cover all those procedures in this review. Nevertheless, a few recent approaches have been described in the following paragraphs which are indicative of the trend of research to overexpress the enzymes in suitable hosts under appropriate genetic controls.

In case of cellulase enzymes, endoglucanases from *Aureobasidium pullulans* ApCel5A, *Gloeophyllum trabeum* GtCel2A, and *Sporotrichum thermophile* StCel5A were expressed at relatively high levels ($\geq$0.3 g/L) in *A. niger*. ApCel5A and StCel5A

hydrolyzed CMC five- and two-times faster, respectively, than *T. reesei* endoglucanase Cel5A. These recombinant strains can, therefore, be promising candidates for developing new cellulase system (Tambor et al. 2012). Again, a Cel7A exoglucanase from *T. reesei* expressed in *A. niger* was able to grow on spent bagasse hydrolysate and spent spruce hydrolysate with higher endoglucanase activity than *T. reesei*. This was done to overcome glucose repression in *T. reesei* strain; the cellulase gene in *A. niger* was under constitutive promoter (Alriksson et al., 2009). The post-translation glycosylation of proteins is known to influence the stability, conformation, secretion, and activity of enzymes (Lis and Sharon, 1993). *Trichoderma reesei* cellulase proteins are known to undergo hyper-glycosylation when expressed in strains such as *A. niger*, *S. cerevisiae,* and *Yarrowia lipolitica*. Recent studies revealed that removal of *N*-glycosylation site via alanine mutation from asparagine-270 slightly increased its activity on crystalline cellulose. In another fungus *Penicillium funiculosum*, removal of three *N*-glycan sites from rCel7A increased enzyme activity by 35%, whereas addition of *N*-glycan at asparagine-194 via mutation of alanine-196 to serine (to create an N-X-S motif) enhanced activity by 85% in *A. niger* (Adney et al., 2009).

Genome sequence of *T. reesei* is now available (Martinez et al., 2008). The cellulase genes are known to be regulated in a coordinated manner. The presence of at least three transcriptional activators, viz. XYR1, ACE 2, and HAH2/3/5 complex; and two repressors, viz. CRE1 and ACE1 has been reported (Seiboth et al., 2012). Up- and down-regulation of certain genes has been shown to result in overexpression of cellulase and xylanase genes. In one of the highest producers of cellulases, viz. *T. reesei* RUT C30, upregulation of *xyr1* gene under a strong *pdc* promoter and downregulation of negative regulator *ace1* increased total protein secretion, filter paper activity, and CMCase activity by 103%, 114%, and 134% respectively (Wang et al., 2013). The *ace1* repressor gene was knocked out in cellulase expression system of *Trichoderma koningii* YC01by ribonucleic acid interference (RNAi) method. The *ace1*-silenced strains had improved cellulase and xylanase production under inducing condition. After 5 days of cultivation, the expression levels of *cbh1*, *cbh2*, *egl1*, *egl2*, and *xyn2* in strain A553 of *T. koningii* were 1.9-, 2.0-, 0.6-, 1.8-, and 1.5-times higher, respectively, than the control strain (Wang et al., 2012). The subject of cellulase regulation in *T. reesei* and its manipulation through genetic engineering approaches have been elaborately discussed recently (Seiboth et al., 2012).

Suitable codon optimization of target genes can also be used to enhance the expression of heterologous protein production. Thus, cellobiohydrolase 1, cellobiohydrolase 2, and β-mannanase from *T. reesei* and xylanase A from *Thermomyces lanuginosus* were individually expressed after codon optimization and under synthetic alcohol oxidase 1 promoter in *P. pastoris*. Such recombinant *P. pastoris* expressed 1.142 g/L of β-mannanase, 6.55 g/L of cellobiohydrolase 2, and 1.2 g/L of xylanase A in fed-batch cultures (Mellitzer et al., 2012). A hyperthermophilic β-glycosidase (CelB) from *Pyrococcus furiosus* was expressed in *Lactobacillus plantarum* NC8 and *Lactobacillus casei* using pSIP system. The specific activity of CelB was 44% higher in *L. plantarum* than in *L. casei*. On cultivation of *L. plantarum* in a bioreactor using whey medium, 33.4 U/mg protein of CelB activity was obtained (Böhmer et al., 2012).

Typically, in aerobic bacteria, cellulolytic enzymes are produced in "free" form and work synergistically during cellulose hydrolysis. In contrast, several anaerobic bacteria have been found to produce a cell-associated enzyme complex called the cellulosome (Bayer et al., 2007). In addition, a few anaerobic fungi, such as *Orpinomyces* and *Piromyces*, have been reported to produce cellulosome (Doi and Kosugi, 2004). Recently, a facultative bacterium *Paenibacillus curdlanolyticus* B-6 was found to produce cellulosome-like multienzyme complexes under aerobic conditions (Waeonukul et al., 2009). Whether cellulase is in monomeric form or displayed on scaffolding protein in cellulosomes, it acts synergistically to attack complex polymeric substrates. This synergy can be achieved between cellulases from different microbial systems, between cellulosomal and noncellulosomal enzymes, between different types of enzymes from different families, and between enzymes that have different modes of action. For example, β-glucosidase (Gluc1C) and β-1,4-endoglucanase (Endo5A) from *Paenibacillus* sp. (isolated from the gut of cotton bollworm) were used to construct and express a bifunctional chimeric protein in *E. coli*. It was found that one of the several constructs, namely, EG5 generated 24% more sugar from alkali-treated rice straw than Endo5A or a mixture of Endo5A–Gluc1C enzymes. The chimeric protein EG5 is reported to have enhanced thermostability, specific activity, substrate affinity, and catalytic efficiency (Adlakha et al., 2012). Similarly, a synergy between different glycoside hydrolases of *Thermobifida fusca* was found. A designer cellulosome was constructed using two *T. fusca* cellulases, Cel48A exoglucanase and Cel5A endoglucanase, and two *T. fusca* xylanases, endoxylanases Xyn10B, and Xyn11A. The final tetravalent cellulosome assembly had ∼2.4-fold enhanced activity on wheat straw, a complex cellulosic substrate (Moraïs et al., 2010). An earlier work showed that desired endoglucanase along with scaffolding protein can be expressed in a host of choice and produce designer mini cellulosomes with specific functions. Endoglucanase Eng B and scaffolding protein mini-CbpA1 from *Clostridium cellulovorans* were expressed and produced as designer cellulosomes in *B. subtilis* WB800 (Cho et al., 2004). Similarly, noncellulosomal cell surface display of hydrolytic enzymes using anchoring proteins can act as whole cell biocatalysts in biomass saccharification. For example, CBH2 (cellobiohydrolase 2) and EG2 (endoglucanase 2) from *T. reesei* and BGL1 (β-glucosidase 1) from *Aspergillus aculeatus* were integrated to C-terminal of cell-surface protein α-agglutinin of *S. cerevisae*. It was observed that increasing the copy number of *CBH2* increased saccharification efficiency by 1.7-fold and increasing the copy number of both *CBH2* and *EG2* improved saccharification efficiency by 2.5-fold of the resulting yeast strain (Matano et al., 2012). Such designer cellulosomes and whole cell biocatalysts may change complicated bioprocesses to cost-effective consolidated bioprocesses in future.

Efforts to overexpress xylanase in the industrial strain of *S. cerevisae* have been made by various groups. For example, endo-β-1,4 xylanase of *A. niger* IME-216 was expressed with 1.5-fold higher yield in *S. cerevisiae* strain YS2-2 compared to the parent (Tian et al., 2013). Endoxylanase from *Geobacillus thermodentrificans* TSAA1 was expressed in *E. coli* BL21 (DE3). It was characterized as thermostable at 70°C and alkalistable at pH 9.0 suggesting suitable application in various industrial processes (Verma et al., 2013). As thermostable enzymes have improved hydrolytic

performance, many potential candidates are being studied for their expression and production of xylanases. Thermophilic and hyperthermophilic xylan-degrading organisms possess a homolog of Xyn10A, a multi-modular endoxylanase. The Xyn10A module is flanked on the N-terminus by a tandem repeat of carbohydrate binding module 22 (CBM 22) and on C-terminus by another tandem repeat of CBM 9 and repeats of surface layer homology (SLH) modules. It was found that N-terminal CBM 22 was critical for imparting thermostability and thermophilicity to xylanases like TsXynA from *Thermoanaerobacterium saccharolyticum* (Lee et al., 1993), TmXynA from *T. maritima* (Meissner et al., 2000) and PbXynC from *Paenibacillus barcinonensis* (Blanco et al., 1999). Partial genome analysis of thermophilic bacterium *Caldanaerobius polysaccharolyticus* predicted that Xyn10A is anchored to the surface of the bacterium. It had two families of CBM 22 at N-terminus and two families of CBM 9 and three SLH modules at C-terminus (Han et al., 2012).

The alcohol oxidase, AOX1, in *P. pastoris* has a strong inducible promoter and therefore is a promoter of choice by most researchers for the expression of xylanase genes in *P. pastoris.* It gives high levels of xylanase expression under methanol induction. Ruanglek et al. (2007) reported an enzyme activity of 3676 U/mL and volumetric productivity of 22,832 U/L · h with methanol induction of the gene product of *xyl*B from *A. niger* when expressed under AOX1 promoter in *P. pastoris*. Similarly, a recombinant thermostable xylanase A from *Bacillus halodurans* C-125 was expressed in *P. pastoris*. On induction by methanol, a very high xylanase activity of 3361 U/mL was achieved in 132 hours (Lin et al., 2013).

Marine microorganisms are potential sources of enzymes which possess unique characteristics such as salt tolerance, hyperthermostability, and cold adaptation (Trincone, 2011). Ribosome engineering was used to introduce mutation in gene *rpsL* (encoding ribosomal protein S12) in marine *Streptomyces viridochromogenes* which produces thermostable xylanase. A mutant M11-1(10) of *S. viridochromogenes* strain M11 selected on streptomycin-containing plates showed 14% higher xylanase activities than that of the wild-type strain. Ribosomal protein S12 plays an essential role in the decoding function and is involved in both inspection of codon–anticodon pairings and response to streptomycin (Liu et al., 2013). An interesting recombinant strain of *Chlamydomonas reinhardtii*, a freshwater, green microalga, was constructed to hyper express xylanase gene *xyn1* from industrial strain of *T. reesei*. The ble-2A is a nuclear expression vector, resistant for zeocin/bleomycin, of *C. reinhardtii*. Linking expression of *xyn1* with *ble2A* expression on the same open reading frame led to a dramatic (100-fold) increase in xylanase activity in cells lysates compared to the unlinked construct. Further, a secretion signal sequence placed between *xyn1* and *ble2A* led to the secretion of xylanase (Rasala et al., 2012). In another approach, the pEXPYR shuttle vector was used for the heterologous overexpression in *Aspergillus nidulans* of the *P. funiculosum* endo-1,4-xylanase (XynC) and the *A. niger* α-L-arabinofuranosidase (AbfB) together. There was synergistic improvement of xylooligosaccharides production from pretreated sugar cane bagasse when both enzymes were combined (Gonçalves et al., 2012).

The strong cbh1 (cellobiohydrolase 1) promoter has been used frequently for heterologous and homologous protein expression in *T. reesei*. However, this promoter

needs induction and is partly regulated by catabolite repression. On the other hand, the mRNA levels of *pdc* (encoding pyruvate decarboxylase) and *eno* (encoding enolase) genes are much higher than other genes under high glucose conditions. Recombinant *T. reesei* strains that homologously expressed xylanase II were constructed by using the promoters of the *pdc* and *eno* whereby they were found to produce 9266 IU/mL and 8866 IU/mL of xylanase, respectively, in the medium containing high glucose concentration (Li et al., 2012). Similarly, other promoters of cellulase system derived from cellobiohydrolase 2 (*cbh2*) and endoglucanase 2 (*egl2*), were used to create an expression platform. A thermophilic xylanase (XynB) from *Dictyoglomus thermophilum* was then expressed synergistically in *T. reesei* RUT-C30. Transformants with the combination of *cbh2* promoter, secretion signal, cellobiohydrolase 2 CBM, and linker as well as *XynB* secreted multiple forms of XynB containing various *N*- and *O*-glycans (Miyauchi et al., 2013). The fusion proteins can also be successfully expressed on the surface of the whole cells like the cell surface display of Cex protein, encoding xylanase, and exoglucanase in *Cellulomonas fimi*, using anchor protein PgsA on *E. coli* surface. The xylanase-surface display achieved the highest activity of 54 U/g dry cell weight at 60 hours and 37°C. The study showed that cell surface display of xylanase embedded in the cell membrane was more thermostable and had a broader pH range than that of the purified enzyme. The optimum temperature shifted from 40°C to 60°C in membrane anchored protein, probably due to the presence of more hydrophobic amino acids (Chen et al., 2012).

Like cellulosomes, designer nanostructured, multifunctional, self-assembling protein complex termed as xylanosomes, are specifically targeted for hydrolysis of complex hemicelluloses. It was reported by McClendon et al. (2012) that a designer xylanosome composed of a xylanase and a bifunctional arabinofuranosidase/xylosidase released 30% more soluble sugars than the corresponding free and unstructured enzymes. Another xylanosome composed of xylanase and a ferulic acid esterase removed 20% more ferulic acid from wheat arabinoxylan than free enzymes. In another study, xylanosome constructed with three endoxylanases of *T. fusca* and a β-xylosidase succeeded in degrading 25% of the total xylan component of wheat straw (Moraïs et al., 2011).

5.4.2 Process Methods

Although efficient and novel genetic methods have been employed by various researchers to overexpress a few components of cellulase and xylanase enzymes in several hosts, information on modification of the properties of the recombinant enzymes is rather scanty (cf. section 5.3). Furthermore, the enzyme components which have been overexpressed are unlikely to hydrolyze cellulose efficiently unless mixed with other components to have a complete enzyme system, which is required for efficient hydrolysis. This is perhaps the reason as to why such recombinant organisms have not been widely studied for production of the enzymes in bench or pilot-scale bioreactors, which is required to assess the economics of their large-scale production. The production of hydrolytic enzymes economically is of prime concern for their subsequent commercial application in bioproduct manufacture. For successful industrial

production of enzymes, the choice of microorganism and the process strategies are the key. Microorganisms that can use agro and industrial wastes as carbon source for cellulolytic enzyme production are potential candidates for industrial-scale production. The following paragraphs describe a few important approaches that have been used for production of cellulase and xylanase enzymes with high titer and productivity in submerged and in solid-state fermentation (SSF) systems.

The microbial production systems are multivariable controlled processes. Media composition and environmental factors can enhance the titer and productivity of enzymes in microbial cell cultures. It is, therefore, essential to study and quantify the effects of key medium components on growth and enzyme production and strike a balance between the two to enhance the enzyme titer and productivity. It is well known that *T. reesei* strains are used for industrial production of cellulases. Its mutant strain *T. reesei* RUT C30 is amongst the top cellulase producers (Kubicek, 2013). Mandels' group was one of the pioneering groups that worked extensively on cellulase system of *T. reesei* during the 1960s and 70s. The group showed that C30 when cultivated on cellulose produced 15 FPU/mL with productivity of 80 U/L · h (Ryu and Mandels, 1980). A six-step mutation in one of its earliest mutants QM9414 increased productivity by two-fold in comparison to RUT C30 (Durand and Clanet, 1988). In another microbe *Acremonium cellulolyticus*, classical mutation technique was used to isolate hyper-cellulase producer. After UV irradiation and NTG treatments, *A. cellulolyticus* CF-2612 mutant produced increased FPase activity of 17.8 IU/mL. Further, in fed-batch mode *A. cellulolyticus* improved cellulase productivity to 140 FPU/L · h (Fang et al., 2009). Several other microbes have also been studied by various laboratories for production of cellulases in bench- and pilot-scale submerged fermenters as summarized in Table 5.2.

Cellulase is an inducible enzyme system for which several carbon sources have been tested to find the best inducer (Mandels, 1975; Seiboth et al., 2012). Cellulose itself has been recognized as one of the best inducers for synthesizing the complete cellulase system; other important inducers include sophorose and lactose. Cellulase biosynthesis is repressed by glucose; however, *T. reesei* grows rapidly on simple sugars like glucose but more slowly with a lag phase on lactose or cellulose. *T. reesei* does not normally produce cellulases when grown in media containing glucose. In *T. reesei* RUT-C30 strain, the mutation in *cre1* (carbon catabolite repressor) released glucose repression and expressed cellulases (Ilmen et al., 1996). Other factors which influence the enzyme production include concentration and quality of the carbon source, growth conditions, aeration, temperature, and pH. The effect of medium composition in *T. reesei* RUT-C30 cultures on cell growth and enzyme production was investigated recently by Ahamed and Vermette (2008a) with the aim of reducing production costs by using cellulose as a main substrate and lactose plus lactobionic acid in the fed-batch phase. Four different media compositions were used to test the production level of cellulase enzyme in *T. reesei* during fed-batch cultures in a 7-L stirred tank bioreactor (STR). *T. reesei* grew very well in cellulose–yeast extract medium with lactose and lactobionic acid as inducers and produced a maximum FPA of 5.02 U/mL, which was two to three times higher than those obtained with other media, that is, corn steep–glucose (1.4 U/mL), cellulose–yeast extract–peptone (2.3 U/mL),

TABLE 5.2 Production of Cellulases by a Few Prominent Microorganisms in Laboratory and Pilot-Scale Bioreactors Under Submerged Fermentation

		Conditions			Enzyme activities			
Strain	Substrate	Temp (°C)	pH	Scale (L)	Cellulase (FPU/mL)	CMCase (U/mL)	β-Glucosidase (U/mL)	Reference
Acremonium cellulolyticus CF-2612	Solka-Floc	30	NA	2	17.8			Fang et al. (2009)
Acremonium cellulolyticus C-1	Milk pack	28	4.5	3	16			Park et al. (2011)
Acremonium cellulolyticus C-1	Solka-Floc	30		50	13.08			Ikeda et al. (2007)
Aspergillus niger A12	Glucose and sugarcane bagasse	30	5	5		0.72		Cunha et al. (2012)
Penicillum funiculosum	Pretreated sugarcane bagasse	30	5	10	1.13	10.25		Maeda et al. (2013)
Streptomyces sp.	CMC	50	6.5	50		148	137	Jang and Chang (2005)
Trichoderma pseudokoingii S-38	Glucose and cellulose powder	28	3.6	2.5		10.86		Duan et al. (2004)
Trichoderma reesei 2U-02	Corn cob	30	4.8	30		5.48		Liming and Xueliang (2004)
Trichoderma viride	Sugarcane bagasse	28		7	0.6	0.6		Lan et al. (2013)
Trichoderma harzianum P49P11	Pretreated sugarcane bagasse and sucrose	29	5	3	1.21		17.32	Delabona et al. (2012)

CMC, carboxymethyl cellulose; NA, not Available.

and cellulose–yeast nitrogen base–CMC (1.4 U/mL). The volumetric enzyme productivity in cellulose–yeast extract medium (69.8 U/L · h) was also approximately two to three times higher than those in the other media (Ahamed and Vermette, 2008a).

STRs are the most common reactors employed for cellulase production. However, it is sometimes difficult to modulate the shear stress levels within these reactors, which often damage filamentous microorganisms such as *T. reesei* and lower enzyme production. Many reactor designs such as air-lift reactors have been suggested to increase the oxygen transfer rate while limiting the deleterious effects of shear stresses on filamentous microorganisms and minimizing power consumption. The absence of impellers, moving parts, and bearings allows extended aseptic operation of these reactors. They have lower power consumption as compared to stirred tanks, good mixing capacity for media containing suspended solids, efficient mass and heat transfer, and better defined flow patterns (Chisti, 1989). Ahamed and Vermette (2010) used a draft tube air-lift bioreactor to produce cellulases by *T. reesei* RUT-C30 in a cellulose medium with lactose and lactobionic acid in fed-batch mode. The airlift reactor was operated without agitation and with agitation where shear was provided by an impeller running at 400 rpm. Significant increases in filter paper activity (from 12 to 17 U/mL) and volumetric enzyme productivity (from 147 to 200 U/mL · h) were obtained in cultures carried out with no mechanical agitation. These results could be explained on the basis of *T. reesei*'s morphology, as mechanical agitation in STR caused more fragmentation of its hyphae. The lowered enzyme activity could also be attributed to the shear sensitive nature of the *T. reesei* cellulase enzyme system.

The association of filamentous fungi such as *Trichoderma* and *Aspergillus*, which are amongst the most important microorganisms used in industry, allows higher production of cellulolytic enzymes by synergistic action. The synergistic reaction occurs as a result of synthesis of some metabolites by one organism which has a positive effect on the other in terms of increased production of cellulolytic enzymes. It was shown by Ghose's group (Ghose et al., 1985; Panda et al., 1987) that there was increased synthesis of both cellulase and xylanase enzymes when the addition of *Aspergillus wentii* culture was phased by 15 hours to a growing culture of *T. reesei*. More recently, Ahamed and Vermette (2008b) have shown that a mixed culture of *T. reesei* and *A. niger* cultured in media containing cellulose–yeast extract in a 3 L fed-batch bioreactor, with lactose and lactobionic acid feeding during fed-batch phase, produced 2.1-fold improved filter paper activity (7.1 U/mL) and enzyme productivity (98.4 FPU/L · h) compared to the monocultures. The enzyme system derived from the co-fermentation of the two species had higher cellulose hydrolysis potential than the enzymes derived from the monocultures.

Recently Lan et al. (2013) studied the production of cellulolytic enzymes by *Trichoderma viride* in a rotating fibrous-bed bioreactor (RFBB) and compared its performance with that of an STR. It was envisaged that the immobilization of *T. viride* mycelia on solid support of polypropylene cloth would eliminate some of the difficulties associated with the use of STRs (such as disintegration of the mycelia by the impeller, fouling of the pH and temperature probes, and clogging of the sample

ports by the mycelia). It was found that the *T. viride* mycelia formed biofilm in the RFBB, which was operated under a relatively low-shear environment, and promoted the enhanced production of enzymes. Under the operating conditions employed, the immobilized mycelia in RFBB produced 35.5% higher FPase activity and gave 69.7% higher saccharification yield of sugarcane bagasse compared to the enzyme produced in STR.

Application of statistical optimization tools like response surface methodology where several parameters are considered simultaneously for their influence and interaction during the bioprocess has resulted in getting increased enzyme titers in xylanase-producing organisms. For example, statistical optimization of xylanase production parameters such as pH, temperature, substrate concentration, cultivation time, and aeration improved enzyme activity by 315% (Bocchini et al., 2002). There was a significant increase of 20-fold xylanase activity to 174 IU/mL by optimizing process conditions in shake flask using response surface methodology in a halophillic eubacterium strain SX15 (Wejse et al., 2003). The xylanase activity of 210 IU/mL was obtained at the end of cultivation in a bioreactor, which was higher than in the shake flask when carbon source was replaced by soluble oat husk extract in *T. lanuginosus* DSM 10635 (Xiong et al., 2004a). A white rot fungus, *Armillaria gemina* SKU2114, secreted lignocellulose-degrading enzymes which showed maximum endoglucanase, cellobiohydrolase, and β-glucosidase activities of 146, 34, and 15 U/mL respectively, and also xylanase, laccase, mannanase, and lignin peroxidase with activities of 1270, 0.16, 57, and 0.31 U/mL, respectively, when grown on rice straw as a carbon source. Application of response surface methodology enhanced the hydrolysis of wood, *Populus tomentiglandulosa*, to an extent of 62% by this enzyme (Jagtap et al., 2013).

In submerged fermentation, the biomass remains evenly dispersed in liquid medium with equal access to the dissolved nutrients and oxygen. Efficient heat and mass transfer also take place. The fungus *Melanocarpus albomyces* which is filamentous, multinucleate, thermophilic, and non-sporulating has been studied with respect to xylanase production in submerged fermentation as it produces thermostable xylanase along with xylosidase and xylan-debranching enzymes. A mutant of *M. albomyces* IITD3A produced 415 IU/mL xylanase on soluble alkaline lignocellulose extract in a 14 L bioreactor with volumetric productivity of 11,530 IU/L · h, which was 8-fold higher than that of the wild-type strain (Biswas et al., 2010a). It was found by response surface methodology that the pH of the medium was critical for the production of xylanase by *M. albomyces* IITD3A. Cyclic maintenance of pH of fermentation medium between 7.8 and 8.2 increased the productivity to 16,670 IU/L · h (Figure 5.1). Cycling of pH in systems such as this one, in which different components of xylanase have different pH optima, seems to be a simple strategy to improve enzyme titer and productivity. Further optimization of process parameters like aeration and agitation in 14 L bioreactor, changed the fungal morphology to a pellet form with an increase in productivity to 22,000 IU/L · h, which is amongst one of the highest reported in filamentous fungi (Biswas et al., 2010b). Maintenance of a particular fungal morphology has been extensively reported to be essential for the production of several secondary metabolites such as antibiotics also.

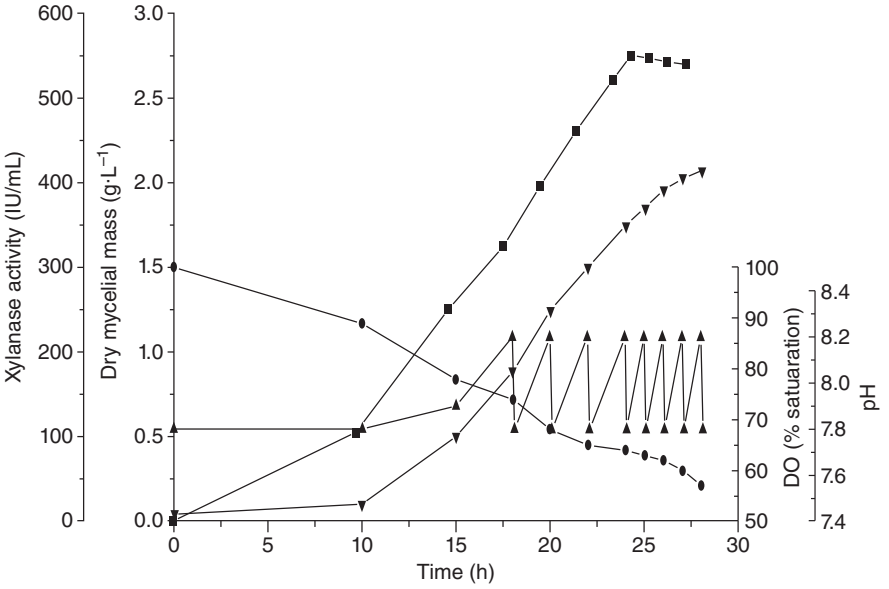

FIGURE 5.1 Production of xylanase by *Melanocarpus albomyces* IITD3A in 14 L bioreactor with pH cycling. Filled square, xylanase activity; filled triangle, pH; filled circle, dissolved oxygen; and inverted filled triangle, dry mycelia mass (Biswas et al., 2010b).

Some microbes have been reported to give better productivity of enzymes in SSF. In SSF, the solid waste residues from agriculture, forest, mills, etc. are used as the support substrates on whose surface the dispersed microbial biomass grows under controlled temperature, moisture, and aeration. It offers several advantages over liquid cultivation, especially for the fungal cultures, as there is higher productivity per unit volume, reduced energy requirements, lower capital investment, low wastewater output, higher concentrations of metabolites obtained, and low downstream processing cost (Narang et al., 2001). During SSF of wheat straw by *M. albomyces*, process conditions were optimized as 600–850 μm particle size of wheat straw, 43 hours inoculum age, 1.37% Tween 80, 86% initial moisture content, 5.1% urea, and 0.74% yeast extract. Under these optimized conditions, xylanase activity of 7760 U/g initial dry substrate was produced (Narang et al., 2001). Production of xylanase by *Fusarium oxysporum* under solid-state culture on corn stover was enhanced by optimization of the type of nitrogen source, initial moisture level, growth temperature, and initial pH (Panagiotou et al., 2003). A marine strain of *A. niger* used water weed, *Eichchornia crassipes*, as main substrate and produced 17.80 U/g dry substrate in 6 days in SSF indicating the potential for economic cellulase production (Xue et al., 2012). Another industrial strain of *A. niger* produced cellulase under different cultivation conditions like biofilms submerged and SSF, and produced 1.77, 1.17, and 1.17 FPU/mL, respectively. Although biofilm cultures had less growth rate than the submerged and solid-state cultures, they gave higher cellulase yields (370, 212, and

217 U/galactose, respectively) and volumetric productivities (24, 16, and 16 U/L · h, respectively). The biofilm cultures also produced higher endoglucanase and xylanase activities (Gamarra et al., 2010).

The SSF in general faces major problems with respect to design and operation of large-scale bioreactors (Ashley et al., 1999). However, various types of bioreactors have been studied for solid-state fermentation processes, including packed beds, rotating drums, gas–solid fluidized beds, and stirred bioreactors but were unable to completely overcome the mass and heat transfer limitations (Mitchell et al., 2000). A novel intermittent agitation rotating drum type bioreactor for thermophilic microorganism *Thermoascus aurantiacus* was used for xylanase production. Important fermentation variables like initial moisture content of the medium, growth temperature, and airflow rate were effectively controlled at the set values through this design and xylanase activity of 4490 U/g of dry wheat straw was achieved (Kalogeris et al., 2003). The operational conditions in SSF like air flow rate, inlet air relative humidity, and initial substrate moisture content influenced the production of biomass-degrading multienzyme in *A. niger* strain 12. An instrumented lab-scale bioreactor equipped with an online automated monitoring and control system maintained initial substrate moisture content of 84%, an inlet air humidity of 70%, and a flow rate of 24 mL/min for *A. niger* fermentation on soya bean meal; it produced 0.55 IU/g FPase, 35.1 IU/g endoglucanase, and 47.7 IU/g xylanase (Vitcosque et al., 2012). The production of xylanases by a few microorganisms in submerged and SSF systems is shown in Table 5.3.

Some microbes such as *A. niger* KK2, *A. pullulans*, *F. oxysporum,* and *T. aurantiacus* simultaneously secrete cellulases and xylanases to digest lignocelluloses. The production of these cellulolytic and xylanolytic enzymes by such organisms has been studied under submerged and solid-state cultivations where they were found to produce these enzymes (Table 5.4). These can be helpful in extracting more sugars from the lignocelluloses and decrease the cost of production of bioproducts. Also, they can be potential candidates for consolidated bioprocesses.

Thermophiles that have faster metabolic rates can result in faster growth lignocellulosic substrates and high productivity of hydrolytic enzymes. Thus, thermophiles producing these enzymes are of great interest for the biocommodity industry. At high temperatures, the reaction rates are faster, the risk of contamination is low, and the process is more energy efficient for pretreatment of biomass. A newly isolated *Geobacillus* sp T1 has been reported to grow on barley and wheat straw to produce cellulase units of 143.5 U/mL after 24 hours of fermentation (Assareh et al., 2012). *Streptomyces* sp CS802, isolated from soil in the Republic of Korea, produced 1482 U/mL of xylanase on corn cob medium at 60°C (Simkhada et al., 2012). An interesting thermophile, *Thermobacillus xylanolyticus*, was able to use glucose and xylans as primary carbon source. Its growth on lignocellulosic biomass like wheat straw and wheat bran induced hemicellulolytic enzyme production, and xylanase was the primary enzyme secreted. Debranching enzyme esterase was produced by the bacterial cultures grown on wheat straw whereas arabinofuranosidase production was significantly higher in bacterial cultures grown on wheat bran. Thus *T. xylanolyticus* seems to have adapted its enzymatic profile to better address the composition of various lignocellulosic substrates (Rakotoarivonina et al., 2012). Multiple acidophilic

TABLE 5.3 Microbial Xylanase Production in Submerged and Solid-State Fermentation

			Fermentation conditions				Xylanase activity		
	Organism	Carbon source	Temp. (°C)	pH	Type	Scale (L)	(IU/mL or IU/g)	Time (h)	References
1.	*Armillaria gemina*	Wheat bran	–		SmF	7.0	1270.0	216	Dhiman et al. (2013)
2.	*Aspergillus oryzae*	Spent sulphite liquor	30	7.5	SmF	15	199.0	48	Chipeta et al. (2008)
3.	*Aspergillus niger*	Corn cob	28	3.5	SmF	20.0	290.0	80	Qi-peng et al. (2005)
4.	*Aspergillus sulphureus*	Wheat bran	32	6.5	SSF	75.0	7.5	72	Wenqing et al. (2003)
5.	*Bacillus coagulans* BL69	Fibrous soy residue	37	–	SSF	0.5	1.5	72	Heck et al. (2005b)
6.	*Bacillus circulans* BL53	Fibrous soy residue	37	–	SSF	12.0	8.25	96	Heck et al. (2005a)
7.	*Bacillus* sp.	Oat spelt xylan	40	8.0	SmF	1.5	65.0	24	Chivero et al. (2001)
8.	*Bacillus subtilis*	Oat spelt xylan	50	6.0	SmF	2.0	1.73	18	Sá-Pereira et al. (2002)
9.	*Melanocarpus albomyces* IIS 68	Wheat straw	45	7.0	SSF	0.5	7760.0	120	Narang et al. (2001)
10.	*Melanocarpus albomyces* IITD3A	Wheat straw extract	45	7.0	SmF	14	550.0	24	Biswas et al. (2010a)
12.	*Neocallimastix frontalis*	Cellulose	39	6.8	SmF	1.2	3.68	144	Srinivasan et al. (2001)
13.	*Penicillium* sp.*WX-Z1*	Wheat bran	30	–	SmF	30	46.5	144	Cui and Zhao (2012)
14.	*Rhizopus oryzae*	Corn cob	35	5.0	SmF	2.0	260.0	120	Bakir et al. (2001)
15.	*Rhodothermus marinus*	Birchwood xylan	61	7.5	SmF	5.0	7.5	72	Gomes et al. (2000a)
16.	*Streptomyces* sp. Ab106	Sugarcane bagasse	50	7.0	SmF	5.0	32.0	144	Techapun et al. (2003)
17.	*Thermoascus aurantiacus*	Sugarcane bagasse	45	–	SSF	0.2	1597.0	240	Milagres et al. (2004)
18.	*Thermoascus aurantiacus*	Solka-Floc	47	4.5	SmF	5.0	208.0	264	Gomes et al. (2000b)
19.	*Thermomyces lanuginosus* SSBP	Xylose	50	6.5	SmF	30.0	405.0	48	Reddy et al. (2002)
20.	*Trichoderma reesei Rut* C-30	Lactose	28	6.0	SmF	2.0	94.7	120	Xiong et al. (2004b)

SmF, Submerged Fermentation (enzyme activity in IU/mL); SSF, Solid-State Fermentation (enzyme activity in IU/g solid subtrate).

TABLE 5.4 Microbial Strains Coproducing Cellulases and Xylanases on Lignocellulosic Residues

Strains	Substrate	SSF/SmF	FPase (U/g or U/mL)	CMCase (U/g or U/mL)	β-Glucosidase (U/g or U/mL)	Xylanase (U/g or U/mL)	β-Xylosidase (U/g or U/mL)	References
Acremonium cellulolyticus CF-2612	Wheat bran	SmF	10.5	356.5	22.6	580.6	1.1	Hideno et al. (2011)
Aspergillus niger KK2	Rice straw	SSF	19.5	129.0	100	5070	193	Kang et al. (2004)
Aureobasidium pullulans	Corn bran	SSF	–	1.05	1.3	5	–	Leite et al. (2007)
Cellulomonas flavigena	Sugarcane bagasse	SmF	–	7.56	–	75.6	–	Rojas-Rejón et al. (2011)
Fusarium oxysporum	Corn stover	SSF	–	211	0.088	1216	0.052	Panagiotou et al. (2003)
Myceliophthora sp IMI 387099	Rice straw	SSF	2.44	32.9	7.48	900.2	–	Badhan et al. (2007)
Penicillium echinulatum 9A02S1	Sugarcane bagasse and wheat straw	SSF	32.89	282.36	58.95	10	–	Camassola and Dhillon (2007)
Thermoascus aurantiacus	Wheat straw	SSF	5.5	1709	79	4490	45	Kalogeris et al. (2003)

SmF, submerged fermentation (enzyme activity in U/mL); SSF, solid-state fermentation (enzyme activity in U/g solid subtrate).

xylanolytic enzymes were produced by *Penicillium oxalicum* GZ-2, and wheat straw was the best inducer, resulting in the highest xylanase (115.2 U/mL) and β-xylosidase (89 mU/mL) activities at 50°C (Liao et al., 2012). *Paenibacillus macerans* IIPSP3 isolated from the gut of the wood-feeding termite produced thermostable xylanase that was active over a broad range of temperatures (40–90°C) with xylanase activity of 100 U/mL on corn cobs. The strain showed maximum xylanase production at its optimum growth temperature, that is, 50°C (Dheeran et al., 2012). Two potential strains of marine fungi obtained from degrading mangrove leaves, *Fusarium* sp KAWIT-A, and *Aureobasidium* sp 2LIPA-M, produced thermophilic xylanase in submerged fermentation supplemented with 1.5% marine salts (Torres and Cruz, 2013). Another thermophilic microorganism, *Streptomyces thermovulgaris* TISTR1948 produced 274.5 U/mL xylanase on rice straw (Chaiyaso et al., 2011). Moreover, several thermophilic cellulase producers like *Paenibacillus barcinoneusis* (Asha et al., 2012), *B. subtilis* strain LFS3 (Rawat and Tewari, 2012), *C. thermocellum* strains CS7 and CS8 (Lv and Yu, 2012), and *Phiahophora* sp G5 (Zhao et al., 2012) have recently been studied for enzyme production with a view to utilize them for production of bioproducts on a large scale.

5.5 ECONOMIC OUTLOOK

Providing cost-effective enzymes is a big challenge for industrial application of enzymes. Serious efforts have been made in this direction by application of both genetic as well as process engineering tools, by the use of waste materials as substrates, and by using the minimum steps in downstream recovery of enzymes. Although significant cost reductions in cellulase production by 20- to 30-fold have been made (Lynd et al., 2005), it is still far to compete with chemical industries for production of ethanol. Many commercial enzyme manufacturers like Novozymes, Genencor, Danisco, Armano Enzyme, etc. are striving to develop highly efficient and less expensive cellulolytic enzyme cocktails (Chandel et al., 2012). Current estimates suggest that the cost of producing cellulosic ethanol is $1.80 per gallon or higher, which is almost twice as high as the cost of producing ethanol from starch. A major emphasis on research is, therefore, on reduction of the cost of hydrolytic enzymes. Current research is being aimed at bringing down the enzyme cost from $0.50 to $0.10 per gallon of ethanol (EERE, DOE, 2010). The economics of production of hydrolytic enzymes will remain an integral part of any successful biocommodity industry. With combined knowledge of protein engineering, metabolic engineering, genomics, and bioprocess engineering, it is expected that cellulases and xylanases can be produced in copious amounts on inexpensive substrates that will make biorefinery industry a reality.

5.6 FUTURE PROSPECTS

The role of cellulase and xylanase enzymes is crucial in realizing the aim of producing ethanol (and other biofuels) and commodity bioproducts from cellulosic feedstocks. The cost of producing cellulase has been brought down significantly over the last

decade, but it still seems to be about two to three times higher than the threshold value projected for economic production of cellulosic ethanol. We believe that the development of more effective cellulases with desirable characteristics such as high catalytic efficiency on crystalline cellulose, end-product inhibition resistance and higher thermostability shall be able to bring out a faster and more complete hydrolysis of celluloses to reducing sugars. To achieve these goals, modern tools of protein and metabolic engineering, together with process engineering and scale-up in suitable bioreactors need to be applied. This will result in the usage of decreased amounts of cellulolytic enzymes, thereby lowering their contribution to the overall production cost of ethanol and other bioproducts. Further, information on genome of the best cellulase producer, *T. reesei*, and its several transcriptional regulators which are involved in cellulase regulation is now available. As our understanding to modulate these regulators in response to various external stimuli increases, it is expected that it shall be possible to overproduce the desired mix of biomass-degrading cellulolytic enzymes with high yield and productivity.

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