

Xu Fang · Yinbo Qu *Editors*

Fungal Cellulolytic Enzymes

Microbial Production and Application

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Preface

The fossil fuel combustion causes air pollution and is one of the primary sources of greenhouse gas emissions. Bioethanol is the most promising renewable and carbon neutral alternative liquid fuel, and then consumption of bioethanol has been rapidly increasing since the Kyoto Protocol was established in February 2005. However, development of bioethanol production from starch-based feedstock, mainly from corn, wheat, and cassava, is limited by food security problem. Lignocellulosic biomass is an inexpensive, abundant, and carbon-neutral renewable bioresource for the production of biofuel and bio-based products. The development of a renewable, biomass-based, fuels and chemicals “biorefinery” process will be crucial if we are to transition to a more environmentally friendly economy. The sugar platform – breaking lignocellulosic biomass into fermentable sugar by cellulolytic enzymes – is the prerequisite for the production of biofuels or biochemical. Except biofuel production, cellulolytic enzymes were used in various industries, including textile, animal feed, bakery, brewing, pulp, and paper. Thus, cellulolytic enzymes have received much scientific attention all over the world.

The typical process of sugar platform is mainly composed of three steps: pretreatment, cellulolytic enzyme production, and enzymatic hydrolysis. In this book, we overviewed the current and updated knowledge of cellulolytic enzymes and their application. Firstly, the progress of pretreatment for enhancing the enzymatic digestion of lignocellulosic material is introduced by Professors B. Yang and Y. He. The major part of this book is on synthesis mechanisms of cellulolytic enzymes, genetic engineering and development of industrial fungal strains, and heterologous expression of cellulolytic enzymes in yeast and fungal strains which are addressed by Drs. H. Inoue, J. Hong, and J. Zhang and Professors D. Zhang, F. Bai, S. Chen, S. Sawayama, X. Fang, X. Zhao, and Y. Qu. Meanwhile, discovery and design of thermophilic cellulolytic enzymes are introduced by Professor F. Li. Moreover, improvement and model of enzymatic hydrolysis and optimization of cellulolytic enzyme system are presented by Drs. F. Sun and J. Hu and Professors F. Bai, J. Bao, J. N. Saddler, X. Fang, and X. Zhao. Finally, industrial applications of cellulolytic enzymes are summarized by Dr. X. Li. We expect that this book will be used as an important reference for graduate students, researchers, and bioengineering experts

who are engaged in lignocellulose biodegradation, biomass utilization, enzyme production, and fungal molecular biology.

On behalf of my coeditor Professor Y. Qu, I express our sincere appreciation to all authors for their contribution and dedication. Also, I greatly appreciate Miss W. Guo for her delicate coordination. Finally, I express my sincere thanks to my colleagues and graduates at Shandong University.

Jinan, China

Xu Fang

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Chapter 1

Pretreatment Process and Its Synergistic Effects on Enzymatic Digestion of Lignocellulosic Material



Yu-Cai He, Cui-Luan Ma, and Bin Yang

Abstract In this chapter, the progress of pretreatment for enhancing the enzymatic digestion of lignocellulosic material is introduced. Furthermore, the pretreatment process and its synergistic effects on enzymatic digestion of lignocellulosic material are discussed. In general, the lignocellulose structure is mainly composed by three major components (hemicellulose, cellulose, and lignin). Cellulose microfibrils are coated with amorphous hemicellulose matrices building holocellulose structures and severely protected by non-sugar lignin outside. To overcome the inherent structural recalcitrance and enhance the sequential enzymatic saccharification of lignocellulosic materials, pretreatment is an indispensable step to be developed for making cellulose more accessible to cellulases. Enzymatic hydrolysis that bioconverts the pretreated lignocellulosic material with cellulases into fermentable sugars is known as the most complex step in this biological process due to enzyme-related and substrate-related effects and substrate-enzyme interactions. Thus, topics are summarized including characteristics of cellulose (e.g., degree of polymerization, crystallinity, and accessible surface area) and other components (e.g., oligomeric xylan and lignin) released from the pretreatment of lignocellulosic material and their effects on the effectiveness of enzymatic saccharification.

Keywords Pretreatment · Biomass · Enzymatic saccharification · Cellulose · Hemicellulose · Lignin

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1 Introduction

The overuse of fossil fuels causes serious fossil shortages and environmental issues. Lignocellulosic material is an inexpensive, abundant, and carbon-neutral renewable bioresource for the production of biofuel and bio-based products (Alvira et al. 2010; Fatih Demirbas 2009; Hamelinck et al. 2005; He et al. 2017a, b, c, d, e; Karmakar et al. 2010; Qing et al. 2017; Ragauskas et al. 2006; Sun and Cheng 2002). The primary energy supply of biomass used worldwide is estimated at about 60 EJ, accounting for over 10% of all energy supplied annually. Of the total biomass supply, contribution of agricultural products and by-products is ~9% (Paudel et al. 2017). The main biofuel and bio based chemicals can be obtained from the different biochemical routes from numerous agricultural biomasses.

The typical process for converting biomass into biofuel and bio based chemicals is mainly composed of three steps: biomass pretreatment, enzymatic hydrolysis, and fermentation (Fig. 1.1). In biomass, hemicellulose and cellulose are densely packed together with lignin, which result in low enzymatic saccharification (He et al. 2017a, b, c, d, e; Leonowicz et al. 1999). An ideal biomass pretreatment process is capable of disrupting recalcitrant lignocellulosic structures, removing lignin to expose the cellulose and hemicellulose, increasing the enzyme accessibility to carbohydrates (hemicellulose and cellulose), and enhancing the yield of fermentable sugars (Ma and Ruan 2015; Wyman et al. 2005a). During the pretreatment of biomass, the removal of hemicellulose and lignin depends on the pretreatment technology, operation conditions, and pretreatment severity (He et al. 2016b; McMillan 1994). Various pretreatments have been attempted to pretreat lignocellulosic materials for enhancing the reactivity of cellulose molecules with cellulases and improving the enzymatic saccharification. The typical goals of pretreatment are to produce highly digestible solids to enhance the yields of fermentable sugars, avoid the degradation

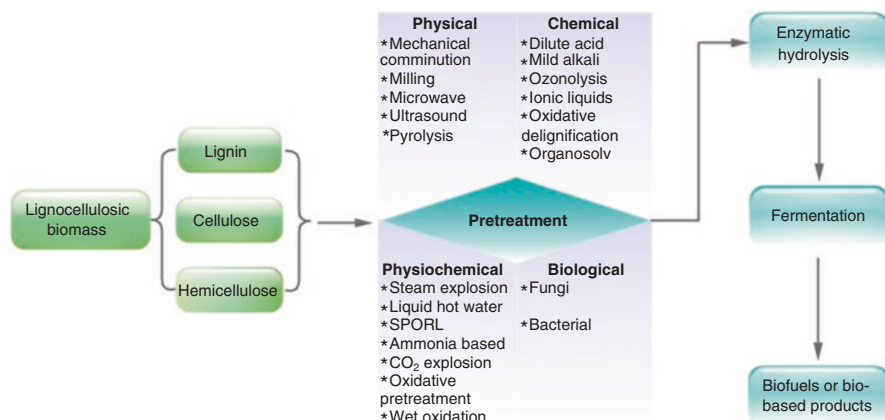


Fig. 1.1 Various pretreatments of lignocellulosic biomass for biological conversion to ethanol

of sugars, and minimize the formation of inhibitors (e.g., furfural and HMF) for subsequent fermentation or biotransformation steps (Alvira et al. 2010; Bhatt and Shilpa 2014; Brodeur et al. 2011; He et al. 2015a; Hendriks and Zeeman 2009).

In this chapter, the progress of various pretreatments for enhancing the enzymatic digestions of lignocellulosic materials is introduced. Furthermore, the pretreatment process and its synergistic effects on enzymatic digestion of lignocellulosic material are discussed.

2 Lignocellulose Compositions

Lignocellulose mainly consists of carbohydrate (cellulose and hemicellulose) and lignin, along with small quantities of proteins, pectins, extractives, and ashes (Di et al. 2018; He et al. 2016a; Jørgensen et al. 2007). These compositions are different from one species to another (Table 1.1) (Ye and Cheng 2002). Additionally, the components in a single lignocellulosic material of plant vary with plant ages, growth stages, and other growth conditions (Pérez et al. 2002). It is known that cellulose is one kind of linear homopolymers consisting of *D*-glucopyranose linked by β -1,4-glycosidic bonds (1,4- β -*D*-glucopyranosyl units) with up to ~10,000 degree of

Table 1.1 Cellulose, hemicellulose, and lignin contents in various lignocellulosic materials^a

Lignocellulosic material	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Bamboo shoot shell	38.5	23.1	11.4
Barley straw	31–45	27–38	14–19
Coastal Bermuda grass	25	35.7	6.4
Cotton seed hairs	80–95	5–20	0
Corncoobs	45	35	15
Grasses	25–40	35–50	10–30
Hardwood stems	40–50	24–40	18–25
Leaves	15–20	80–85	0
Nutshells	25–30	25–30	30–40
Newspaper	40–55	25–40	18–30
Paper	85–99	0	0–15
Rice straw	32–47	18–28	5.5–24
Softwood stems	45–50	25–35	25–35
Solid cattle manure	1.6–4.7	1.4–3.3	2.7–5.7
Sorted refuse	60	20	20
Sugarcane gabasse	32–48	19–24	23–32
Swine waste	6	28	Na
Switchgrass	45	31.4	12
Waste paper from chemical pulps	60–70	10–20	5–10
Wheat straw	33–45	20–32	8–20

^aAdapted from Dai et al. (2017); Jørgensen et al. (2007); Saini et al. (2015); Tye et al. (2016)

polymerization (DP) (Hallac and Ragauskas 2011). The cellulose chains have a strong tendency to form intramolecular and intermolecular hydrogen bonding by the hydroxyl (OH-) groups on these linear glucan chains (Pu et al. 2011), which promote cellulose aggregations and lead to supramolecular structures with crystalline and amorphous domains (Xu and Huang 2014). Hemicellulose (also known as polyose) is any of several **heteropolymers** of pentoses and hexanoses (mainly xylose and mannose) that are linked together and frequently have various branching and substitution groups (Xu and Huang 2014). Lignin is one kind of complex **organic polymers** that form important structural materials in the support tissues of **vascular plants** and some **algae**. Chemically, lignin is a complex, racemic, cross-linked, and highly heterogeneous aromatic macromolecule based on hydroxycinnamyl monomers with different degrees of methoxylation (Foston et al. 2012). Generally, a matrix of lignin and polysaccharides intimately associates with each other result in the effective utilization of lignocellulosic material (He et al. 2016c; Lawoko et al. 2005; Ralph et al. 2004; Xu and Huang 2014).

3 Pretreatment of Lignocellulosic Materials

Many retreatments include physical (milling, chipping, and grinding), chemical (concentrated alkali, concentrated alkali, dilute alkali, dilute acid, oxidizing agents, and organic solvents) (Alvira et al. 2010; Veluchamy and Kalamdhad 2017), physico chemical (steam pretreatment/autohydrolysis, hydrothermolysis, and wet oxidation) (Hendriks and Zeeman 2009; Veluchamy and Kalamdhad 2017), biological (Dai et al. 2017), or their combination (Brodeur et al. 2011; He et al. 2016c; Hendriks and Zeeman 2009; Sindu et al. 2016; Sun et al. 2015) (Fig. 1.1). The following pretreatment processes show promise for cost-effective pretreatment of lignocellulosic biomass for bioconversion to biofuels and bio-based chemicals.

4 Physical Pretreatment

4.1 Mechanical Pretreatment

Chipping, grinding, and milling are common mechanical pretreatment processes (Chang et al. 1997; Kim et al. 2013; Lin et al. 2010; Paudel et al. 2017; Zakaria et al. 2014). The main intention of mechanical pretreatment is to disintegrate the solid particles of biomass (Alvira et al. 2010; Sun and Cheng 2002), subsequently releasing biomass fragments with small particle size and permeability inside the overall lignocellulosic biomass structure (Paudel et al. 2017). To decrease the crystallinity of cellulose in biomass and enhance its saccharification, vibratory ball milling (VBM) was found to be more cost-effective than ordinary ball milling (OBM) (Millett et al. 1976). The particle size and physical characteristics of biomass can be

used to determine the total energy requirement for the mechanical comminution of biomass (Cadoche and López 1989). Chipping can be used for reducing the particle size to 1–3 cm only while milling and grinding can be used to reduce the biomass size up to 0.2 mm (Kumar and Sharma 2017). However, no significant effect on the enzymatic saccharification rates is found when the particle size of biomass is below 0.4 mm. Grinding and milling can effectively reduce the particle size and cellulose crystallinity because of the shear forces generated during milling, while chipping can significantly reduce the limitations of mass and heat transfer (Kumar and Sharma 2017). Wet disk milling (WDM) has been used as a popular mechanical pretreatment for the pretreatment of biomass due to its low energy consumption (Hiden et al. 2009). WDM enhances the saccharification of biomass by producing fibers and is more effective than hammer milling (HW) which can produce finer bundles (Zhang et al. 2012). After sugarcane bagasses were pretreated by WDM and ball milling (BM), it was found that BM pretreatment was better than WDM pretreatment based on the yields of carbohydrate (glucose and xylose) (Silva et al. 2010). After oil palm frond fiber (OPFF) was pretreated with BM, glucose and xylose could be obtained at 87% and 82% yields, respectively, while empty fruit bunch (EFB) produced glucose and xylose at 70% and 82% yields, respectively (Zakaria et al. 2014).

4.2 *Pyrolysis*

Pyrolysis, a thermal degradation process, has been employed for pretreating lignocellulosic materials in biorefinery processes (Case et al. 2015). Pyrolysis pretreatment is divided into slow, intermediate, fast, and flash pyrolysis based on the required heating rate and residence time (Roy and Dias 2017). Fast pyrolysis can be used for the production of a higher quantity and quality of biooils than slow pyrolysis. Besides production of high value-added bio-based chemicals, pyrolysis pretreatment is widely used in thermal industries due to easy storage, combustion, transport management, and retrofitting (Kumar and Sharma 2017). In addition, it is flexible in production and marketing. Pyrolysis is known as a thermal decomposition process, occurring in the absence of oxygen (Roy and Dias 2017), which can convert lignocellulosic materials into carbon-rich solids and liquids. Pyrolysis is found to be more efficient when performed in the presence of oxygen at lower temperatures (Kumar et al. 2009). Sulfuric acid (1 M) was used for the pyrolysis-pretreated biomass at 97 °C for 2.5 h, and the yield of reducing sugars reached ~85%. However, the glucose yield was obtained at >5% (Fan et al. 1987).

4.3 *Irradiation*

Recently, various radiation pretreatments under γ -rays (Yang et al. 2008), ultrasound, ultrasonic (Velmurugan and Muthukumar 2011), electron beam (Bak et al. 2009), UV (Dunlap and Chiang 1980), and microwave heating (Ma et al. 2009) have

been employed to pretreat lignocellulosic biomass for enhancing its saccharification (Kapoor et al. 2017).

High-energy radiation pretreatments (e.g., UV pretreatment and γ -rays pretreatment) are conducted via emitting rays at the raw lignocellulosic materials, which can reduce the polymerization degree of cellulose, loose cellulose structure, increase moisture, and enhance subsequent enzymatic hydrolysis. High-intensity radiation results in high degree of damage to cellulose structure; nonetheless, high-energy electron radiation method is costly, thus large-scale industrial production is difficult (Galbe and Zacchi 2007).

Microwaves are known as a form of [electromagnetic radiation](#) (ER) with long [wavelengths](#) ranging from 1 mm to 1 m in free space and [frequency](#) between approximately 300 GHz (0.1 cm) to 300 GHz (100 cm) (Singh et al. 2016). In microwave heating, the vibration of polar molecules and movement of ions cause the rapid heat and extensive collisions. Microwave irradiation is widely employed for biomass pretreatment due to its simplicity, low energy consumption, high heating capacity in short-duration time, and minimum generation of fermentation inhibitors (Chen et al. 2011; Kumar and Sharma 2017; Lu et al. 2011). Microwave-assisted alkali pretreatment of switchgrass yielded 70–90% sugars (Hu and Wen 2008). Microwave-assisted NaOH pretreatment of coastal Bermuda grass (CBG) produced 87% glucose and 59% xylose (Keshwani and Cheng 2010).

Ultrasound waves can alter the morphology of lignocellulosic materials. During the ultrasound pretreatment leads to formation of small cavitation bubbles which rupture the cellulose and hemicellulose fractions in biomass thereby increasing the accessibility to cellulases for the effective breakdown into reducing sugars (Gogate et al. 2011; Kumar and Sharma 2017; Rehman et al. 2013; Tang et al. 2005; Yachmenev et al. 2009). Duration of sonication and frequency of sonication have significant effect on pretreatment of biomass and enzymatic saccharification. Sonication of corn starch slurry (CSS) for 40 s increased the yield of reducing sugar by over fivefolds as compared to control without sonication (Montalbo-Lomboy et al. 2010). Ultrasound with frequency of 10–100 kHz for biomass pretreatment can be effective for cell breakage and polymer degradation (Gogate et al. 2011). Based on the biomass and slurry characteristics, power and duration of sonication need be optimized to meet the desired pretreatment objectives (Kumar et al. 2017).

5 Chemical Pretreatment

5.1 Ozonolysis

In biomass, lignin's association with cellulose protects it from enzymatic hydrolysis. Ozone (O_3) pretreatment can be used to degrade lignin and remove hemicellulose in lignocellulosic materials (e.g., bagasse, cotton straw, green hay, peanut, pine, poplar sawdust, rice straw, corn stover, wheat straw, etc.) (Ben-Ghedalia and Miron 1981; Benghedalia and Dror 1983; Neely 1984; Vidal and Molinier 1988). Ozone

pretreatment can be used to reduce lignin content significantly. However, hemicellulose in biomass can be removed slightly. After wheat straw was pretreated with ozone, 60% of lignin was removed, and enzymatic saccharification rate increased by fivefolds (Vidal and Molinier 1988). By decreasing the lignin content in poplar sawdust from 29% to 8% by ozone pretreatment, its enzymatic saccharification increased from 0% to 57% (Vidal and Molinier 1988). Ozone pretreatment can be carried out at room temperature under normal pressure (Quesada et al. 1999). A disadvantage of ozone pretreatment is that a large quantity of O_3 is required, which restrict its application due to its high economic cost (Shi et al. 2015).

5.2 Acid Pretreatment

Inorganic acids (e.g., sulfuric acid, hydrochloric acid, hydrofluoric acid, phosphoric acid, nitric acid, etc.) are known as common catalysts for the biomass pretreatment (Ramesh et al. 2018). Concentrated sulfuric acid is firstly used for saccharification of cellulose (Agbor et al. 2011).

Concentrated acid pretreatment allows to obtain high yield of reducing sugars from biomass at low temperature (Rabemanolontsoa and Saka 2016). Acid hydrolysis rate of crystalline cellulose is slower than that of amorphous hemicellulose in biomass due to their different intrinsic properties. After acid hydrolysis is carried out by concentrated acid in one step, pentoses and hexoses from hemicellulose in biomass are more susceptible for degrading into furfural and 5-hydroxymethylfurfural (5-HMF), which can inhibit the subsequent fermentation of reducing sugars. Additionally, concentrated acid pretreatment has the disadvantages including high consumption of acid, corrosion of the equipment, toxicity to the environment, and energy consumption for acid recovery.

Compared to concentrated acid pretreatment, dilute acid pretreatment has the merit of lower acid consumption. However, high temperature is required to obtain high yield of glucose from biomass, resulting in an extensive degradation of hemicelluloses (Rabemanolontsoa and Saka 2016). To avoid the disadvantages of concentrated and diluted acid pretreatments, a two-stage process has been performed with sulfuric acid: In the first stage, concentrated sulfuric acid (H_2SO_4 , 70%) at moderate temperature (30–40 °C) is used for hydrolyzing hemicellulose and decrystallizing cellulose in biomass. H_2SO_4 is then diluted to be about 35% with hot water, which is prepared for the second-stage pretreatment at 90–95 °C for the hydrolysis of the decrystallized cellulose (Rabemanolontsoa and Saka 2016). After these pretreatments, an ion exchange column (IEC) is used for separating H_2SO_4 from the hydrolysates obtained after the two-stage pretreatment, and the recovered H_2SO_4 is recycled for the next run acid hydrolysis pretreatment.

Recently, organic acids are found as alternatives to inorganic acids, which avoid equipment corruptions and low energy consumption for acid recovery. Dicarboxylic acids (e.g., maleic acid, succinic acid, oxalic acid, fumaric acid) and monocarboxylic acid (e.g., acetic acid) have been used for pretreatment. At same pH values,

monocarboxylic acids have lower catalytic performance than dicarboxylic acids due to the different pK_a values. Maleic acid can hydrolyze cellulose to glucose as effectively as sulfuric acid. However, glucose decomposition is much lower during the acid hydrolysis of cellulose with maleic acid in comparison to sulfuric acid, but hemicellulosic sugars are more extensively decomposed. Organic acids can be used for biomass pretreatment with high recovery of cellulose. However, low recovery of hemicellulose is obtained (Rabemanolontsoa and Saka 2016).

5.3 Alkaline Pretreatment

In biorefinery, alkaline pretreatment (AP) is essentially employed for the reduction in the degree of polymerization and crystallinity, swelling of the fibers, as well as disruption of the lignin structure removing lignin in biomass, which improves the enzymatic accessibility of the polysaccharides (Chong et al. 2018a). Alkaline pretreatment of biomass originates from soda pulping. NaOH, KOH, NH₃·H₂O, Ca(OH)₂, and oxidative alkali have been developed as main alkali agents for biomass pretreatments (Bali et al. 2015; He et al. 2016d; Janu et al. 2011; Millett et al. 1976). Alkaline pretreatment can be performed at lower temperature under the normal pressure, resulting in a higher sugar recovery than acid pretreatment. However, the pretreatment is required for a relatively long time (e.g., several hours or days or even weeks) (Bali et al. 2015). After alkaline pretreatment, the cell walls of biomass are swollen, resulting in the substantial increase of pore volume and internal surface area. During the pretreatment, the alkali agents can saponify the uronic ester linkages of 4-*O*-methyl-*D*-glucuronic acid (GlcA/MeGlcA) attached to the xylan backbone (Rabemanolontsoa and Saka 2016), forming charged carboxyl groups and cleaving the chemical linkages between lignin and some hemicelluloses (McMillan 1994). Scanning electron microscope (SEM), X-ray diffraction (XRD), and Fourier transform infrared spectrometer (FTIR) have been used for the characterization of alkaline-pretreated biomass. Compared with untreated biomass, the pretreated one had an increase in porosity and a greater surface area (Janu et al. 2011). NH₃·H₂O and Ca(OH)₂ pretreatment can increase the cellulose accessibility (Bali et al. 2015). Aqueous ammonia shows potential application for biomass pretreatment at mild pretreatment temperature, and the remaining ammonia can be used as nitrogen source for microbial growth during biofuels production (Yoo et al. 2011). Aqueous ammonia pretreatment is used to selectively remove lignin from biomass while the major part of polysaccharides (cellulose and hemicellulose) is still in biomass (Chong et al. 2017a, b; Liu et al. 2013), and the accessible surface of polysaccharides to cellulases can increase (Pryor et al. 2012). 48.5% of lignin in bamboo shoot shell was removed by aqueous ammonia (25 wt%) at 50 °C for 1 day (Chong et al. 2017a, b). After sugarcane bagasse was pretreated with aqueous ammonia (28%) at 160 °C for 1 h under near 1.0 MPa, high enzymatic saccharification (87%) was obtained (Aita et al. 2011). After switchgrass was treated with aqueous ammonia

(15%) at 120 °C for 1 day, hemicellulose (34.6%) and cellulose (2.4%) in switch-grass could be bioconverted into the complex of oligomers and lignin-carbohydrate (Gupta and Lee 2010).

Recently, alkalic salt (He et al. 2017a, b, c, d, e; Qing et al. 2016) pretreatment has been used to promote enzymatic saccharification rate due to its low corrosivity, and this pretreatment alters the biomass ultrastructure, porosity, and chemical composition (Mendes et al. 2011), with the consequent increase in enzyme accessibility to cellulose (Chong et al. 2018b; Kumar et al. 2011; Mendes et al. 2015; Zhu et al. 2009). After $\text{Na}_2\text{CO}_3/\text{H}_2\text{O}_2$ (40% Na_2CO_3 , 15% H_2O_2) was used for pretreating corn stover at 120 °C for 1 h, the total sugar yield achieved at 79% (Gong et al. 2015). After corn stover was pretreated with $\text{Na}_3\text{PO}_4/\text{Na}_2\text{S}$ (4% Na_3PO_4 , 10% sulfidity) at 120 °C for 40 min, most of cellulose and hemicellulose were hydrolyzed into soluble sugars (Qing et al. 2016). It is known that the sulfite (SO_3^{2-}) can cleave α -alkyl ether linkages, α -benzyl ether linkages, and β -benzyl ether linkages on phenolic lignin units as well as the sulfonation of lignin, thus enhancing the enzymatic saccharification (Liu et al. 2016; Yang et al. 2013).

5.4 Ionic Liquid Pretreatment

Ionic liquids (ILs) are one kind of organic salts which generally exist in liquid state at room temperature under normal pressure because of their low melting point (Andanson and Costa Gomes 2015; He et al. 2015b; Rabemanolontsoa and Saka 2016). IL pretreatment of lignocellulosic materials has gained a great attention due to IL's virtues of excellent solvency, low melting point, nonvolatility, designability, and recyclability (He et al. 2016b). An asymmetric organic cation with an organic or inorganic counterpart can compose IL (Rabemanolontsoa and Saka 2016). Cellulose-dissolving ILs with anions of chloride, formate, acetate, or alkylphosphonate have been prepared for the pretreatment of biomass because they form strong hydrogen bonds with cellulose at elevated temperature (He et al. 2016c; Zhao et al. 2009a, b). Solubility of cellulose has been found up to 39 and 25% (w/w) for IL 3-methyl-*N*-butylpyridinium chloride ([BMpy]Cl) and 1-*N*-butyl-3-methylimidazolium chloride ([Bmim]Cl), respectively (Li et al. 2009). 1-Alkyl-3-methylimidazolium chloride ([Amim]Cl) has been used to pretreat various woods. IL can selectively extract the components of biomass to decrease the crystalline structure of biomass, even when the biomass is not completely dissolved with IL. During IL pretreatment, the extraction of lignin occurs simultaneous with the decrease in residual cellulose crystallinity (Xu et al. 2016a). The decrease in non-productive binding of cellulases on lignin can effectively enhance the enzymatic saccharification efficiency. IL 1-ethyl-3-methylimidazolium acetate ([Emim]Ac) can be effectively used to liquefy wood (Clough et al. 2015). IL 1-ethylpyridinium bromide ([Epy]Br) is more reactive toward lignin than cellulose and hemicellulose (Kanbayashi and Miyafuji 2015). Despite IL's promising chemical properties, ILs are expensive and require tedious recycling (de Oliveira and Rinaldi 2015; He et al.

2016b), which restrict their application. Additionally, their toxicity and biodegradability of ILs are not yet well understood (Rabemanolontsoa and Saka 2016).

Recently, another family of ionic fluids, deep eutectic solvents (DES), is employed for the pretreatment of lignocellulosic biomass (Procentese et al. 2015; Xu et al. 2016b). DES is typically synthesized by the combination of hydrogen bond acceptor molecules (e.g., quaternary ammonium salts) and hydrogen bond donor molecules (e.g., organic acid or alcohol). Choline chloride (ChCl) and glycerol (Gly), ethylene glycol (EG), formic acid (FA), glycolic acid (GA), lactic acid (LA), malonic acid (MA), and oxalic acid (OA) have been used for pretreating lignocellulosic materials (Dai et al. 2017), and these solvents can effectively remove hemicellulose and lignin (Xu et al. 2016b; Zhang et al. 2016).

5.5 Organosolv Pretreatment

Organosolv pretreatment can be efficiently used for the extraction and removal of lignin in lignocellulosic biomass (He et al. 2016a; Ostovareh et al. 2015). Organosolv pretreatment can be conducted in various organic or aqueous-organic solvent media with or without added catalysts at 100–250 °C (Ostovareh et al. 2015). Different organic solvents including low boiling points alcohols (e.g., methanol and ethanol), higher boiling point alcohols (e.g., ethylene glycol and glycerol), and other organic compounds (e.g., *N*-methylmorpholine-*N*-oxide, dimethyl sulfoxide, ethers, and ketones) have been attempted to pretreat lignocellulosic biomass (He et al. 2016a; Ostovareh et al. 2015; Zhang et al. 2007; Zhao et al. 2009a, b).

Non-derivatizing solvent *N*-methylmorpholine-*N*-oxide (NMMO) has gained a great interest to pretreat biomass because of its low volatility and flammability (Biganska and Navard 2009). NMMO can be used for dissolving cellulose in biomass due to the high polarity of its N–O bond (Kuo and Lee 2009), which cleaves the hydrogen bond network of the cellulose and forms new hydrogen bonds with the solute (He et al. 2013). NMMO pretreatment can be conducted below 100 °C under atmosphere pressure. Additionally, NMMO can be recovered due to its low vapor pressure (Li et al. 2012). Cellulose withdrawn from NMMO solutions has also generated increased saccharification rates by cellulases thus implying its potential use in pretreating lignocellulosic materials for biofuels (He et al. 2013).

Low boiling point alcohols (e.g., methanol and ethanol) have attracted more attention due to the high effectiveness on the pretreatment of lignocellulosic biomass (Ostovareh et al. 2015), as well as the simplicity of their recovery by distillation (Mesa et al. 2011; Taherzadeh and Keikhosro 2008; Zhao et al. 2009a, b). Various acids have been added to the pretreatment media for enhancing the efficiency of organosolv pretreatment with low boiling point alcohols (Koo et al. 2012; Teramoto et al. 2008). Eucalyptus wood pretreated with ethanol-water solvent containing acetic acid as catalyst yielded reducing sugar with the yield of 100% (Teramoto et al. 2008). An ethanol-based organosolv pretreatment can significantly

remove lignin, decrease the degree of polymerization, reduce the crystallinity of cellulose, and enhance the digestibility of cellulose (Hallac et al. 2010; Ostovareh et al. 2015; Teramoto et al. 2008).

Recently, high boiling organic solvent pretreatments have also gained a great interest to avoid the high-pressure operation without highly volatile and flammable solvents (He et al. 2016b). Glycerol (boiling point 290 °C) and ethylene glycol (EG) (boiling point 197.6 °C) are known as the most favored high boiling solvents for pretreating biomass (He et al. 2016a). Aqueous glycerol (80%) was used to pretreat sugarcane bagasse at ~200 °C for 2.5 h, and 81% of delignification was obtained (Novo et al. 2011). Dilute HCl (0.1%) could assist organosolvlysis of pulp at 180 °C for 6 min, and the sugar was obtained at 53% yield (Liu et al. 2010). Under the microwave irradiation (200 W) at 100 °C for 5 min, the lignin in corn stover could be effectively removed with EG-HClO₄-water (88.8:1.2:10, w/w/w) media (He et al. 2015c).

6 Physical-Chemical Pretreatment

6.1 Steam Explosion

Steam explosion pretreatment, a typical combination of mechanical forces and chemical effects, is one of the most commonly used physico chemical methods for pretreating lignocellulosic materials (Agbor et al. 2011; Grous et al. 1986; Jacquet et al. 2012). Lignocellulosic materials are subjected to high pressure saturated steam at the temperatures of 160–260 °C and the pressure of 0.7–4.8 MPa for given pretreatment time (Kumar and Sharma 2017), resulting in the hydrolysis and release of hemicellulose. The steam enters the lignocellulosic biomass expanding the cell walls of fibers leading to partial hydrolysis and increasing the cellulose accessibility to cellulases (Kumar and Sharma 2017; Rabemanolontsoa and Saka 2016). During steam explosion, the hydrolysis of hemicellulose into soluble sugars (glucose and xylose) is conducted by acetic acid produced from the cleavage of acetyl groups in hemicellulose (Mosier et al. 2005). The main factors that affect steam explosion pretreatment are residence time, pretreatment temperature, chip size, and moisture content. Lower pretreatment temperature and longer residence time are more favorable (Rabemanolontsoa and Saka 2016). Lower pretreatment temperature (190 °C) and longer residence time (10 min) were found to be better as compared to high temperature (270 °C) and lower residence time (1 min) due to less generation of fermentation inhibitors in the earlier process (Kumar and Sharma 2017; Wright 1988). Limited use of chemicals, low energy consumption, and environment friendly are the advantages of steam explosion. However, the possibility of formation of fermentation inhibitors at high temperature and incomplete digestion of lignin-carbohydrate matrix are disadvantages of steam explosion (Agbor et al. 2011).

6.2 *Liquid Hot Water Pretreatment*

Liquid hot water (LHW) is known as one of the most promising pretreatment methods due to the advantages of no additive chemicals requirement, which is also termed aqueous fractionation, aquasolv, solvolysis, hydrothermolysis, and hydrothermal pretreatment (Agbor et al. 2011; Rabemanolontsoa and Saka 2016). LHW pretreatment is conducted with water at 170–230 °C under the high pressure up to 5 MPa, which can remove most of hemicellulose and part of lignin in biomass (Rabemanolontsoa and Saka 2016). A two-step hydrolysis by hot compressed water was carried out as follows (Kumar and Sharma 2017): First stage is conducted at low pretreatment severity for hydrolysis of hemicellulose in biomass, while second stage is performed at high pretreatment severity for depolymerizing cellulose (Abdullah et al. 2014). During LHW pretreatment, no fermentation inhibitors (e.g., furfural and HMF) were found (Yang and Wyman 2004). Although LHW can be conducted at low temperature with low cost of the solvent, a large quantity of water is required to be recovered in downstream processing (Agbor et al. 2011).

6.3 *Oxidative Pretreatment*

It involves pretreatment of biomass with oxidizing agents including hydrogen peroxide (H_2O_2), ozone (O_3), oxygen (O_2), or air (Alvira et al. 2010; Lucas et al. 2012; Nakamura et al. 2004; Saha and Cotta 2007; Yu et al. 2009). During the oxidative pretreatment, a series of chemical reactions (e.g., electrophilic substitution, side chain displacements, and oxidative cleavage of aromatic ring ether linkages) may happen, which help in the conversion of biomass. Lignin can be catalyzed by the peroxidase in the presence of H_2O_2 (Cao et al. 2012; Hammel et al. 2002). The pretreatment of sugarcane bagasse with H_2O_2 greatly enhanced its susceptibility to enzymatic saccharification (Azzam 1989; Bjerre et al. 1996). Wet oxidation is considered as one of the simple processes for pretreatment of lignocellulosic biomass where the air/oxygen along with H_2O or hydrogen peroxide (H_2O_2) can be used for pretreatment of biomass at over 120 °C (Chaturvedi and Verma 2013; Varga et al. 2003). Wet oxidation is an aqueous high-temperature high-pressure pretreatment method that uses oxidative agents (Arvaniti et al. 2012; Szigártó et al. 2009). The mechanism lies on formed hydroxyl radicals and autocatalyzing by formed organic acids (Arvaniti et al. 2012). Wet oxidation, which has been effectively applied to pretreat wood, wheat straw, corn stover, organic household waste, sugarcane bagasse, and other biomass materials, is a pretreatment strategy that can be used for clover-ryegrass mixtures (Martín et al. 2008). Three main factors including oxygen pressure, reaction temperature, and reaction time have significant influence the efficiency of wet oxidation. When wet oxidation is conducted at over 170 °C, water molecule can act as an acid molecule that catalyzes the hydrolysis of biomass.

During the pretreatment, hemicellulose can be hydrolyzed from lignocellulosic biomass under the acidic condition resulting in the release of smaller pentose monomers, and the degradation of lignin in biomass undergoes oxidation by oxidizing agents, while wet oxidation pretreatment has a slight influence on the structure of cellulose. Wet oxidation combined with base could readily oxidize lignin in wheat straw, thus enhancing its enzymatic hydrolysis (Kumar and Sharma 2017). Sodium carbonate (Na_2CO_3) and alkaline peroxide in wet oxidation decreased the pretreatment temperature, increased the degradation of hemicellulose, and avoided the formation of inhibitors (e.g., furfurals and HMF) (Banerjee et al. 2011). Alkaline peroxide-assisted wet air oxidation (APAWAO) approach solubilized 67% of hemicellulose and 88% of lignin in rice husk (Banerjee et al. 2011). Wet oxidation of rice husk at 185 °C for 15 min under 0.5 MPa pressure yielded 67% of cellulose, and 89% of lignin and 70% of hemicellulose were removed (Banerjee et al. 2009).

6.4 SPORL Pretreatment

To overcome recalcitrance of lignocellulosic biomass, sulfite (SO_3^{2-}) pretreatment (SPORL) can be used as an efficient pretreatment strategy for the enhancement of lignocellulosic biomass (Xu et al. 2016c). It can be conducted in a combination process: Biomass is pretreated with CaSO_3 or MgSO_3 for removing hemicellulose and lignin fractions. Subsequently, the particle size of the pretreated lignocellulosic biomass can be reduced significantly with mechanical disk miller. After spruce chips was pretreated by SPORL pretreatment using 8–10% bisulfite and 1.8–3.7% H_2SO_4 at 180 °C for 0.5 h (Zhu et al. 2009), over 90% of substrate was converted with 15 FPU cellulase/g substrate plus 23 CBU β -glucosidase/g substrate, and fermentation inhibitors, 0.5% HMF and 0.1% furfural, were detected. Switchgrass was pretreated with SPORL process on performed at 163–197 °C for 3–37 min with 0.8–4% H_2SO_4 0.6–7% Na_2SO_3 (Zhang et al. 2013). Parts of hemicellulose and lignin in switchgrass were removed, and the hydrophobicity of lignin was decreased after sulfonation. Reducing sugars were obtained from SPORL-pretreated switchgrass at 83% yield with 15 FPU cellulase/g substrate and 30 CBU β -glucosidase/g substrate. SPORL pretreatment has been regarded as a popular method because of its versatility, efficiency, and simplicity (Idrees et al. 2013). However, sugar degradation, requirement of large quantity of water for post-pretreatment washing, and high recovery cost of pretreatment chemicals need to be addressed for making SPORL a cost-effective pretreatment technology (Kumar and Sharma 2017). Jiang et al. developed one-pot pretreatment with dilute alkali salts $\text{Na}_2\text{SO}_3/\text{Na}_3\text{PO}_4$ (0.4% Na_3PO_4 , 0.03% Na_2SO_3) and hot water (DASHW) for pretreating sugarcane bagasse by autoclaving at 110 °C for 40 min (Jiang et al. 2017). Furthermore, the reducing sugars and glucose from the enzymatic in situ saccharification of 50 g/L $\text{Na}_2\text{SO}_3/\text{Na}_3\text{PO}_4$ pretreated sugarcane bagasse in dilute $\text{Na}_2\text{SO}_3/\text{Na}_3\text{PO}_4$ (0.27% Na_3PO_4 , 0.02% Na_2SO_3) media were obtained at 33.8 and 21.8 g/L, respectively.

6.5 Ammonia Fiber Explosion (AFEX) and Ammonia Recycle Percolation(ARP)

AFEX and ARP have been effectively pretreated biomass with aqueous ammonia (AA) (Kim and Lee 2005; Sun and Cheng 2002). AFEX can be normally performed at ambient temperature, while ARP is preferably carried out at a high temperature (Alizadeh et al. 2005). Over 90% of cellulose and hemicellulose in biomass pretreated with AFEX can be hydrolyzed into fermentable sugars. At high pressure and given temperature, ammonia can cause swelling and phase change the cellulose crystallinity in biomass, which results in the increase in the reactivity of leftover carbohydrates (Kumar and Sharma 2017). Notably, AFEX pretreatment does not produce inhibitors (e.g., furfural and HMF). No extra detoxification is required. It is highly desirable in the practical application. Additionally, ammonia can be recovered and recycled for the decrease of overall pretreatment cost. Ammonia recycle percolation (ARP) process preferably solubilizes hemicellulose, but cellulose remains unaffected (Alvira et al. 2010). The disadvantage of ARP is high energy requirement for maintaining operation temperature.

6.6 CO₂ Explosion

CO₂ explosion is a carbon dioxide-based pretreatment of biomass via supercritical CO₂ (Kyoungheon and Hong 2001). The supercritical CO₂ fluid is forced into a high pressure vessel containing lignocellulosic materials at the required temperature and time (Hendriks and Zeeman 2009). CO₂ can enter the lignocellulosic materials at high pressure, and subsequently it forms carbonic acid (H₂CO₃) which hydrolyzes hemicellulose. The disrupted structure of biomass provides more accessible surface area (Zheng et al. 1995). It was found that the higher moisture content in lignocellulosic biomass resulted in higher saccharification yield (Kyoungheon and Hong 2001). CO₂ explosion requires the low cost of CO₂ to pretreat the high solid loading of biomass at low temperature. During the CO₂ explosion, no toxin forms, which makes it an attractive for biomass pretreatment. However, high cost of high-pressure equipment is required, which restricts its practical application on a large scale (Srinivasan and Ju 2010).

7 Biological Pretreatment

It is known that hemicelluloses, lignin, and polyphenols can be utilized by biological pretreatment (BP) involving the action of bacteria and fungi with high substrate specificity, low energy cost, and no generation of toxic chemicals (Aguilar and Ferraz 2008; Aguilar et al. 2006, 2013; Cianchetta et al. 2014; Dai et al. 2015; Guerra

et al. 2003; Kandhola et al. 2017a, b; Monrroy et al. 2011; Sindu et al. 2016; Larran et al. 2015). Fungi are highly efficient degradation candidates of biomass and play an essential role in the global carbon cycle and ecology (Kandhola et al. 2017a, b; Koray Gulsoy and Eroglu 2011; Mäkelä et al. 2014). Fungal pretreatment has been used as an effective way for enhancing enzymatic saccharification of lignocellulosic biomass for producing biofuel or bio based products (Mäkelä et al. 2014; Monrroy et al. 2011; Ryu et al. 2013). White-rot and brown-rot fungi are known to be good candidates for pretreating biomass. White-rot fungi are major lignin degraders in nature (Ryu et al. 2013). White-rot fungi (*Phanerochaete chrysosporium*, *Phlebia radiata*, *Trametes versicolor*, etc.) can degrade lignin either selectively or non-selectively through oxidases. Lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase are extracellular lignin degradation enzymes of white-rot fungi (Aguiar and Ferraz 2008; Aguiar et al. 2006; Guerra et al. 2003). LiP executes the H_2O_2 -dependent C_α - C_β cleavage of lignin model compounds and subsequently catalyzes the partial depolymerization of methylated lignin in vitro (Saratale et al. 2010). MnP can oxidize Mn^{2+} to chelated Mn^{3+} using H_2O_2 as oxidant on phenolic or non-phenolic lignin units. Laccase belongs to the family of blue multi-copper oxidases that can catalyze the one-electron oxidation of aromatic amines, phenolics, and other electron-rich substrates via the reduction of O_2 to H_2O (Sánchez 2009). Brown-rot fungi preferentially degrade wood carbohydrates and partially oxidize lignin (Monrroy et al. 2011; Schilling et al. 2009).

Recently, several bacteria (e.g., *Pseudomonas* and filamentous bacteria known as *Actinomycetes*) can be used to delignify plant cell wall. During the biological pretreatment, catalase, demethylase, LiP, MnP, peroxidase, and phenol oxidase excreted from bacteria have been identified (Rabemanolontsoa and Saka 2016). Some bacteria with lignin depolymerization enzymes can degrade lignin and its derivatives because of their immense biochemical versatility and environmental adaptability (Chong et al. 2018c; Salvachúa et al. 2015). Biological pretreatment with bacteria results in cross-linking, cleavage of C_α - C_β bonds, alkyl-aryl cleavage, solubilization of lignin, and demethylation. Various high value-added products including 4-ethoxy-3-methoxybenzaldehyde, vanillin, vanillic acid, guaiacol, and protocatechuic acid can be produced under mild conditions (Godden et al. 1992; Rabemanolontsoa and Saka 2016).

However, most biological pretreatments are found to be a very slow pretreatment process that requires careful process control (Dai et al. 2017). To effectively pretreat lignocellulosic biomass, the combination with BP and another pretreatment has been successfully be more. Combining *E. taxodii* with dilute H_2SO_4 (0.25%) to pretreat water hyacinth (Ma et al. 2010), the saccharification could be increased to twofold than that of H_2SO_4 -pretreated one. Combination of bacterial pretreatment with NaOH/Urea pretreatment at low temperature (-20 to -10 °C) was conducted to enhance enzymatic saccharification of rice straw (Dai et al. 2015). These studies indicated that combination of biological and other pretreatment is a promising strategy for improving enzymatic hydrolysis of biomass (Dai et al. 2017).

8 Summary of Biomass Pretreatment Methods

Many kinds of lignocellulosic biomass have been used as cheaper starting materials for the production of biofuels. One pretreatment technology to help in the rapid and efficient for one type of lignocellulosic material might be not suitable for pretreating another material. As shown in Table 1.2, major advantages and disadvantages of these common technologies for pretreating lignocellulosic materials are summarized. The choice of the pretreatment technology to help in effective conversion of a particular lignocellulosic biomass depends on its composition and the by-products produced as a result of pretreatment. These factors significantly affect the costs associated with a pretreatment method. There have been some reports comparing

Table 1.2 Summary of several pretreatment processes

Pretreatment process	Advantages	Limitations and disadvantages
Acid pretreatment	Hydrolyzes hemicellulose to xylose and other sugars; alters lignin structure	High cost; equipment corrosion; formation of inhibitors
Alkaline pretreatment	Removes hemicelluloses and lignin; increases accessible surface area	Long residence times required; irrecoverable salts formed
AFEX	Increases accessible surface area of biomass, removes lignin and hemicellulose to an extent; does not produce inhibitors	Not efficient for biomass with high lignin content
Biological pretreatment	Degrades lignin and hemicelluloses; low energy consumption	Low hydrolysis rate; long pretreatment time
CO ₂ explosion	Increases accessible surface area; cost-effective; does not cause formation of inhibitory compounds	Does not modify lignin or hemicelluloses
Ionic liquid	Perform with thermal stability, inflammability, low volatility and recyclability; remove lignin effectively	High recovery cost; potential toxicity
Liquid hot water	Requires low cost of solvent and produces minimum of inhibitors at low pretreatment temperature	Requires high energy consumption in downstream process
Mechanical comminution	Reduces cellulose crystallinity	High energy consumption
Organosolv	Hydrolyzes lignin and hemicelluloses	Solvents need to be drained from the reactor, evaporated, condensed, and recycled; high operation cost
Ozonolysis	Reduces lignin content; does not produce toxic residues	Large amount of ozone required
Steam explosion	Causes hemicellulose degradation and lignin removal	Destruction of a portion of the xylan fraction; incomplete disruption of the lignin-carbohydrate matrix; generation of inhibitor at high temperature

various pretreatment methods for biomass (Agbor et al. 2011; Brodeur et al. 2011; He et al. 2016b; Hendriks and Zeeman 2009; Rabemanolontsoa and Saka 2016; Rosgaard et al. 2007; Silverstein et al. 2007; Sindu et al. 2016; Wyman et al. 2005b). Cost-effective pretreatments for enhancing the enzymatic saccharification deserve in-depth investigation.

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Chapter 2

Metabolic Engineering of Fungal Strains for Efficient Production of Cellulolytic Enzymes



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Abstract Filamentous fungi are widely used for production of cellulase and other cellulolytic enzymes. Metabolic engineering of filamentous fungal strains has been applied to improve enzyme production, and rapid progress has been made in the recent years. In this chapter, genetic tools and methods to develop superior enzyme producers are summarized, which includes establishment of genetic modification systems, selection and redesign of promoters, and metabolic engineering using either native transcription factors or artificial ones. In addition, enhancement of cellulase production through morphology engineering was also discussed. Emerging tools including CRISPR-Cas9-based genome editing and synthetic biology are highlighted, which are speeding up mechanisms elucidation and strain development, and will further facilitate economic cellulolytic enzyme production.

Keywords Filamentous fungi · *Trichoderma reesei* · Cellulase · Promoter engineering · Artificial transcription factor · Genome editing · Metabolic engineering

1 Introduction

Lignocellulosic biomass are abundant in nature, which includes agricultural residues, forestry wastes, as well as energy crops. In China, it was estimated that 600–700 million tons of agricultural residues are produced annually (Xie et al. 2010).

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Production of biofuels, especially cellulosic ethanol (Zhao et al. 2016), as well as biochemicals from these cellulosic biomass (Zhang et al. 2016a) has received considerable interest in the past decades. To release fermentable sugars from lignocellulosic biomass, enzymatic hydrolysis is commonly used. Therefore, efficient production of cellulolytic enzymes is important for the process economy.

Filamentous fungi are major cellulase producers and are also widely utilized for producing other industrial enzymes. Cellulase production from the fungal species of *Trichoderma reesei* (Bischof et al. 2016), *Neurospora crassa* (Znameroski et al. 2012), *Aspergillus niger* (Stricker et al. 2008), and *Penicillium oxalicum* (Li et al. 2017b) is of special interests and have been intensively studied. In recent years, filamentous fungi have gained attention as cell factories for enzyme production. However, high cost of cellulase production is still one of the bottlenecks for industrialization of lignocellulosic bioconversion.

More than 70 years ago, the ascomycete *T. reesei* was isolated, and its potential to encode cellulase and hemicellulase for biomass degradation was investigated (Bischof et al. 2016). Despite the fact that some other filamentous fungi can produce efficient cellulose-hydrolyzing enzyme cocktail, *T. reesei* is still widely used for industrial cellulase production. Besides, genetic engineering of *T. reesei* has been conducted for more than 30 years, which benefits regulatory mechanisms studies on enzyme production and have achieved powerful cellulolytic mixtures by the fungus (Peterson and Nevalainen 2012). Therefore, in this chapter, we would mainly focus on genetic engineering of *T. reesei* for cellulase production.

In the past decades, the regulatory network of cellulase expression and enzyme secretion has been investigated in details. Metabolic engineering of the widely used cellulase producer *T. reesei* has been reviewed previously (Kubicek et al. 2009). In the recent years, development of high-throughput sequencing technologies has led to remarkable advancement in systems biology studies, and abundant data have been obtained including genomic sequences, global transcriptomic profiles from RNA sequencing, proteomics data and so on (Liu et al. 2013). On the other hand, new methods and tools have been developed to improve the efficiency of genetic engineering of filamentous fungi. In this context, it is optimistic to see the new era of strain development, which is based on deeper understanding of the regulatory mechanisms of enzyme production and advanced technologies of genetic engineering.

In this chapter, we summarized recent progress of genetic tools and methods to develop superior enzyme producers, which includes establishment of genetic modification systems, selection and redesign of promoters, and metabolic engineering using either native transcription factors or artificial ones. Enhancement of cellulase production through morphology engineering was also discussed. Emerging tools including genome editing and synthetic biology are highlighted, which are speeding up studies on mechanism elucidation and strain development, and will enable economic cellulase production for bioconversion of lignocellulosic biomass.

2 Selective Markers for Genetic Engineering of Filamentous Fungi

Establishment of transformation methods is the first step for genetic engineering of filamentous fungi. The transformation methods mainly include protoplast transformation, electroporation, and *Agrobacterium*-mediated transformation. The detailed transformation methods were described elsewhere (Malmierca et al. 2015; Chakraborty 2015; Frandsen 2015). Besides the transformation methods, efficient selective markers used in filamentous fungi are also key factors for obtaining the target transformants. Many dominant selectable markers including the nutritional markers and positive selection resistance markers are being employed, which were summarized in Table 2.1.

Nutritional markers of *pyrG* gene (*A. nidulans*) or *pyr4* gene (*N. crassa*) encoding orotidine-5'-phosphate decarboxylase were used to complement uracil auxotrophs (Gruber et al. 1990; Gao et al. 2012). *pyrG/pyr4* deletion mutants were easily selected by 5-fluoroorotic acid (5-FOA) resistance, whereas the wild-type strains could not grow on the toxic compound 5-FOA. Similar systems employing the *trpC* (Yelton et al. 1984) or *acuD* (Beri and Turner 1987) genes as the auxotrophic markers are also available. In addition, the *niaD* marker encoding the nitrate reductase was widely used to obtain the chlorate-resistant mutant, which has been successfully used in transformation of *A. niger* (Unkles et al. 1989). Moreover, the *amdS* gene (encoding acetamidase) was also employed as the nutritional markers for *Aspergillus* (Kelly and Hynes 1985) and thermophilic *Myceliophthora* (Liu et al. 2017) species by selecting for acetamide utilization, since acetamide is a poor nitrogen source for the wild-type strains.

Table 2.1 Representative selective markers used in the transformation of filamentous fungi

Marker gene	Transformed fungi	References
<i>acuD</i>	<i>A. nidulans</i>	Beri and Turner (1987)
<i>amdS</i>	<i>A. niger</i> and <i>Myceliophthora</i> sp.	Kelly and Hynes (1985) Liu et al. (2017)
<i>asl1</i>	<i>T. reesei</i>	Derntl et al. (2013)
<i>bar</i>	<i>A. niger</i>	Ahuja and Punekar (2008)
	<i>N. crassa</i>	Matsu-ura et al. (2015)
<i>hisB</i>	<i>A. niger</i>	Fiedler et al. (2017)
<i>hph</i>	<i>N. crassa</i>	Honda and Selker (2009)
<i>niaD</i>	<i>A. niger</i>	Unkles et al. (1989)
<i>ptrA</i>	<i>T. reesei</i>	Wang et al. (2013a)
<i>pyrG</i>	<i>T. reesei</i>	Liu et al. (2015)
<i>Shble</i>	<i>T. reesei</i>	Chen et al. (2016)
<i>trpC</i>	<i>A. nidulans</i>	Yelton et al. (1984)
<i>ura5</i>	<i>T. reesei</i>	Liu et al. (2015)

The use of nutritional markers is important in the progress of genetic engineering of filamentous fungi. However, auxotrophic selection requires strains with specific mutations and culturing in specialized media, which may have negative effects on cell growth. In contrast, the positive resistance markers are nowadays widely applied in the studies of filamentous fungi transformation with the advantage of that the genotype of the recipient strains need not to be known in advance (Ruiz-Díez 2002). The transformation method with the resistance markers is easy to operate, and vectors carrying the resistance marker gene cassette can be transformed into the recipient strains directly, and the mutants tolerate no background growth in the standard media. The hygromycin-B resistant marker was used in the early study of *A. nidulans* transformation (Punt et al. 1987) and *T. reesei* (Zhang et al. 2017; Li et al. 2017a). The other frequently used selection resistance marker genes include pyri-thiamine gene (*ptrA*) (Wang et al. 2013a), phleomycin gene (Chen et al. 2016), and phosphinothricin gene (Ahuja and Puneekar 2008). However, the major drawback of using these kinds of resistance markers is that the resistance of the wild-type strains may not show significant dominance, resulting in selection difficulties. Moreover, some of those antibiotics are often expensive.

Although the transformation methods and useful resistance markers have been established currently, the lack of selective markers and the problem of false-positive transformants are still the obstacles of filamentous fungi genetic engineering. Screening the positive transformants with two selection markers, for example, with fluorescent protein and hygromycin-B as double reporter genes, would be helpful (Noh et al. 2010).

In cellulolytic synthesis filamentous fungi, such as *Trichoderma* sp. and *Penicillium* sp., no stable autonomously replicating plasmid was found so far. Therefore, once the resistance marker was introduced, it would be integrated into the host genomic DNA, which was difficult to remove for a second round of genetic engineering. The Cre-*loxP* system has been developed as an important molecular tool to overcome these genetic limitations and also should reduce public concerns involving environmental release of resistant strains. The Cre recombinase from bacteriophage P1 of *Escherichia coli* catalyzes recombination between the two 34 bp *loxP* sites, each of them has two 13 bp Cre-binding sites which are interrupted by an 8 bp spacer region (Sternberg and Hamilton 1981). The system was first investigated in *E. coli* (Sternberg and Hamilton 1981) and utilized widely later in fungi, such as *T. reesei* (Steiger et al. 2011) and *Aspergillus* sp. (Forment et al. 2006). The expression of Cre recombinase was usually controlled by inducible promoters thus providing a means to switch on or off. In filamentous fungi, the xylanase promoter was frequently employed which can be induced by many kinds of carbohydrates, such as xylose and cellulose (Steiger et al. 2011; Forment et al. 2006). Moreover, Tet-on and Cre-*loxP*-based systems were successfully employed in *P. oxalicum* and Cre recombinase was activated by doxycycline which was controlled by the Tet-on system (Jiang et al. 2016).

The FLP/*FRT* recombination system was an alternative to the Cre-*loxP* system. Similar with the Cre-*loxP* system, heterologous expression of FLP recombinase from 2 μ m plasmid of *Saccharomyces cerevisiae* could facilitate resistance marker

gene disruption between the two specific DNA sequences in a single cell. The system has been successfully tested and optimized in fungi *P. chrysogenum* (Kopke et al. 2010). The resulting marker genes deletion strain can be used as a recipient strain for further genetic engineering reapplying the same system.

3 Promoter Engineering to Improve Cellulase Production

T. reesei can secrete many types of cellulolytic enzymes, of which cellulohydrolase 1 (CBH1) is the major component of all secreted proteins. Therefore, *cbh1* promoter has been widely used to induce hyper production of target proteins in *T. reesei*. It is well-known that β -glucosidase (BGL) activity in *T. reesei* is very low. Therefore, overexpression of BGL and other cellulase components in *T. reesei* using the strong inducible promoter of *cbh1* and *cbh2* have been reported, which can significantly improve the corresponding gene expression and ultimately can significantly improve the overall hydrolase activities (Ma et al. 2011; Li et al. 2017a). Improved enzyme production was also reported using the artificial four-copy *cbh1* promoter with repeated positive transcriptional elements and deletion of possible glucose repressor-binding sites (Zhang et al. 2010). Through the rational design of the promoter, it will also significantly affect the transcription and expression of the corresponding genes, such as the engineered *cbh1* promoter, in which the cellulase negative regulatory factor Cre1 binding sites were replaced by transcriptional activator Ace2 and Hap2/3/5 complex binding sites, and the engineered *cbh1* promoter intensity was increased by 5.5 and 7.4 times under induced and inhibited conditions, respectively (Zou et al. 2012).

Wang et al. developed a promoter collection for the expression of alkaline cellulase genes in *T. reesei*, which was employed to construct different expression systems based on several promoters and terminators of *T. reesei*, and produced the most efficient enzyme expression system for bio-stoning, which relied on the action of two enzymes synergistically working to modify the fabric surface (Wang et al. 2014). Therefore, it is necessary to dig out more strong promoters through the transcriptomic data to increase the expression level of the target glycoside hydrolase gene without affecting the main metabolic pathways and the expression of main glycoside hydrolases.

Despite some progress presented above, promoters of filamentous fungi have been only limitedly studied. For advanced metabolic engineering and synthetic biology studies of these important fungal species, artificial promoters such as hybrid promoters, as well as synthetic minimal promoters, which have been successful in budding yeast strains of *S. cerevisiae* (Blazeck et al. 2012; Redden and Alper 2015), can be explored, which will provide different expression levels of key enzymes and regulators for strain development. Construction of modular vectors containing synthetic promoters is the basis for synthetic biology manipulation of filamentous fungi (reviewed by Gupta et al. 2016), and design or screening promoter library can also be combined with different regulators which will be described in the following section to generate functional diversities.

4 Engineering of Transcription Regulators to Improve Cellulase Production

It is well known that cellulase production is regulated on transcription level, and abundant transcription regulators have been identified in different filamentous fungi (Benocci et al. 2017). Comparative transcriptome analysis showed that different types of glycoside hydrolases were induced under different induction conditions. For example, the components of extracellular secretion proteins are different significantly for *T. reesei* when the cellulases were induced by the pretreated lignocellulose, xylan, and sophorose (Hakkinen et al. 2012). In addition, environmental factors, such as pH (Li et al. 2013) and light (Schmoll et al. 2012), also affect cellulase induction. Therefore, understanding the regulatory network is important to engineer cellulase production in filamentous fungi.

Regulation of biomass-degrading enzymes in *T. reesei* was recently reviewed (Gupta et al. 2016), and new findings are also emerging in the later studies. The cel-lulolytic and xylanolytic gene expression in *T. reesei* is coordinately regulated by the action of at least four transcriptional activators (Xyr1, Ace2, Ace3, and Hap2/3/5 complex) and three repressors (Cre1, Ace1, and the recently reported regulator Rce1) (reviewed by Seiboth et al. 2012; Cao et al. 2017). Xyr1 (xylanase regulator 1) is the key positive transcriptional activator; and lack of *xyr1* eliminates the induction of cellulase and xylanase by all known inducers (Stricker et al. 2006). Cre1 is the major negative regulator mediating carbon catabolite repression (CCR) by inhibiting both basal and the inducible expressions, and moreover also prevents *xyr1* gene expression. In *T. reesei*, *cre1* transcription was autoregulated under non-inducing conditions, and *xyr1* gene was therefore shown at a low basal level (Lichius et al. 2014). Overexpression of *xyr1* improved glycoside hydrolase production under inducing conditions, while deletion of *cre1* eliminated CCR, but the catabolic de-repression was not sufficient to increase glycoside hydrolase production, suggesting that hyperproduction is still inducer dependent (Wang et al. 2013b; Nakari-Setälä et al. 2009).

RNA interference was used to regulate the expression of Cre1 in *T. koningii*, causing the transcription of *cre1* gene to be disturbed to varying degrees, and finally increased the total cellulase activity by more than two times (Wang et al. 2013b). In addition, Wang et al. also carried out overexpression of positive regulator Xyr1 in *T. reesei* and RNA interference of Ace1 at the same time. Under the double effect, extracellular protein secretion and filter paper activity from *T. reesei* Rut-C30 mutant are improved more than one time (Wang et al. 2013c).

Transcription factor proteins are consisted of DNA-binding domain and effector domain. Previous study showed that the chimeric transcription activator, which contains two DNA-binding domains from repressors Cre1 and Ace1 and effector domain from activator Ace2, could bind to both Cre1 and Ace1 binding sites in promoters of cellulase genes to activate gene expression (Su et al. 2009). Additionally, lots of *xyr1* and *cre1* binding sites exist in the promoter of major cellulase and xylanase gene (Castro et al. 2014). In *T. reesei* Rut-C30, Cre1 is truncated and loses its

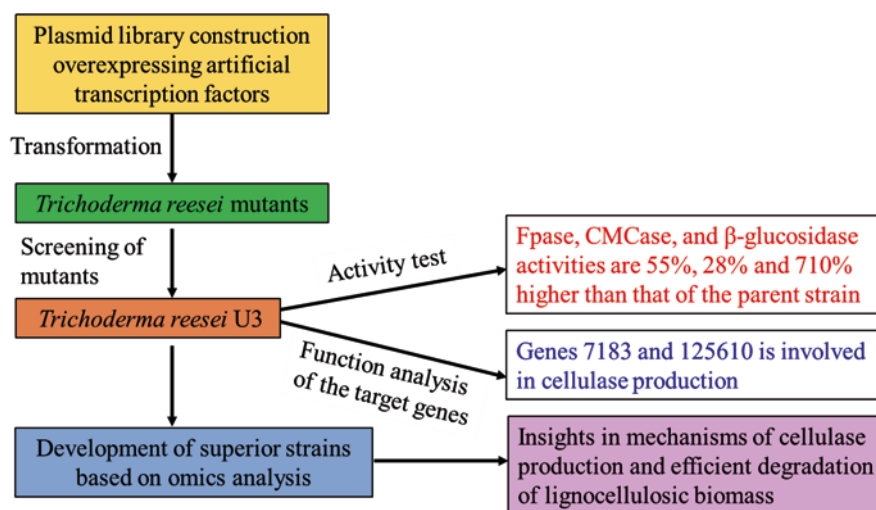


Fig. 2.1 Flow diagram for cellulase production by *T. reesei* Rut-C30 derivative strains engineered by an artificial transcription factor (ATF) library. Detailed information was described in the main text in Sect. 4

DNA binding ability, making the binding sites in the promoter idled (Seidl et al. 2008). In a recent report from our group, constitutively expression of an artificial activator containing both the Cre1 DNA-binding domain, the Xyr1 DNA-binding domain and effector domain, enhanced cellulase production in the presence of glucose (Zhang et al. 2017).

In addition to manipulating natural transcription factors, artificial transcription factor was also attempted to improve cellulase production in *T. reesei*. In the recent studies in our group, the designed artificial zinc finger transcription factor containing various DNA-binding domain and Gal4 effector domain was introduced into *T. reesei* Rut-C30, and cellulase hyper producer was screened. One of the mutants showed a 55% rise in filter paper activity and an 8.1-fold increased β-glucosidase activity (Zhang et al. 2016c). The scheme of the strain development using the artificial zinc finger protein was elucidated in Fig. 2.1. Interestingly, we found elevation of *Trvib-1* in the selected mutant, and we tested further the effect of its overexpression on cellulase production in *T. reesei* Rut-C30. *Vib-1* was reported to exert control of cellulase production in *N. crassa* (Xiong et al. 2014), and it was found that *Trvib-1*, the homologous gene of *vib-1* from *T. reesei*, can complement the deletion of *vib-1* in *N. crassa*. We found improvement of cellulase production in *T. reesei* Rut-C30 by overexpression of *Trvib-1* (Zhang et al. 2018), and the involvement of this gene in regulation of cellulase production was also reported by another group (Ivanova et al. 2017). Therefore, novel regulatory mechanisms can be revealed by studying the mutant carrying the artificial transcription factor. It can also be expected that in the future, more artificial transcription factors can be used to modulate cellulase production in filamentous fungi.

It is worth noting that the regulation of cellulase production is not only restricted at the transcription level but also affected by chromatin remodeling (Mello-de-Sousa et al. 2015). Studies on epigenetic control of cellulase production will offer novel metabolic engineering strategies to develop superior fungal strains.

5 Improvement of Cellulase Production by Morphology Engineering

Filamentous fungi exhibit complex morphological changes during submerged culture, which is of great interest for process optimization. Freely suspended mycelia and pellets with varying sizes can be found commonly in different filamentous fungal cultures. Although there are still some debates on the relationship of morphology and enzyme productivity, positive correlation of morphology with enzyme production was reported in *T. reesei* (Novy et al. 2016; Yu et al. 2012), *N. crassa* (Sun et al. 2014), and *A. niger* (Driouch et al. 2011). For example, relatively higher agitation speed increased pellet diameter and cellulase productivity of *T. reesei* Rut-C30, and on the other hand, cellulase activity and cell viability were sensitive to impeller shear (Yu et al. 2012). It is therefore important to control balanced pellet morphology, which is influenced by many process parameters, including medium composition, inoculation size, culture mode, pH value, temperature, osmolality, aeration, and so on (Krull et al. 2013). It was discussed that properly controlled pellet size and inner structures allowed a reduced viscosity and facilitate oxygen mass transfer (Driouch et al. 2011). So far many related studies of morphological control focused on process optimization, and very little is known on the underlying molecular mechanisms and regulatory networks. Recently, studies using 95 morphology mutants of *N. crassa* revealed that improved cellulase production and protein secretion was achieved in pellet-forming mutants, but not in the mutants with long hyphae (Sun et al. 2014). Until now, no similar report on other filamentous fungi was found. It will be interesting to explore whether genetic engineering can be applied to finely modulate the morphology of filamentous fungi for efficient production of cellulosic enzymes.

6 Metabolic Engineering of Filamentous Fungi Using CRISPR-Cas9-Based Genome Editing System

The type II CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 (CRISPR-associated protein 9) system has recently been developed to enable rapid genome editing in various organisms (Doudna and Charpentier 2014), including various filamentous fungal species (Fuller et al. 2015; Liu et al. 2015; Matsuura et al. 2015; Nødvig et al. 2015; Pohl et al. 2016; Liu et al. 2017).

Table 2.2 Examples of CRISPR-Cas9 systems in cellulase-producing filamentous fungi

Strain name	Genome editing strategy	References
<i>T. reesei</i>	In vitro transcription of gRNA, and transformed into <i>T. reesei</i> overexpressing codon-optimized Cas9	Liu et al. (2015)
<i>A. niger</i>	In vitro synthesis of sgRNA and transformed into <i>A. niger</i> strain carrying the Cas9 plasmid	Novy et al. (2016)
<i>P. chrysogenum</i>	Transforming the CRISPR-Cas9 ribonucleoproteins (RNPs) either by in vitro assembly of the Cas9-sgRNA complex or by expression the complex using the AMA1 based vector	Pohl et al. (2016)
<i>N. crassa</i>	In vivo expression of Cas9 and single crRNA:tracrRNA chimeric gRNA in separate circular plasmids which were cotransformed together with circular donor plasmids	Matsu-Ura et al. (2015)
<i>Myceliophthora</i> sp.	Expressing sgRNA using endogenous U6 promoter, and Cas9 using tef1 promoter, and then transforming the sgRNA cassette, Cas9 expression cassette, and the PCR amplified donor DNA into the cell	Liu et al. (2017)

The CRISPR-Cas9 system contains two components: the effector protein, which is the endonuclease Cas9, and a single chimeric guide RNA (sgRNA). In addition to Cas9, new effector protein Cpf1 was also developed and employed for genome editing (Zetsche et al. 2015). Although so far there is no report using Cpf1 in filamentous fungi, it is expected that novel effector proteins including Cpf1 will be utilized to improve efficiency of genome engineering of this important group of fungi. The sgRNA provides a 17–20 bp guide sequence that defines the target DNA, and the guide sequence was found adjacent a DNA motif (the PAM, protospacer adjacent motif) of three bases (NGG or TTN). The sgRNA binds to the effector protein and targets a specific locus in the recipient genome, where a double-strand break (DSB) will be introduced. The DSB can then be repaired by the host cell repair systems. In most cases, DSB is fixed by the error-prone non-homologous end-joining (NHEJ) mechanism (Fuller et al. 2015; Pohl et al. 2016). This can lead to random insertions or deletions within the target sequence. If a DNA share homology flanks closing to the DSB (a so-called donor DNA, dDNA) is available, homologous recombination (HR) will happen. And then the donor DNA can either replace or modify the target gene. Thus, the CRISPR systems can be used both for the deletion and insertion of genes (Pohl et al. 2016; Liu et al. 2017), resulting in marker-free gene disruption, deletion, or insertion (Doudna and Charpentier 2014).

The first example of genome editing in filamentous fungi was reported by Liu et al. (2015), which established the CRISPR-Cas9 system in *T. reesei*. Later on, such system was also successful in different filamentous fungal species, and Table 2.2 summarized related studies on cellulase-producing filamentous fungi, and the different genome editing methods were also briefly reviewed.

The efficient application of the CRISPR system requires the heterologous expression of the effector protein (Cas9 or Cpf1) fused to a nuclear localization signal (NLS) and simultaneous expression of the sgRNA (Schuster et al. 2016). For the expression of Cas9, two main strategies, in vitro and in vivo, have been established. In the case of in vitro, the Cas9-sgRNA ribonucleoprotein complex was formed

in vitro to edit the target genes (Pohl et al. 2016). In the case of in vivo, the codon of Cas9 gene followed by a stronger NLS should be optimized for expression in filamentous fungi (Matsu-Ura et al. 2015; Fuller et al. 2015; Liu et al. 2015). In addition, different promoter systems also influence the expression of Cas9. When the strong constitutive promoters were used, the efficiency of genome editing should be improved; however, this way may lead to possible off-target effects. In order to realize the controllability of this system, some inducible promoters have been selected to inhibit Cas9 expression for minimal off-target effects (Liu et al. 2015; Pohl et al. 2016).

In addition to optimizing the expression of Cas9 gene, looking for optimal functional sgRNA modules was also very important (Schuster et al. 2016). The synthetic sgRNA needs to be transcribed using RNA polymerase III promoters (Nødvig et al. 2015). However, these promoters are poorly defined in filamentous fungi. For the expression of sgRNAs, two main strategies, in vitro and in vivo, have also been established. In some organisms, such as the *T. reesei* and *A. niger*, sgRNAs were generated in vitro and then co-transformed to the protoplasts together with a Cas9 gene expression cassette or the Cas9 protein (Zhang et al. 2016b). This strategy is suitable for almost all organisms. However, the stability of sgRNAs should be taken into consideration. It has been reported that RNA polymerase III promoters such as SNR52 and U6 and some tRNA promoters can be applied to transcribe sgRNAs in some of filamentous fungi, such as *N. crassa* and *P. chrysogenum* (Fuller et al. 2015; Matsu-Ura et al. 2015; Schuster et al. 2016). However, due to uncertainty of genome sequence and the complexity of genetic background, the endogenous RNA polymerase III promoters from filamentous fungi are difficult to be identified or not suitable for sgRNA transcription. Thus, the most common method to express sgRNAs in vivo is to use 5'-end hammerhead (HH) and 3'-end hepatitis delta virus (HDV) to flank the sgRNA (Nødvig et al. 2015), and then RNA polymerase II promoter can be used to express sgRNAs.

Compared with the traditional genetic engineering technology applied in filamentous fungi, CRISPR-Cas9 system has clear advantages. Firstly, it is simple. The same Cas9 can be used to target different genes, only sgRNA is different. Secondly, it is efficient. The CRISPR-Cas9 system not only can be used to rapidly manipulate single gene but also to modify multiple sites at the same time. Third, it is flexible. It enables marker-free engineering of the strain, therefore facilitating metabolic engineering of the strain by manipulating multiple genes. Fourth, it is not toxic. No difference of cell growth and sporulation was observed when cells were expressing Cas9 (Liu et al. 2017).

The disadvantage of the CRISPR-Cas9 system is its off-target effect. Therefore, precise genome editing will be developed, which can be achieved by high-fidelity CRISPR-Cas9 nucleases (Kleinstiver et al. 2016) and optimization of sgRNA design (Doench et al. 2016). Second, only deletion and replacement were achieved in filamentous fungi. Activation or repression of gene expression using this system as well as epigenome editing will also be pursued, which has been successful in mammalian system (Hilton et al. 2015; Konermann et al. 2015). It can be expected that the CRISPR-Cas9 system as well as similar genome editing method will be further

improved in the near future in filamentous fungi, and functional genomic studies of filamentous fungi will be greatly promoted by these genome editing technologies, which will further enhance the efficiency of metabolic engineering manipulations.

7 Conclusion and Future Prospects

The economy of lignocellulosic biomass bioconversion is still hampered by high production cost of cellulase. In the past decades, significant progress has been made in understanding the induction and regulation of cellulase biosynthesis through functional genomic studies as well as multi-omics analysis. On the other hand, efficient genetic engineering methods have been developed to modify filamentous fungi, and these novel methods include marker-reuse techniques, promoter engineering, artificial transcription factor library, and genome editing using CRISPR-Cas9 systems. Development of systems biology and synthetic biology is providing new targets and concepts for construction of hyper producers for cellulase production.

Establishment of efficient genetic engineering platform of filamentous fungi is of great importance for development of hyper producing strains. Nowadays, most related reports only focused on single gene function; there is a lack of comprehensive metabolic engineering studies on filamentous fungi. It is expected that more progress of metabolic engineering of filamentous fungi will be made in the near future including but not being restricted in the below aspects:

1. Functional genomic studies of filamentous fungi

It is vital to deepen the functional genomic studies of filamentous fungi, and various databases describing omics data, mutant phenotypes, function of transcription regulators, metabolites, and so on, should be developed for researchers to obtain detailed information for further functional genomic analysis. Metabolic engineering of filamentous fungi will rely on not only efficient genetic engineering and genome-editing methods, but also advanced techniques on analysis of gene expression and metabolites.

2. Exploration of various genetic elements for metabolic engineering

These elements include promoters (both natural and synthetic ones), integration sites, selection markers, as well as transcription factors (both endogenous and artificial ones) for efficient metabolic engineering and synthetic biology design of filamentous fungi. The Cas9-based toolkit for different filamentous fungi will be developed, as has been achieved in the budding yeast *S. cerevisiae* (Reider et al. 2017).

3. Development of genome editing techniques

The fast-growing knowledge on genome editing of mammalian cells will also lead to optimization of the genome editing techniques of filamentous fungi. So far most studies focused on metabolic engineering of filamentous fungi through transcriptional

control of cellulase biosynthesis. Taking advantage of the CRISPR/dCas9 system, epigenetic regulation of cellulase production will also be manipulated to achieve precise control on chromatin dynamics.

The results obtained from the above mentioned studies will not only allow production of low-cost and efficient enzyme cocktails toward a better process economy, but also will lead to deeper understanding of the complex regulatory network of filamentous fungi, which is essential to further develop this important group of microorganisms as powerful microbial cell factories.

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Chapter 3

Lignocellulase Formation, Regulation, and Secretion Mechanisms in *Hypocrea jecorina* (*Trichoderma reesei*) and Other Filamentous Fungi



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Abstract *Trichoderma reesei* is the anamorph form of *Hypocrea jecorina* (*H. jecorina*) and belongs to a soft rot ascomycetal fungus that is used in commercial applications, such as the production of enzymes; this fungus is an efficient cell factory for protein production, a property that is exploited by the enzyme industry. The most important property of *H. jecorina* for commercial applications is that it can secrete a variety of cellulases involved in lignocellulose hydrolysis, a property that has made *H. jecorina* the most widely used cellulase in the world, producing filamentous fungi for research and other applications. In this chapter, the functions of transcription factors that regulate cellulase and hemicellulase will be introduced, and the expression of cellulases and hemicellulases regulated by chromatin remodeling will also be discussed. Furthermore, the transcriptional regulation of cellulase and hemicellulase in *H. jecorina* by external signals will be discussed.

Keywords *Hypocrea jecorina* · Cellulase · Transcriptional factors · Chromatin remodeling

1 Introduction

In nature, there are many microbes that can efficiently decompose and utilize cellulose. Endogenous cellulase genes are also found in microorganisms, plants, and animal species. However, compared to bacteria, plants, and animals, filamentous

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fungi have the highest cellulase activity and secrete a variety of degradative enzymes. The filamentous fungi species that have been widely used in the industrial production of enzymes include *Trichoderma* sp., *Aspergillus* sp., *Penicillium* sp., *Rhizopus* sp., and *Myrothecium* sp., Although the genome of *H. jecorina* contains more than 200 genes responsible for glycoside hydrolysis (Samuels 1996), which is far less than the number of such genes in other filamentous fungi, the ability to secrete cellulase is greater in *H. jecorina* than in other filamentous fungi (Martinez et al. 2008; Saloheimo and Pakula 2012). According to statistics, 80% of the cellulase used for cellulosic ethanol production in the world is from *H. jecorina* (Bischof et al. 2016).

In the production of cellulosic ethanol, the lignocellulosic biomass is degraded into fermentable sugar under the synergistic effect of enzymes, including three major classes of enzymes: (1) endo-beta-1,4-glucanases (EGs), also known as 1,4-b-D-glucan-4-glucan hydrolases (EC 3.2.1.4); (2) cellobiohydrolases (CBHs), also known as exoglucanases, or exogenous glucosidases (exo-β-1,4-glucanases, EC 3.2.1.91); and (3) β-glucosidases (BGs), also known as cellobiases (cellobiase, CB, EC 3.2.1.21). In addition, recent studies have found that enzymes that degrade cellulose through oxidative mechanisms are classified as copper-dependent lytic polysaccharide monooxygenases (LPMOs). LPMOs comprise four auxiliary activity (AA) families of carbohydrate-degrading enzymes (AA9, AA10, AA11, and AA13). Among them, AA9 (formerly GH61) are eukaryotic LPMOs which are related to the degradation of cellulose, whereas AA10 (formerly CBM33) are bacterial (Eibinger et al. 2014). For more details, please see Chap. 10.

The expression of the abovementioned enzymes is regulated by different factors, including a variety of physiological and environmental factors, such as transcription factors, chromatin remodeling and external signals.

2 Transcriptional Mechanisms that Regulate Cellulase and Hemicellulase Gene Expression

Efficient production of cellulases and hemicellulases is achieved through precise gene regulation including carbon-dependent transcription factors and other regulatory factors. The identification of genes responsible for the transcriptional regulation of cellulase gene expression may result in a major breakthrough in the understanding of the mechanisms of cellulase production.

In this section, we summarized the transcriptional regulators of cellulase and hemicellulase production in various filamentous fungi, as shown in Table 3.1.

Table 3.1 Transcriptional regulators of cellulase and hemicellulase production

<i>H. jecorina</i> (T. reesei)		<i>Penicillium oxalicum</i>		<i>Neurospora crassa</i>		<i>Aspergillus niger</i>		Function	Positively/negatively regulate cellulase expression
Gene ID	Gene name	Gene ID	Gene name	Gene ID	Gene name	Gene ID	Gene name		
120117	Cre1	PDE_03168	CreA	NCU08807	CreA	An02g03830	CreA	Carbon catabolite regulation	–
75418	Ace1	PDE_01988	AceA	NCU09333	AceA	An16g02040	Ace1	A repressor of cellulase expression	–
72611	Rce1	PDE_03127 ^a	No	NCU03643 ^a	No	ND	No	A repressor of cellulase expression	–
52368	BgIR	PDE_03964	AmyR	NCU07788	Col-26	ND	No	β-glucosidase regulator	–
122208	Xyr1	PDE_07674	XlnR	NCU06971	XlnR	An15g05810	XlnR	Xylanase regulator 1	+
26163	Clr2	PDE_05883	ClrB	NCU08042	Clr-2	An12g01870	ClrB	Cellulose degradation regulator-2	+
78445	AceII	ND	No	NCU06907	Ace2	An02g07000	Ace2	Cellulase transcriptional activator	+
77513	AceIII	PDE_09536 ^a	No	ND	No	ND	No	Cellulase transcriptional activator	+
54675	Vib1	ND	No	NCU03725	No	ND	No	Vegetative transcription factor	+
27600	Clr1	ND	No	NCU07705	Clr-1	An16g06200	ClrA	Hypothetical transcription factor	N
41617	Lae1	PDE_00584	LaeA	NCU00646	LaeA	An01g12690	LaeA	Methyl transferase	+
124286	Hap2	ND	No	NCU03033	Hap2	An15g03650	Hap2	Protein complex subunit HAP2	+
122284	Vel1	ND	No	NCU01731	Ve-1	An08g05100	No	Velvet protein complex subunit	+

^aMeans uncharacterized
“ND” means that no homolog was detected
“N” means unknown
“+/-” means the value for cellulase activity is up regulated or down regulated

2.1 Transcriptional Factors

In *H. jecorina*, Xyr1 (xylanase regulator 1), the homolog of XlnR in *A. niger*, plays an important role in the xylose-mediated induction of *xyn1*. Xyr1 is a zinc binuclear cluster protein that binds to an inverted repeat (GGCTAA motif) in the *xyn1* promoter (Rauscher et al. 2006). Sequential researches showed that in addition to the inverted repeats, single 5'-GGC(A/T)₄-3' motif was also critical in Xyr1-mediated gene expression. Stricker et al. (2006) showed that Xyr1 is necessary for the expression of the main cellulase and hemicellulase genes, including *cbh1*, *cbh2*, *egl1*, and *xyn1* (xylanases 1), regardless of the structure of the inducer or the expression pattern (basal, derepression or induction at the transcriptional level). The Δ *xyr1* strain grew poorly on both D-xylose and xylan compared to the parent strain. Those results indicated that Xyr1 plays an important role in the expression of the genes encoding the xylanolytic and cellulolytic enzymes and in D-xylose metabolism in *H. jecorina* (Stricker et al. 2006). Moreover, it was shown that Ace1 directly inhibits Xyr1 function by competing for one of its binding sites (the right GGCTAA box) in the *xyn1* promoter (Rauscher et al. 2006; Stricker et al. 2008a). It was hypothesized that the intense interplay between Ace2 and Xyr1, including several phosphorylation steps and likely heterodimerization, as well as the recruitment of additional proteins, is vital for the production of an active xylobiose-dependent *xyn2* transcriptosome (Stricker et al. 2008a, b). Portnoy reported that the complete expression of Xyr1 and Ace1 requires the presence of *cre1* under lactose culture conditions (Portnoy et al. 2011).

In 2001, Aro et al. (2001) reported that Ace2 in *H. jecorina* has a typical zinc binuclear cluster DNA-binding domain near the N-terminal and is identified as a positive transcription factor; disruption of the *ace2* gene led to reduced expression of *xyn2*, *cbh1*, *cbh2*, *egl1*, and *egl2* in *H. jecorina* grown on a medium containing cellulose. Ace2 also affected xylanase expression in *H. jecorina*; transcription of *xyn2* was reduced in the *ace2* deletion strain. However, cellulase induction by sophorose was not affected in the *ace2* deletion strain (Aro et al. 2001). Further study by Stricker showed that in the *ace2* deletion strain, a faster initial inducibility was observed under inducing conditions (xylan, xylobiose), but final levels of *xyn2* transcripts and xylanase activity did not reach those of the parent strain. Phosphorylation and dimerization were shown to be prerequisites for the binding of Ace2 to the *xyn2* promoter. Moreover, Ace2 was shown to influence *xyn2* expression by binding to the *xyn2* promoter or by affecting the regulation of *xyr1* transcription (Stricker et al. 2008a, b).

Coradetti and Glass (2012) reported that CLR-1 and CLR-2 in *Neurospora crassa* (*N. crassa*) were identified by transcriptional profiling with next-generation sequencing methods and were shown to be indispensable for the induction of all major cellulase and some major hemicellulase genes in *N. crassa* (Coradetti and Glass 2012). Moreover, CLR-1 was found to be necessary for *clr-2* expression and for efficient utilization of cellobiose. And it was suggested that CLR-1 and CLR-2 are conserved in the genomes of most filamentous ascomycete fungi which are

capable of degrading cellulose by phylogenetic analyses (Coradetti et al. 2013; Li et al. 2015). In *A. nidulans*, ClrB, which is the CLR-2 homolog in *N. crassa*, failed to induce cellulase gene expression, and the ClrB deletion strain lacked cellulolytic activity on Avicel (Coradetti and Glass 2012). In *P. oxalicum*, the *clrB* gene was identified as the *clrB* homolog in *N. crassa*, and it was found to encode a protein of 780 amino acid residues (Coradetti et al. 2012). Li et al. (2015) reported that ClrB could directly bind to the *cbh1* promoter region and that a dose-dependent effect of *clrB* transcriptional abundance is important for the high expression for cellulases (Li et al. 2015). To date, the ClrB homologous protein and its functions in *H. jecorina* have not been reported.

In 2014, Ace3 was reported to be essential for cellulase and hemicellulase production in *H. jecorina*. Compared to the parental strain, the expression level of the genes *cbh1*, *cbh2*, *egl1*, *axe1*, and *xyn3* was almost undetectable in the *ace3* deletion strain. Besides, the expression level of *bxl1*, *xyn1*, *xyn2*, *bgl1*, and *xyr1* was also lower than the parental strain. Moreover, xylanase activity and the expression of xylan-degrading enzymes were also significantly reduced in the *ace3* deletion strain (Häkkinen et al. 2014). The function of Ace3 should be investigated in more detail in the future.

Recently, another transcription factor, VIB1, which is also involved in cellulase induction, was identified in *N. crassa*. VIB1 neither directly regulates hydrolytic enzyme gene expression nor functions in cellulosic inducer signaling/processing, but it affects the expression level of CLR2 (Xiong et al. 2014). Ivanova et al. (2017) reported that a homolog of *vib1* exists in the genome of *H. jecorina* and is a key regulator of cellulases in *H. jecorina*, although its exact mechanism remains to be elucidated. Deletion of the *vib1* gene resulted in reduced cellulase expression in both strains QM9414 and Rut-C30. And QM9414 is a moderate-producing strain, and Rut-C30 is a high-producing strain. In contrast, the overexpression of *vib1* had no effect on cellulase production both in QM9414 and Rut-C30, and it was suggested that *vib1* is already expressed at an optimal level under normal conditions (Ivanova et al. 2017).

Carbon catabolite repression (CCR) is a mechanism that preferred carbon source (usually glucose) and prevents microorganisms from the utilization of secondary sources, which is primarily regulated by transcription factors (Stülke and Hillen 1999). CCR occurs in filamentous fungi, and CreA (Cre1), CreB, CreC, and CreD have been identified as the major regulatory proteins affecting CCR. CreA (Cre1) is a global transcription factor required for the repression of many genes subject to CCR (Bernhard et al. 2011), and CreB, CreC, and CreD are involved in ubiquitination/deubiquitination networks (Lockington and Kelly 2001, 2002; Boase and Kelly 2004).

In the initial study on CCR in filamentous fungi, Dowzer and Kelly (1989) reported that CreA mediated CCR in *Aspergillus nidulans* (*A. nidulans*) (Dowzer and Kelly 1989). They showed that in the presence of glucose, expression levels for certain cellulase genes were low. Then, Drysdale discovered CreA in *Aspergillus niger* (*A. niger*) in 1993 (Drysdale et al. 1993). In 1995, Strauss et al. (1995) reported that Cre1 was the carbon catabolite repressor in *H. jecorina*. Cre1 in *H. jecorina*

shares 46% sequence identity with CreA in *A. nidulans* by amino acid alignment, and Cre1 has zinc fingers of the C2H2 type (Ilmen et al. 1996). EMSA (electrophoretic mobility shift assay) and *in vitro* footprinting revealed the binding of Cre1 to the sequence 5'-GCGGAG-3' in *H. jecorina*, which matches well with the *A. nidulans* consensus sequence for CreA binding (5'-SYGGRG-3') (Strauss et al. 1995). The functional analysis of the *cbhl* promoter revealed that there were two reverse repeats of Cre1 binding sites approximately 700 bp upstream of the transcription initiation site, and mutations at this site resulted in the derepression of certain genes when the mutant was grown in a glucose medium (Ilmen et al. 1996). However, it should be noted that Cre1 does not participate in the regulation of certain cellulases and hemicellulase genes, such as *cbh2*, *xyn2*, or *bglI* (Margolles-Clark et al. 1997; Zeilinger et al. 2003). In terms of function, Cre1 is a very important protein in cellulase regulation, because Cre1 regulates not only the expression of cellulase enzymes but also transcriptional factors in *H. jecorina*. Demonstrating the function of Cre1 as a domain-wide repressor of particular cellulase genes, it was found that there was growth inhibition in the $\Delta cre1$ strain (Nakarisetälä et al. 2009). Furthermore, it was reported that the complete expression of the positive transcription factors Xyl1 and Ace1 requires the presence of Cre1 under lactose culture conditions (Portnoy et al. 2011). Thus, the deletion of *cre1* in *H. jecorina* is not a wise strategy for improving cellulase production.

Although Cre1 in *H. jecorina* is homologous to Mig1 in *Saccharomyces cerevisiae* (*S. cerevisiae*), there is a significant difference between their CCR mechanisms. In yeast, Mig1 enters and exits the nucleus, and the subcellular localization of Mig1 is regulated by glucose; Mig1 enters the nucleus when glucose is present, and after glucose has been consumed, Mig1 is transported back to the cytoplasm (De Vit et al. 1997). Furthermore, phosphorylation has an important effect on the nuclear localization of Mig1 in yeast cells. Treitel reported that Mig1 is negatively regulated by the Snf1-kinase and is transported to the cytoplasm in response to glucose (Treitel et al. 1998). In *H. jecorina*, Cziferszky et al. (2002) found that Cre1 is also a phosphorylated protein and that the conserved site that is phosphorylated by the casein kinase CKII (HSNDEDD) is found near the S241 residue of the acidic site, thereby enabling Cre1 to be phosphorylated at position S241 (Cziferszky et al. 2002). The S241E mutation of Cre1 in *H. jecorina* mimics phosphorylation, whereas a S241A mutant protein leads to the permanent binding of Cre1 to DNA, independent of phosphorylation. Thus, it was suggested that phosphorylation is required to release Cre1 from an inactive conformation wherein S241 is unphosphorylated. The site that is phosphorylated by casein kinase CKII is required to be in an acidic environment; therefore, a S241 V mutation is neither phosphorylated nor capable of binding to DNA to eliminate CCR (Cziferszky et al. 2002). Nguyen et al. (2016) found that the phosphorylation of S241 fluctuated over time when sophorose was used as the carbon source (Nguyen et al. 2016).

It was shown that the CreA protein in *A. nidulans* binds to ubiquitin to form ubiquitinated protein. The ubiquitinated form of CreA is relatively unstable and is

susceptible to protease degradation. CreB is a de-ubiquitinated protein which can stabilize the CreA protein (Lockington and Kelly 2001; Alam and Kelly 2017). The filter paper enzyme activity, endogenous cellulase activity, xylanase activity, exo-cellulose content, and the content of extracellular protein were found to be improved in the *creB* deletion strain of *Penicillium oxilicium* (*P. oxilicium*, formerly *P. decumbens*) (Zhou et al. 2012). Similar work was later done in *Aspergillus oryzae* and *H. jecorina*. Denton and Kelly (2011) reported that in *H. jecorina*, the disruption of *cre2*, which encodes an ubiquitin C-terminal hydrolase, resulted in increased cellulase activity (Denton and Kelly 2011).

Enzymes involved in sugar transport and phosphorylation play an essential role in signal generation by different mechanisms in CCR. The underlying mechanisms of the regulation were different among different bacteria. The mechanism of lactose-glucose diauxie in *Escherichia coli* has been reinvestigated and was found to be caused mainly by inducer removal (Stülke and Hillen 1999). In addition, the gene encoding HPr kinase was discovered recently which was reported to be a key factor of CCR in many bacteria (Deutscher et al. 1995; Boël et al. 2003).

Subsequently, the transcription factor Ace1 in *T. reesei* was identified (Saloheimo et al. 2000). The *ace1* gene encodes a DNA-binding protein containing three Cys₂-His₂ zinc finger structures. It was shown via EMSA that there are at least eight binding sites for Ace1 in the *cbh1* promoter. In addition, Saloheimo et al. (2000) reported that the growth of a *H. jecorina* strain on a cellulose-containing medium was retarded when the *ace1* gene was disrupted (Saloheimo et al. 2000). Furthermore, Aro et al. (2003) reported that the deletion of *ace1* resulted in an increase in the expression of all the main cellulase genes (including *cbh1*, *cbh2*, and *eg1*) and two xylanase genes (*xyn1* and *xyn2*) in sophorose- and cellulose-induced cultures. Therefore, it was suggested that Ace1 also acts as a transcriptional repressor of cellulase and xylanase expression. At same time, it was suggested that Ace1 was specific to filamentous fungi, because there is no homologous protein for Ace1 in *S. cerevisiae* (Saloheimo et al. 2000).

Rce1, a transrepressor recently found in *H. jecorina*, regulates the expression of cellulases (Cao et al. 2017). Rce1 has a typical zinc binuclear cluster DNA-binding motif (Zn(II)₂Cys₆) at its N-terminus (Macpherson et al. 2006). Rce1 was screened by a yeast-based one-hybrid method. Cao et al. (2017) found that the expression of cellulase genes was significantly improved in the *rce1* deletion strain, but the expression of xylanases induced by xylan remained unchanged. Additionally, it was shown that Rce1 was not involved in Cre1-mediated catabolite repression. The mechanism through which Rce1 regulates cellulase gene expression is to compete for the binding sites of Xyr1 on the *cbh1* promoter; this mechanism was discovered using competitive binding assays (Cao et al. 2017).

In addition, the β -glucosidase regulator BglR was identified as an activator for efficient β -glucosidase expression (except for *bgl1*) in *H. jecorina*. The *H. jecorina* strains lacking the *bglr* gene exhibited elevated cellulase production during growth on cellobiose due to the accumulation of intracellular cellobiose (Nitta et al. 2012).

2.2 Other Regulatory Factors

The Hap2/3/5 complex proteins in *T. reesei* are the homologs of HapB/C/E complex proteins in *A. nidulans*. The CCAAT box, which is one motif of CAE (*cbh2* activating element), is a common cis-acting element found in the promoter and enhancer regions in many eukaryotic genes. So far, all identified CCAAT box-binding proteins belong to the HAP-like factors in filamentous fungi and yeast (Zeilinger et al. 2001). Zeilinger reported that in *H. jecorina*, the CCAAT box in the sequence of CAE (*cbh2* activating element) is bounded by Hap complex (Zeilinger et al. 2001).

Seiboth et al. (2012) reported that the putative methyltransferase Lae1, which is the LaeA orthologue from *Aspergillus* sp., controls cellulase synthesis in *H. jecorina*. A complete loss of expression including all seven cellulases, β -glucosidases, xylanases, and auxiliary factors for cellulose degradation was observed in the *lae1* deletion strain (Seiboth et al. 2012). The results showed that in *H. jecorina*, Ve1 has an important role in cellulase gene expression (Karimi et al. 2014), and Liu et al. (2016) proposed that the velvet family proteins, Ve1, Ve2, and Ve3, in *H. jecorina*, which are the homologs of VeA, VeB, and VeC in *A. nidulans*, are involved in sporulation, morphogenesis, and cellulase expression. The three velvet-deficient strains showed that Ve3 plays a minor role, whereas Ve1 and Ve2 play major roles in cellulase expression.

3 Chromatin Remodeling

Chromatin is a highly organized structure that results from the compaction of eukaryotic genomic DNA through conserved histone proteins. DNA, which has a negative charge, is wrapped around histones (Ries et al. 2013). The transcription process in eukaryotic begins with chromatin remodeling. To interact with histones and facilitate the assembly of transcriptional complexes, DNA needs to be free so that RNA polymerase and the transcription factors can access the promoter region and thus initiate transcription. Neutralizing the positive charge in histones may be able to free the DNA strand. To accomplish that, the acetylation of specific lysine residues can be catalyzed by histone acetyl transferase (HAT) within the N-terminal tails of core histones. This process is considered to be vital to chromatin remodeling (Csordas 1990; Xin et al. 2013).

The ScGcn5 protein in *S. cerevisiae* is one of the most well-studied eukaryotic HATs (Georgakopoulos and Thireos 1992). Transcription activation depends on the HAT activity of Gcn5; mutation of the histone acetyl transferase domain of Gcn5 eliminated transcription activation (Wang et al. 1998). The homolog of ScGcn5 in *H. jecorina* (TrGcn5) was identified and studied at the chromatin level, and it was shown that the histone acetylation activity of TrGcn5 is essential for the efficient induction of cellulase gene expression in the *cbh1* promoter by the acetylation of

histone H3 (Xin et al. 2013). Gcn5 also plays an important role in the growth and morphogenesis of filamentous (Xin et al. 2013).

Nucleosome localization in *H. jecorina* has been studied within the promoter region of *cbh2* (Zeilinger et al. 2003) and the *cbh1* promoter region (Ries et al. 2013). It was found that under all of the conditions tested, Cre1 was essential for strict nucleosome positioning in the 5' regulatory sequences of *cbh2*, and induction could occur in a promoter that lacks positioned nucleosomes. Additionally, Cre1, the Hap2/3/5 complex, and the GTAATA-binding protein were all shown to be involved in nucleosome assembly on the *cbh2* promoter. The GTAATA-binding protein and the Hap2/3/5 complex respond to inducing conditions by relocating nucleosomes (Zeilinger et al. 2003). Cre1 was shown to bind to several consensus recognition sequences in the *cbh1* promoter region *in vitro*, and it was also found that Cre1 was vital for correcting nucleosome positioning within the *cbh1* coding region under repressing conditions (Ries et al. 2013).

It has been shown that chromatin status changes in response to the change of carbon source. Chromatin opens during sophorose-mediated induction, and chromatin gets denser during D-glucose-conferred repression. Further study was performed with the *xyl1* deletion strain, and it was shown that *xyl1* influenced the status of chromatin concerning different carbon resources (Mello-de-Sousa et al. 2015). There are additional regulators found in the transcriptional data on lignocellulose-derived materials. They belong to different InterPro domains, including IPR013056, IPR000953, IPR008251, IPR000182, IPR 013178, IPR001214, IPR000330, IPR001025, IPR 001487, and IPR000210 (Hakkinen et al. 2014). Studies showed that chromatin-level regulation also occurs in the regulation of the CAZy genes of *H. jecorina* (Karimiaghcheh et al. 2013). The putative methyltransferase LAE1 of *H. jecorina*, a homolog of LaeA in *A. nidulans*, which can globally affect the expression of multiple secondary metabolite gene clusters, also functions at the chromatin level to regulate the secondary metabolism in *H. jecorina*, although the exact mechanism is not fully understood (Karimiaghcheh et al. 2013).

4 Transcriptional Regulation of Cellulase and Hemicellulase in *H. jecorina* by External Signals

It is well-known that the expression of cellulolytic genes in *H. jecorina* is induced in the presence of cellulose and several disaccharides, such as δ -cellobionono-1,5-lactone, cellobiose, sophorose, and lactose (Zeilinger et al. 2003; Castro et al. 2014). Sophorose is the strongest cellulase inducer. Sophorose is assumed to be formed by a transglycosylation reaction in *H. jecorina* during cellulose hydrolysis and strongly triggers the expression of the *cbh1* gene transcript (El-Gogary et al. 1989). It should be noted that cellulase induction by sophorose was not affected in *H. jecorina* when *ace2* was disrupted (Aro et al. 2001). In contrast, the expression of cellulolytic

genes is repressed when easily metabolized carbon sources, such as glucose, fructose, and sucrose, are added into the culture media (Chambergo et al. 2002).

The regulation of cellulase gene expression is at the transcriptional level and is in a coordinated manner (Castro et al. 2014). Consequently, comparisons of the transcriptional patterns from cultures with various carbon sources can be used to identify cellulase or hemicellulose expression-related genes. In previous research, the transcriptomes of *H. jecorina* grown on glycerol, glucose, and lactose cultures were compared. The results showed that the transcription of all cellulase genes and most of the hemicellulase genes was enhanced on lactose. Additionally, many genes encoding putative transporters which belongs to the major facilitator super (MFS) family were upregulated when lactose was the carbon source. In *H. jecorina*, the transcriptome was richer in the diversity of chitinases, polygalacturonases, and xylanases when the culture was grown on cellulose compared to lactose (Tisch et al. 2011b; Kubicek 2013). By comparing the transcriptomes and differential secretomes (2D-DIGE) of cultures grown on sophorose, cellulose, or glucose as the sole carbon source, Castro et al. (2014) used gene regulatory network analyses to identify 107 and 75 genes that were expressed specifically when cellulose or sophorose was the carbon source, respectively (Castro et al. 2014). A total of 30 proteins were found to be exclusive to sophorose and 37 to cellulose by 2D-DIGE analyses. Thus, it was proposed that the transcriptional regulation of cellulase and hemicellulase in *H. jecorina* is more complicated on cellulose than on sophorose (Dos Santos Castro et al. 2014).

Interestingly, cellulase gene expression is also stimulated by light in *H. jecorina*, and this stimulation is regulated by the ENVOY protein, which contains a PAS/LOV domain. ENVOY shows moderate similarity to Vivid, which is a light desensitization protein in *N. crassa*. In *H. jecorina*, the induction of cellulase transcription was enhanced by light in the QM9414 strain when cellulose was used as carbon source. However, cellulase genes were not expressed in the absence of an inducer under light conditions, so it was suggested that Envoy involved in connecting the light response to carbon source signaling, and light was considered to be an additional external factor that influences the expression of cellulase in *H. jecorina* (Schmoll et al. 2005).

In addition, Schuster et al. (2007) found that ENVOY not only acts as a light-independent repressor for several genes but also influences the growth of *H. jecorina* on several carbon sources under light condition. Moreover, ENVOY impacts the light-dependent regulation of numerous genes in various ways (Schuster et al. 2007). The function of ENVOY, with respect to cellulase expression, is both light and inducer-dependent. The pathway of heterotrimeric G-protein signaling is also involved in this mechanism, especially for GNA1 and GNA3 which were two G-protein alpha subunits likely to transmit nutrient signals. The constitutive activation of the Ga subunit GNA1 enhances the transcription levels of *gna1* when glycerol was used as carbon source, indicating a feedback loop, and the constitutive activation of GNA3 leads to increased transcription of *gna3* on cellulose in light condition. Interestingly, the transcript levels of *gna3* were found to be influenced by ENVOY (Schmoll et al. 2009; Seibel et al. 2009). Castellanos et al. (2010)

demonstrated that light is sensed by BLR proteins (BLR1/BLR2) which comprised the main photoreceptor complex, and they are necessary for regulation of the expression of *env1* and cellulase-encoding genes and mycelia growth under constant illumination. In addition, ENVOY establishes a regulatory negative feedback which is necessary for consequently tolerance to light and photo adaptation (Castellanos et al. 2010). Moreover, in *Trichoderma atroviride*, a connection between BLR-1 and BLR-2 which are both blue light receptors and the cAMP signaling pathway has also been noticed. The extracellular signal is sensed through a receptor, and then the signal is transduced. Tisch et al. (2011a, b) investigated the basic interaction of signal transduction pathways to understand the regulatory networks that make up this fine-tuning mechanism between nutrient signaling and light response, and they found that ENV1 connects the light response pathway with nutrient signaling by the heterotrimeric G-protein cascade by adjusting the transcript levels of *gna1* and *gna3* and acting on cAMP levels – presumably by inhibiting phosphodiesterase (Tisch et al. 2011a). Furthermore, Tisch et al. (2011b) reported that the expression of the genes in *H. jecorina* that have an important role in transcription, translation, signal transduction, transport, and metabolism is regulated by light. Schuster et al. (2012) demonstrated that adenylate cyclase 1 (ACY1) and cAMP-dependent protein kinase A (PKA) which are the crucial components of the cAMP pathway were involved in the regulation of cellulase gene expression in *H. jecorina* (Schuster et al. 2012; Nguyen et al. 2016).

In addition, it was shown that the regulatory mechanism of the velvet complex in *A. nidulans* is light-dependent. In dark conditions, VeA binds to VelB, and then VelB is transported into the nucleus. The complex of VeA, VelB, and LaeA is formed and performs regulatory functions in the nucleus. In contrast, in light conditions, VeA does not enter the nucleus; therefore the velvet complex is not able to be formed (Bayram et al. 2008). Similarly, investigations on *H. jecorina* showed that deletion of the *vel1* locus caused a complete loss of conidiation and impaired the formation of perithecia which is light-independent. Besides, sexual and asexual development of the strain was controlled by VEL1, and this effect is independent of light (Karimi et al. 2014). Liu et al. (2016) suggested that light induces sporulation in *H. jecorina* through velvet family proteins, since compared to dark conditions, more spores were produced under light conditions in both parent and $\Delta vel3$ strains. Moreover, under dark conditions, the colony sizes of the $\Delta vel2$ transformants decreased, and the edges of the colonies became irregular; however, under light conditions, the sizes did not change significantly. Furthermore, their results indicated that there may be an unidentified pathway that overcomes the growth inhibition in the *vel3* deletion strain under light, since the expression of cellulase-coding genes in the *vel3* deletion strain was downregulated in the dark, but their expression in light conditions was unaffected (Liu et al. 2016). Cells sense their surrounding environments and react to external signals via signal transduction pathways (Wang et al. 2013a). The mitogen-activated protein kinase (MAPK) signal transduction pathways also participate in the regulation of cellulase formation. Three MAPKs have been reported to exist in *H. jecorina*. Tmk3 is involved in resistance to high osmolality via the derepression of genes which were involved in the osmotic stabilizer

biosynthesis and cellulase transcription. In addition, cellulase production was found to be decreased in the *tmk3* deletion strain. Tmk3 was also shown to be involved in the regulation of cellulase formation (Wang et al. 2013c), and further study showed that Tmk2 is involved in repressing cellulase formation (Wang et al. 2014). Cellulase formation requires the involvement of all MAPKs. The formation of cellulase was negatively affected by Tmk1 and Tmk2 through repressing growth and maintaining cell wall integrity, respectively. Whereas Tmk3 is the only MAPK involved in the regulation of cellulase expression at the transcriptional level (Wang et al. 2017). Although extensive researches have sought to determine how *H. jecorina* senses the inducer and transport the induction signal, that mechanism has not been elucidated.

It has been reported that changes in culture conditions, such as the agitation rate, impact cellulase production. The activities of all major cellulases were shown to improve when the agitation rate was increased from 150 to 250 rpm in *P. oxilicum*. However, the transcription levels of both *ace1* and *xlnR* were downregulated when the agitation rate was increased. It was suggested that the molecular mechanism for improved transcription of cellulase was the competition between CreA-mediated derepression and CreA-mediated deactivation which was induced by the downregulation of *ace1* and the downregulation of *xlnR*, respectively (Wang et al. 2013a).

Moreover, it is reported that Erp, the p24 γ protein homolog, is a key factor in *H. jecorina* which is involved in protein maturation and secretion (Wang et al. 2013b). Sporulation was hampered in the *erp* deletion strain. In the Δ *erp* Δ *p* δ strain, inefficient protein transport caused a “traffic jam” in the endomembrane system and caused subsequent secretion stress. It was further proposed that cells in the Δ *erp* Δ *p* δ strain attempted to degrade proteins that cluttered the endomembrane system in response to secretion stress, and the p24 heterodimer mediates protein transport, particularly the transport of cellobiohydrolase. It is suggested that *P. oxilicum* and *H. jecorina* have different responses when secretion stress “traffic jams” occur (Wang et al. 2013b). Cellulase transcription was activated in *P. oxilicum* but repressed in *H. jecorina* in response to secretion stress (Wang et al. 2013b).

Figure 3.1 shows a schematic representation of the transcriptional regulation of cellulase and hemicellulase in *H. jecorina* by external signals (Schuster et al. 2012). However, the pathway of induction and inhibition signaling on cellulase and hemicellulase gene expression has yet to be elucidated.

5 Prospects

Understanding of the mechanisms underlying the formation of cellulase and hemicellulase in filamentous fungi has greatly increased, but there are still many scientific questions worthy of further study. For example, it is important to establish a direct correlation between transcription factors and various signaling pathways. In terms of signal transduction, the questions of what the direct receptor of the received signal is and how the signal is transmitted downstream remain unclear. However, it

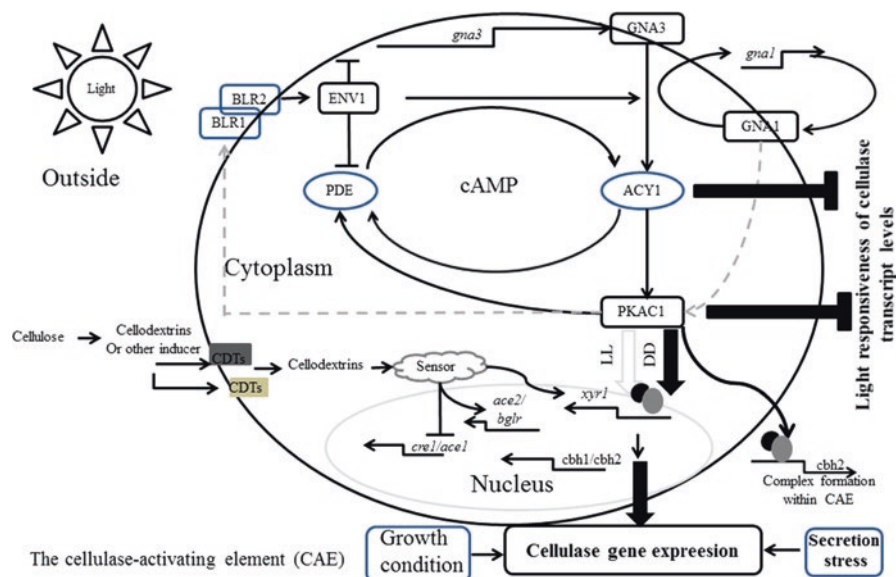


Fig. 3.1 Schematic representation of the transcriptional regulation of cellulase and hemicellulase in *H. jecorina* by external signals

is believed that through unremitting efforts, a better understanding of the entire synthesis of cellulase and hemicellulase regulatory networks in filamentous fungi will be obtained, and a solid theoretical basis will be provided for the further genetic transformation of industrial strains.

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Chapter 4

Development of Highly Efficient, Low-Cost Lignocellulolytic Enzyme Systems in a *Penicillium*: From Strain Screening to Systems Biology



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Abstract Some *Penicillium* species have been reported to produce enzyme systems with good performances in lignocellulose degradation. *Penicillium oxalicum* (formerly classified as *P. decumbens*) strains, which produce more balanced native lignocellulolytic enzyme systems than *Trichoderma reesei*, have been studied for more than 30 years. The original *P. oxalicum* isolate 114 was obtained from decayed straw-covered soil in 1979 and has been improved through a series of mutagenesis and screening over the years. The whole genome sequence of the *P. oxalicum* was finished in 2009. Comparative and functional genomics studies between *P. oxalicum* mutant JU-A10-T and wild-type strain 114-2 were performed to decipher how strain improvement has significantly improved the production of the lignocellulolytic enzyme system. Further, the transcriptomes and secretomes of the *P. oxalicum* were determined. The applications of genomic, transcriptomic, and proteomic analysis methods make it possible in evaluation of the native enzyme system, discovery of novel auxiliary proteins, understanding of regulatory mechanisms by key transcription factors, and exploration of the cellular network controlling lignocellulolytic enzyme synthesis. A single-gene disruptant library for 470 transcription factors was constructed, and several activators and repressors were identified to play essential roles in regulating lignocellulolytic enzyme production. Redesigning the regulatory pathway substantially improves lignocellulolytic enzyme production up to the industrial level by combinational manipulation of three key genes to amplify the induction along with derepression. By combining systems biology tools, engineered fungal strains are expected to produce high levels of optimized lignocellulolytic enzyme systems.

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1 Introduction

Lignocellulose is the most abundant renewable biomass on earth. Lignocellulose, composed of cellulose, hemicelluloses, and lignin, can be converted to chemicals and fuels, displacing a significant portion of the current demand for fossil fuels. In a generally accepted biorefinery scheme, lignocellulose is treated with physical or chemical method to release cellulose and hemicelluloses, which then are hydrolyzed by lignocellulolytic enzymes to produce fermentable sugars. Then, the sugars can be converted to various products (Lynd et al. 2008).

In the process of biorefinery, the high cost of lignocellulolytic enzymes is one of the bottlenecks, hindering commercialization of cellulosic fuels (Klein–Marcuschamer et al. 2012). Industrial cellulases are mainly produced by ascomycete fungi. The most widely used commercial lignocellulolytic enzymes are produced by *Trichoderma reesei* strains that have been improved through over 40-year mutagenesis and screening (Kubicek et al. 2009). However, the lignocellulolytic enzyme system of *T. reesei* needs to be supplemented with several exogenous enzymes to achieve more effective hydrolytic activity on natural complex lignocellulosic materials (Berlin et al. 2007). Recently, many documents have shown *Penicillium* species also have the ability of producing high-activity lignocellulolytic enzymes that are valuable for white biotechnology (Gusakov and Sinitsyn 2012). Lignocellulolytic enzyme system produced by *Penicillium pinophilum* was found to produce more balanced native lignocellulolytic enzyme systems than *T. reesei* and be more efficient in the hydrolysis of corn cobs compared with commercial cellulases because of its high β -glucosidase activity (Sahare et al. 2012).

In this chapter, *Penicillium oxalicum* (formerly classified as *Penicillium decumbens*) strains and their lignocellulolytic enzyme systems will be introduced, including (1) comparative and functional genomics studies between *P. oxalicum* mutant JU-A10-T and wild-type strain 114-2, to decipher how strain improvement has significantly improved the production of the lignocellulolytic enzyme system; (2) the transcriptomes and secretomes of the *P. oxalicum*; (3) and the applications of genomic, transcriptomic, and proteomic analyses, to discover novel auxiliary proteins, understand regulatory mechanisms by key transcription factors, and explore the cellular network controlling lignocellulolytic enzyme synthesis.

2 Genome and Transcriptome Analyses of *P. oxalicum* Wild-Type Strain 114-2 and Mutant JU-A10-T

The original *P. oxalicum* isolate 114 was isolated from decayed straw-covered soil in 1979 (Qu et al. 1984). After a long-term strain improvement process including mutagenesis and screening, a high cellulase productivity of 160 filter paper units L⁻¹

h^{-1} was achieved in *P. oxalicum* mutant strain JU-A10-T (Liu et al. 2010). When the strains were cultivated in cellulose-wheat bran (CW) medium, an optimal lignocellulolytic enzyme-inducing medium for *P. oxalicum*, mutant JU-A10-T has ninefold higher filter paper activity (FPA, indicating total cellulase activity), eightfold higher xylanase (indicating hemicellulase) activity, and fourfold higher total secreted proteins than wild-type strain 114-2. Although the cellulase activities of JU-A10-T increased remarkably compared with the wild type, the molecular mechanisms behind the strain improvement for higher lignocellulolytic enzyme production are poorly understood. So, comparative genomics studies of *P. oxalicum* mutant JU-A10-T and wild-type strain 114-2 were performed to decipher how strain improvement has significantly improved the production of the lignocellulolytic enzyme system (Liu et al. 2013a).

2.1 Global Comparison of Genomes of 114-2 and JU-A10-T

P. oxalicum 114-2 has a 30.19 Mb genome. Telomeric repeats (primarily 5'-TTAGGGG-3') were found at the ends of eight large scaffolds, indicating the eight chromosomes. A smaller circular scaffold, more likely mitochondrial genome with 26.36 kb, was observed. The number of putative chromosomes is different from that in *P. chrysogenum*, in which four chromosomes were assayed by pulsed-field gel electrophoresis (Fierro et al. 1993), but is the same as some *Aspergillus* species, such as *A. nidulans* (Galagan et al. 2005) and *A. niger* (Andersen et al. 2011). There are 10,021 protein-coding genes predicted in *P. oxalicum* 114-2 genome, and the number of protein-encoding genes are comparable to those of other sequenced ascomycete fungi. *P. oxalicum* JU-A10-T has 30.69 Mb genome, with 96.3% sequences mapped onto the eight chromosomes and the mitochondrial genome of 114-2. There are 10,473 protein-coding genes predicted in JU-A10-T. 114-2 and JU-A10-T shared 9599 proteins including 6699 proteins with 100% identity. The aligned DNA had an average of 1.4 single variations per kilobase (SNVs/kb). Interestingly, proteins with sequence differences were enriched for transcription factors, that 140 of the 522 predicted transcription factors contained amino acid variation. Of them, we noticed the gene *creA* (PDE_03168), which encodes the key carbon catabolite repressor, a homolog of *T. reesei cre1* (Ilmen et al. 1996). *creA* gene had a frameshift mutation at the C-terminus in JU-A10-T. The *cre1* with a truncation mutation in *T. reesei* hyper-producing mutant Rut-C30 also enhances cellulase production. This coincidence suggests that *creA* is a key target in strain engineering strategies for higher productivity. Some strain-specific genes were also found in a few genomic regions, encoding transposases, vegetative incompatibility proteins, and some unknown function proteins. The characters of strain-specific genes suggest that the parent isolate 114 was likely a heterokaryon, and current strains 114-2 and JU-A10-T have been purified by subsequent transferring or screening processes (Liu et al. 2013a).

There are in total 371 carbohydrate-active enzymes (CAZymes) annotated in *P. oxalicum* 114-2, of which 81 enzymes were predicted to be involved in lignocellulose degradation including 18 cellulases and 51 hemicellulases. Eighteen cellulases include 11 endo- β -1,4-glucanases (EGs), 3 cellobiohydrolases (CBHs), and 4 polysaccharide monooxygenases. Four secreted β -glucosidases were predicted, including one in GH family 1 and three in GH family 3. Especially, *P. oxalicum* 114-2 possessed six EGs belonging to GH family 5, whereas *T. reesei* has only three. Among them, PDE_00507, which seemed to be acquired by a horizontal gene transfer event, had no ortholog in all other species with published genome sequences in the family *Trichocomaceae*. *P. oxalicum* 114-2 was also rich in hemicellulases. Fifty-one related hemicellulose-degrading enzymes were dispersed in 20 CAZyme families and were divided into 11 types according to their substrate specificities. More pectinases (25 genes) were observed in the *P. oxalicum*114-2 genome than those in *T. reesei* (6 genes). *P. oxalicum* 114-2 contained some enzymes that are important for lignocellulose degradation but absent in *T. reesei* (Martinez et al. 2008). These included five feruloyl esterases, which can facilitate the cellulose hydrolysis by hydrolyzing the ester bonds cross-linking lignin and xylan (Tabka et al. 2006). In the *P. oxalicum* 114-2 genome, there are 23 genes encoding proteins with cellulose-binding domain (family 1 carbohydrate binding module, CBM1). The number was much higher than those 14 genes in *T. reesei*. Significantly, *P. oxalicum* 114-2 contained the highest number of CBM1 proteins among all sequenced *Penicillium* and *Aspergillus* species. JU-A10-T has 66 of cellulases and hemicellulases, and the shared proteins were mostly identical. However, three adjacent hemicellulase genes in 114-2 scaffold 8 (PDE_08036, PDE_08037, and PDE_08038) were not observed in JU-A10-T genome. There are only 26 amino acid differences in 16 cellulases or hemicellulases between 114-2 and JU-A10-T, all occurring at non-conserved positions that are not expected to affect enzyme activities. The genome sequence analysis shows that mutations in catalytic amino acid residues are not the reason for increased lignocellulolytic enzyme activities in the mutant. This is not a surprise because the same conclusion has been found in *T. reesei* mutant strains with overexpressed cellulolytic enzymes (Liu et al. 2013a, b).

2.2 Comparative Transcriptome Analysis of 114-2 and JU-A10-T

Compared with 114-2, 567 genes significantly upregulated in JU-A10-T. Among upregulated gene set, many genes predominantly coded extracellular hydrolases. The genes involved in pentose phosphate pathway (PPP) were also upregulated in JU-A10-T. For example, the expression of putative glucose-6-phosphate dehydrogenase (PDE_01924) gene upregulated ninefold compared with 114-2. Meanwhile, the genes involved in channeling xylose and arabinose into PPP for consumption were also overexpressed in JU-10A-T. For amino acid biosynthesis, the genes

involved in lysine and cysteine biosynthesis, such as homocitrate synthase gene (PDE_03916) and homoserine dehydrogenase gene (PDE_04799), were upregulated by two and sevenfold, respectively; it was assumed to facilitate the synthesis of the three major (hemi-)cellulolytic enzymes (Cel7A-2, Cel6A, and Xyn10A) rich in lysine and/or cysteine. Ribosomal protein genes and five genes involved in protein folding were significantly enriched. For example, putative protein disulfide-isomerase gene (PDE_06215), molecular chaperone BipA gene (PDE_08980), and Hsp70 family chaperone gene (PDE_00412) were upregulated by 6.0-, 2.7-, and 3.0-fold, respectively, which may positively correlate with the hyper production of secreted proteins in JU-A10-T (Liu et al. 2013b).

Compared with 114-2, 1447 genes significantly downregulated in JU-A10-T. Among the downregulated gene set, oxidoreductases involved in metabolism was significantly enriched. There included 35 enzymes involved in amino acid (especially aromatic amino acids) degradation and 29 genes involved in secondary metabolite biosynthesis. Indeed, of 314 genes which were predicted to be involved in secondary metabolism, 104 genes were significantly downregulated, while only 11 were significantly upregulated in JU-A10-T. Comparative transcriptome analysis of 114-2 and JU-A10-T showed that JU-A10-T had more genes transcribed at low levels than 114-2. The result revealed that some biological processes might be less active in JU-A10-T than those in 114-2 (Liu et al. 2013a).

3 Secretome Analysis of *P. oxalicum* Wild-Type Strain 114-2 and Mutant JU-A10-T

In fungal genomes, hundreds of genes encoding secreted enzymes can be observed. However, profiling of the secretomes under specific growth conditions is required because secreted protein will vary according to different culture media. Quantification of the abundance of individual components, especially the extracellular lignocellulolytic enzyme, is important to understand and improve lignocellulolytic enzyme systems. Two-dimensional electrophoresis (2DE)-based and liquid chromatography (LC)-based proteomic technologies were used to analyze the components in lignocellulolytic enzyme systems in different fungi, such as *T. reesei* (Herpoel-Gimbert et al. 2008), *N. crassa* (Phillips et al. 2011), and *Aspergillus* (Sharma et al. 2011). Compared with the LC-based experiment, the 2DE-based experiment has advantages in analyzing post translational modifications (PTMs) on proteins. However, LC-based experiment could generally identify more proteins and identify multi-enzyme complexes (Gonzalez-Vogel et al. 2011). According to *P. oxalicum* genome analysis, there predicted 512 secreted proteins, including 84 of the 114 plant cell wall-degrading enzymes. Proteases, chitinases, oxidoreductases, and 183 proteins with unknown function were also included in the predicted secretome. To assess the expression levels of these secreted proteins, the secretomes of *P. oxalicum* wild-type strain 114-2 and mutant JU-A10-T under different carbon resource were analyzed

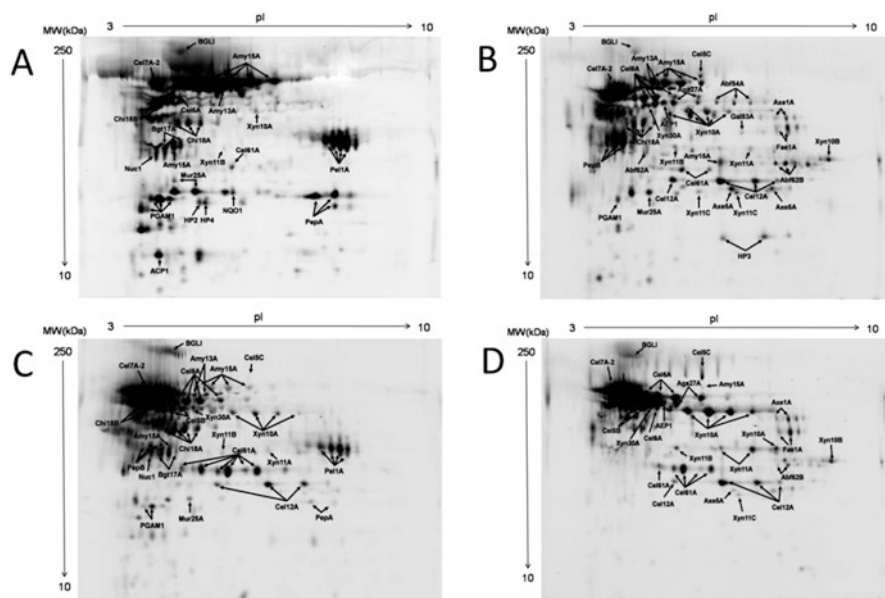


Fig. 4.1 DIGE analysis of *P. oxalicum* secretomes. Extracellular proteins of *P. oxalicum* 114-2 grown in glucose medium (A) and in cellulose-wheat bran medium (B) for 48 h. Extracellular proteins of *P. oxalicum* mutant JU-A10-T grown in glucose medium (C) and in cellulose-wheat bran medium (D) for 48 h. The proteins were analyzed at equal protein loadings

using 2DE-based and LC-based methods (Liu et al. 2013b). The study represents the first genome of a high lignocellulolytic enzyme-producing species in the genus *Penicillium*.

The protein composition of the extracellular proteome of the wild strain *P. oxalicum* 114-2 and its mutant strain JU-A10-T grown on glucose and cellulose-wheat bran was explored (Fig. 4.1). Totally, 101 protein spots on the DIGE gels were successfully identified and assigned to 37 protein models. A total of 37 proteins were identified in the extracellular proteome. The majority of these extracellular proteins are biomass degradation enzymes involved in degradation of cellulose, hemicellulose, protein, starch, pectin, and chitin. The glucose medium is a repressing condition. When *P. oxalicum* was cultivated in glucose medium, few of cellulase and hemicellulase was produced by 114-2. The abundance of amylases (Amy15A, Amy13A), pectinase (Pel1A), and protease (PepA) was high. Of them, amylase Amy15A accounted for 40% of the total proteins in the glucose medium (Fig. 4.1A). When *P. oxalicum* 114-2 was cultivated, cellulose-wheat bran (CW) medium, cellulases, and hemicellulases increased remarkably compared with those in the glucose medium (Fig. 4.1B). There included two cellobiohydrolases (Cel7A-2, Cel6A), three endoglucanases (Cel5C, Cel12A, Cel61A), one β -glucosidase (BGL1), six xylanases (Xyn10A, Xyn10B, Xyn11A, Xyn11B, Xyn11C, Xyn30A), and other enzymes related with hemicellulose degradation (Abf62A, Abf62B, Abf54A,

Fae1A, Axe1A, Axe5A). Notably, the cellobiohydrolase Cel7A-2 accounted for 16% of the total proteins in the CW medium, much higher than that in the glucose medium (2.5%). Interestingly, the proportions of BGLI, the only detected β -glucosidase in the secretome, were almost identical in the two secretomes. A metalloprotease PepA mainly expressed in the glucose medium, and an aspartic protease PepB mainly expressed in the CW medium, suggesting that protease synthesis is in response to different media (Liu et al. 2013b).

The secretome of *P. oxalicum* in the CW medium was compared with the secretome of *T. reesei* (Herpoel-Gimbert et al. 2008). Both secretomes showed a prominent cellobiohydrolase, belonging to GH family 7, identified as the most abundant component. It was also notable that more kinds and higher amounts of hemicellulases, including six endo- β -1,4-xylanases, exist in the *P. oxalicum* secretome than those in *T. reesei*. Also, large amounts of amylases (Amy15A, Amy13A) and protease were present in the *P. oxalicum* secretome but only a little in that of *T. reesei*. In the future, deletion or downregulation of these irrelevant proteins was expected to improve the synthesis levels of lignocellulolytic enzyme by release pressure of protein synthesis and secretion in *P. oxalicum* (Liu et al. 2013c).

Compared to wild strain, the mutant strain showed obviously derepressed cellulase synthesis under glucose condition (Fig. 4.1C). The amount of prominent cellulases (Cel7A-2, Cel6A, Cel5B, Cel5C, Cel12A) and hemicellulases (Xyn10A, Xyn11A, Xyn11B, Xyn30A) were significantly increased, and more types of cellulases and hemicellulases were detected, while the production of amylases, proteases, and other proteins decreased remarkably in the JU-A10-T secretome (Fig. 4.1D), according to peptide spectral counts of LC-MS/MS. Compared to wild strain, the mutant strain appeared overproduction on cellulose-wheat bran condition. Extracellular protein concentration and cellulase and xylanase activities were significantly increased. The amounts of several cellulases (Cel7A-2, Cel6A, Cel5C) and hemicellulases (Xyn10A, Xyn11A, Xyn30A) were significantly increased, while others were almost decreased, suggesting that there are some difference in regulation of cellulase and hemicellulase expression. In addition, in the extracellular proteome of mutant stain JU-A10-T, only trace amylase was detected, and no protease was detected, suggesting that some genetic variations affect the related regulatory system (Liu et al. 2013b).

4 Improving Lignocellulolytic Enzyme Production by Redesigning the Regulatory Pathway

4.1 Transcription Factors

As abovementioned, transcription factors were enriched for proteins with sequence differences when the genome of wild-type 114-2 and the genome of mutant JU-A10-T was compared; 140 of the 522 predicted transcription factors contained amino acid variations. Transcriptional regulation is central in lignocellulolytic

gene expression. Overexpression of some activators or deletion of some repressors is an efficient way to improve lignocellulolytic gene expression (Coradetti et al. 2013). In *P. oxalicum*, a total of 522 genes encoding sequence-specific regulators were predicted according to the genome analysis. Using a *P. oxalicum* *pku70* deletion mutant (non-homologous end joining (NHEJ)-deficient background) which has high homologous recombination frequency (Li et al. 2010), a transcription factor mutant library, which bears a single-gene deletion for 470 transcription factor genes in *P. oxalicum*, was successfully constructed. The transcription factor deletion strains were screened for cellulose degradation on cellulose plates. Twenty transcription factors including ClrB, ClrB-2, CreA, XlnR, Ace1, StuA, and FlbC that displayed putative roles in cellulase production were identified (Li et al. 2015).

4.1.1 ClrB and XlnR, Transcriptional Activators for Cellulolytic Gene Expression

The *P. oxalicum* *clrB* gene (PDE_05999) encodes a protein of 780 amino acid residues, with a Zn₂Cys₆ binuclear cluster DNA-binding motif. ClrB has 39% of identity to the homolog of *N. crassa* and 56% of identity to that of *A. nidulans* (Coradetti et al. 2012). *clrB* deletion strain (Δ *clrB*) showed identical phenotype on glucose, xylan, or potato dextrose agar (PDA) plates compared with *P. oxalicum* wild-type 114-2 but displayed significantly reduced growth on cellulose plate. mRNA levels of cellobiohydrolase gene *cel7A-2* and endoglucanase gene *cel5B* in the Δ *clrB* mutant significantly decreased and could hardly be detected. When *clrB* was overexpressed (OE*clrB*), OE*clrB* showed almost 2.5-fold increase in FPA, 2.5-fold increase in pNPCase activity, and 8.7-fold increases in CMCase activity when grown on cellulose for 48 h. Northern blot analyses also showed that the mRNA levels of cellobiohydrolase gene *cel7A-2* and endoglucanase gene *cel5B* in mutant were much higher than those in 114-2 on cellulose.

The results of RNA-Seq showed 121 genes have higher transcription levels in the Δ *clrB* mutant than in 114-2. Of them, only seven genes encoding CAZy proteins, including two predicted hemicellulase genes. No cellulase, β -glucosidase, and xylanase gene was observed. A total of 224 genes were differentially expressed between the Δ *clrB* and the wild-type strains on cellulose. Of these genes, 103 genes showed lower expression levels in the Δ *clrB* than in 114-2. Among these downregulated genes, gene encoding putative maltose permease, gene encoding cellodextrin transporter CdtC, and 24 genes encoding transporters were enriched, suggesting that ClrB might also be involved in the cellodextrin and maltose metabolisms. In addition, 32 genes encoding carbohydrate-active enzymes (CAZymes) were also enriched, including 9 cellulase genes and 2 β -glucosidases. However, only 6 of the 51 hemicellulase genes showed obvious downregulation in Δ *clrB*, suggesting ClrB might play important role in cellulase gene activation but has different role in xylanolytic gene expression regulation (Li et al. 2015).

P. oxalicum XlnR, with a Zn2Cys6 binuclear cluster motif, is another transcription activator for cellulolytic gene expression. XlnR was identified previously along with the orthologs *N. crassa* Xlr-1 and *T. reesei* Xyr1 (Mach-Aigner et al. 2008; Sun et al. 2012). The deletion of in *P. oxalicum* ($\Delta xlnR$) led to repressed growth on cellulose or xylan media. Significant expression downregulation of cellobiohydrolase gene *cel7A-2*, endoglucanase gene *cel5B*, and xylanase gene *xyn1* was observed in the $\Delta xlnR$ mutant compared with that in wild-type strain when the strains were cultivated in the cellulose-containing medium (Li et al. 2015). These data showed that *P. oxalicum* XlnR is a general transcription factor that regulates both cellulolytic and xylanolytic gene expressions. Moreover, simultaneously deletion of both ClrB and XlnR ($\Delta clrB \Delta xlnR$) could cause greater repression of cellulolytic and xylanolytic gene expression than sole gene absence under cellulose growth conditions, suggesting ClrB and XlnR had additive effects on activating the cellulolytic and xylanolytic gene expressions (Li et al. 2015).

4.1.2 CreA and AmyR, Transcription Repressors for Cellulolytic Gene Expression

The *P. oxalicum* *creA* gene (PDE_03168) encodes a protein of 417 amino acids and shows 80% and 83% identities to the orthologs of *A. nidulans* FGSC A4 and *A. niger* CBS 513.88. CreA was thought as the major carbon catabolite repressor. The *creA* deletion mutant ($\Delta creA$) degraded cellulose faster than the wild-type strain. Similar results were observed in *T. reesei* *cre1* or *N. crassa* *cre-1* deletion strains (Portnoy et al. 2011; Sun and Glass 2011). When the strains were cultivated on cellulose for 96 h, *P. oxalicum* $\Delta creA$ exhibited increased lignocellulolytic enzyme activities. The activity of FPA, pNPCase, CMCcase, and xylanase showed almost 7.2-, 2.2-, 8.0-, and 4.4-fold increases compared with 114-2, confirming the negative effect of CreA on cellulase expression.

P. oxalicum *amyR* (PDE_03964) was also considered tightly associated with cellulolytic gene expression. AmyR was previously reported in *A. niger* as amylase-specific regulator; it also regulated the expression of β -glucosidase and galactosidase gene expression (vanKuyk et al. 2012). *amyR* mutation in *A. oryzae* led to downregulation of amylases whereas upregulation of cellulase. Carbon catabolite repression (CCR) involved mechanism was proposed to explain the mechanism that AmyR activated the amylase synthesis that facilitated converting starch to glucose, and the latter inhibits the production of cellulolytic enzymes through CCR (Watanabe et al. 2011). *P. oxalicum* *amyR* deletion ($\Delta amyR$) strain displayed reduced amylase under cellulose growth conditions, while the PFA has 1.6-fold improvement compared with that in the wild type. $\Delta amyR$ also showed high expression of cellobiohydrolase gene *cel7A-2* and endoglucanase gene *cel5B* but deficient in transcribing the major amylase gene *amy15A* and *amy13A*. The results implied that AmyR is the main activator for amylase expression but functions negatively in the expression of cellulase genes (Li et al. 2015).

4.1.3 BrlA and FlbC Transcription Factors Involved in Both Asexual Development and Cellulolytic Gene Expression

As conidia are always used as starters in the first step of fermentation, condition is essential for most industrial fungi. Interestingly, some mutant fungal strains with high expression of cellulolytic gene were found with decreased conidiation ability. For example, the conidiation ability of *T. reesei* mutant Rut-C30 decreased much compared with its parent strain, with the conidia color change from dark green to light green (Nakari-Setälä et al. 2009). In the mutant strain of *A. oryzae* with high product of amylase which were used for production of sweet rice wine in Japan, many genes silenced, including conidial wall synthesis genes and pigment synthesis genes (Masayuki et al. 2008). In ascomycetous filamentous fungi, conidiation was precisely monitored by a variety of proteins at multi-levels. Activation of BrlA was a key step that governs conidiation-specific gene expression. Together with the AbaA and WetA proteins, which are responsible for conidia maturation, the three regulatory factors together constitute the central regulatory pathway of conidiation in *Aspergillus*. BrlA was shown to be regulated by other transcription factors; FlbA, FlbB, FlbC, FlbD, and FlbE, which are collectively called Flbs, are required during normal activation of BrlA (reviewed in Park and Yu 2012). However, whether these transcription factors play the roles in the regulatory network of cellulolytic gene expression is still unknown, the regulatory interrelationship between the asexual conidiation and cellulase production remains unclear.

BrlA is a C2H2-type zinc finger transcription factor. The deletion of *P. oxalicum* *brlA* ($\Delta brlA$) not only blocked conidiation but also regulates the expression of cellulolytic gene expression. Expression levels of the main cellulolytic genes (*cel5A*, *cel6A*, *cel7A-2*, *cel7B*, and *cel3A*) and the glucoamylase gene (*amy15A*) were upregulated compared with the wild-type strain. Researchers investigated the 5' upstream regions (750 bp) of the six main cellulolytic genes (*cel5A*, *cel5B*, *cel6A*, *cel7A-1*, *cel7A-2*, and *cel7B*) and the five main amylase genes (*amy15A*, *amy15B*, *amy13A*, *amy13B*, and *amy13C*). All of them were found with putative BrlA-binding sites (C/A)(G/A)AGGG(G/A) or putative AbaA-binding sites (CATTC(C/T) (Chang and Timberlake 1993; Andrianopoulos and Timberlake 1994), suggesting that BrlA might have functions in regulating the expression of cellulolytic genes (Qin et al. 2013). However, the regulation of cellulolytic gene expression by BrlA is not notable as the other regulators, such as CCR gene *creA*, and no evidence supported BrlA binds on cellulolytic gene promoters in vivo. Therefore, whether BrlA plays a direct role in the regulation of cellulolytic gene expression needs to be further investigated.

FlbC, another C2H2-type transcription factor, was also reported to be involved in cellulolytic gene expression. *P. oxalicum* FlbC was crucial for the normal growth and asexual development. The deletion of *flbC* ($\Delta flbC$) led to asexual conidiation impair. When the strains were cultivated for 30 h, no spore formation was observed in $\Delta flbC$, while about 2×10^6 spores per square centimeter were found in wild type. $\Delta flbC$ also showed much reduced cellulase and hemicellulase productions. No FPase activity (representing overall cellulolytic activity) was detected in $\Delta flbC$ after

120 h cultivation, and only 5% of that of WT was detected at the end of fermentation. In addition, CMCase, pNPase, and xylanolytic activity also significantly decreased in $\Delta flbC$ compared with those in WT. Comparative transcriptome analysis revealed a global downregulation of lignocellulolytic genes including cellulase genes (*cel5A*, *cel5B*, *cel6A*, *cel7A-1*, *cel7A-2*, and *cel7B*), hemicellulase genes (10 xylanase genes belonging to GH10, GH11, and GH30), and others (*bgl1*, cellulose-active LPMO gene) with functions in lignocellulose degradation. However, the expression of key transcription factors, including ClrB, XlnR, and CreA, was not observed to change significantly, except AmyR. In addition, direct binding between FlbC protein and the promoter region of cellobiohydrolase gene *cel7A-1* was not detected, suggesting FlbC participated in regulation of cellulolytic gene expression in with an indirect mode. Interestingly, when *flbC* was overexpressed (*OEflbC*), similar defect was observed in *OEflbC*, suggesting that normal expression of the *flbC* was required in the production of cellulolytic enzymes (Yao et al. 2016).

4.2 Other Important Regulatory Factors Involved in Regulation of Lignocellulolytic Gene Expression

4.2.1 Bgl2, An Intracellular β -Glucosidase Playing a Negative Role in Cellulase Gene Expression

β -glucosidases (EC 3.2.1.21), involved in hydrolysis of terminal nonreducing residues in β -D-glucosides with release of glucose, are crucial in lignocellulosic biomass degradation (Lynd et al. 2002). Deletion of *T. reesei* major extracellular β -glucosidase gene *bgl1* led to decreased growth and cellulase production on cellulose (Fowler and Brown 1992). The intracellular β -glucosidases were also observed to be involved in the induction of cellulose genes by cellulose in *T. reesei* (Zhou et al. 2012).

In *P. oxalicum*, there were at least five extracellular and six intracellular β -glucosidase genes. The major extracellular β -glucosidase Bgl1 has been characterized and observed to improve hydrolysis of corncob residue by using it as cellulase supplementation (Chen et al. 2010). As the major intracellular β -glucosidase, Bgl2 was classified into glycoside hydrolase family 1 (GH 1), according to the results of BlastP analysis. The *bgl2* deletion ($\Delta bgl2$) led to about 69% of the intracellular β -glucosidase activity lost compared with that of 114-2. The extracellular FPA, EG, CBH, and xylanase activities of $\Delta bgl2$ increase dramatically on CW medium. However, the production of extracellular β -glucosidase Bgl1 was not improved synchronously with those of cellulose-induced enzymes. Recombinant Bgl2 (rBgl2, expressed in *E. coli*) showed high activity in hydrolyzing cellodextrins to glucose. rBgl2 could also hydrolyze sophorose (β -1,2-linked disaccharide) or gentiobiose (β -1,6-linked disaccharide), although the hydrolyze rates were lower than cellobiose as substrate. In *T. reesei* and *Penicillium purpurogenum*, sophorose and gentiobiose were considered to be the “true” inducers for their strong inductive

effects on lignocellulolytic gene expression (Kurasawa et al. 1992; Saloheimo et al. 2002). In $\Delta bgl2$, there observed more accumulation of intracellular cellobiose compared with that in wild type. However, cellulase induction by cellobiose in *P. oxalicum* is not mediated by the formation of gentiobiose and sophorose. The regulatory role of *P. oxalicum* Bgl2 in cellulase gene expression was similar to that of *N. crassa* (Znameroski et al. 2012), however, different from that of *T. reesei* (Zhou et al. 2012). The transcription levels of cellobiohydrolase gene *cel7A-2*, endoglucanase gene *cel5B*, and xylanase *xyn10A* in $\Delta bgl2$ improved 263.0-, 371.6-, and 347.9-fold on cellobiose respectively, compared with the wild type. So, it was assumed that the deletion of *bgl2* might cause cellodextrin accumulation in the cytoplasm, which resulted in higher level of lignocellulolytic gene expression and continuous efficient production of lignocellulolytic enzymes (Chen et al. 2013). Like the key transcription factors, Bgl2 could also be a promising target to engineer industrial strains for higher yields of lignocellulolytic enzymes.

4.2.2 LaeA, a Putative Methyltransferase, Playing Different Roles in the Production of Extracellular Cellulase and β -Xylosidase

In addition to the key transcription factors, other regulators such as Lae1 were also found to play important roles in regulating lignocellulolytic enzyme expression. The *lae1/laeA* (loss of *afIR* expression) gene was first characterized in *A. nidulans* (Bok et al. 2005). As LaeA possesses S-adenosyl-methionine-binding (SAM) motifs, its function is always thought to the epigenetic control by its putative protein (specifically for histone tails) methyltransferase function. *lae1/laeA* was proven to play the key roles in morphological development and the secondary metabolite biosynthesis in many filamentous fungi, such as in *Aspergilli*, *Penicillium*, and *Fusarium* (Kosalková et al. 2009; Oda et al. 2011; Butchko et al. 2012). LaeA was also proven to regulate lignocellulolytic enzyme gene expression in *T. reesei*. The expression of all seven cellulases and auxiliary factors for cellulose degradation was downregulated in *T. reesei laeA* deletion strain, with low *xyl1* transcript levels observed (Seiboth et al. 2012).

P. oxalicum laeA gene (PDE_00584) encodes a protein of 348 amino acids. LaeA shows 75% and 70% identities to the orthologs of *A. niger* CBS 513.88 and *A. nidulans* FGSC A4, respectively. LaeA extensively affected lignocellulolytic enzyme gene expression. The expression of genes that encoded the top ten glycoside hydrolases (Amy15A, Amy13A, Cel7A-2/CBHI, Cel61A, Chi18A, Cel3A/BGLI, Xyn10A, Cel7B/EGI, Cel5B/EGII, Cel6A/CBHII) was downregulated significantly especially in later phases of prolonged batch cultures in *laeA* deletion strain ($\Delta laeA$). The regulon of $\Delta laeA$ compared with that in WT was enriched in cellulose binding, aspartic-type endopeptidase activity, cellulase activity, monooxygenase activity, heme binding, electron carrier activity, and cellulose 1,4- β -cellobiosidase activity. As abovementioned, ClrB and XlnR are transcription activators for lignocellulolytic enzyme gene expression. So, it was expected that *clrB* overexpression strain (OE*clrB*) and *xlnR* overexpression strain (OE*xlnR*) have improved cellulolytic ability than that of the wild type. Meanwhile, as CreA is a transcription repressor for

lignocellulolytic enzyme gene expression, its deletion ($\Delta creA$) was also observed to exhibit higher cellulolytic ability than the wild type. However, in the absence of *LaeA*, the FPA of all the three mutants *OEclrB $\Delta laeA$* , *OExlnR1 $\Delta laeA$* , and *$\Delta creA\Delta laeA$* decreased remarkably compared with their parent strains *OEclrB*, *OExlnR*, and *$\Delta creA$* , respectively. The *clrB* or *xlnR* overexpression could not rescue the impairment of cellulolytic enzyme gene expression by *LaeA* absence. The results suggested the coexistence of transcription activators (*ClrB* and *XlnR*), and *LaeA* was required for proper cellulolytic enzyme gene expression. *LaeA* not only regulates the expression lignocellulolytic enzyme gene but also the expression of lignocellulolytic enzyme-related transcription factor. *creA* expression level was considerably increased in *$\Delta laeA$* , suggesting *LaeA* had a negative role in regulating *creA* expression (Li et al. 2015).

P. oxalicum *LaeA* has positive roles in regulating cellulase and hemicellulase gene expression. However, the extracellular β -xylosidase formation was negatively regulated by *LaeA*. Especially, combination of *laeA* deletion and *xlnR* overexpression (*OExlnR $\Delta laeA$*) activated extracellular β -xylosidase synthesis. The extracellular β -xylosidase activities improved over fivefold in the *OExlnR $\Delta laeA$* mutant. The expression of prominent β -xylosidase gene *xyl3A* was significantly upregulated. So, the cumulative effect of *LaeA* and *XlnR* has potential applications in the production of more β -xylosidase. Indeed, β -xylosidase gene *xyl3A* has been overexpressed using the *OExlnR $\Delta laeA$* mutant h as the parent strain, which improved by over 20-fold compared with that of the WT (Li et al. 2015).

4.2.3 HepA, a Heterochromatin Protein, Involving in Lignocellulolytic Enzyme Gene Expression

Just as above described, transcription factors participate in the process of transcription activation or repression for lignocellulolytic enzyme gene expression. In the generally accepted model, transcription activators were thought to recruit nucleosome modifiers that help transcriptional machinery binding at the promoter to activate transcription, while transcription repressors recruit different types of nucleosome modifiers that block transcriptional machinery binding at the promoter to repress transcription (Voss and Hager 2014). For example, in *T. reesei* strain with transcription activator *Xyr1* (ortholog of *XlnR* in *P. oxalicum*) disrupted, the degrees of chromatin opening are strongly reduced, and the two prominent cellobiohydrolase gene *cbh1* and *cbh2* expressions downregulated significantly (Mello-de-Sousa et al. 2015). *T. reesei* *Cre1* (ortholog of *CreA* in *P. oxalicum*) was also reported to be involved in correct nucleosome positioning within *cbh1* promoter (Ries et al. 2014). There are two types of nucleosome modifiers, those that add or delete chemical groups for histone tails, such as histone methyltransferases or demethyltransferases and histone acetyltransferases or deacetyltransferases (Iizuka and Smith 2003), and those remodel the nucleosomes, such as SWI/SNF complex with ATP-dependent activity (Khavari et al. 1993). These modifications can “loosen” or “tighten” the chromatin structure, rendering it more accessible or inaccessible for the transcriptional machinery binding (Bernstein et al. 2006). For example, *T. reesei* *Gcn5*, a

histone acetyltransferase, was proven to be required in lignocellulolytic enzyme gene expression; the acetylation levels of histone H3K9 and H3K14 in the cellulase gene promoter dramatically decreased in the absence of Gcn5 (Xin et al. 2013). The lack of acetylation enables the mono-, di-, or tri-methylation of H3K9 (H3K9me), and this histone modification could be recognized by the heterochromatin protein 1 (HP1) (Haldar et al. 2011).

HepA (ortholog of HP1 in *Drosophila melanogaster*) is a small nonhistone chromosomal protein and was reported as a dominant suppressor of position-effect variegation (PEV) on heterochromatin gene silencing (Eissenberg et al. 1990). The best-studied HP1 homologues in fungi were the *Schizosaccharomyces pombe* SWI6, *Aspergillus nidulans* HepA, and *N. crassa* Hpo (Hiragami and Festenstein 2005; Honda and Selker 2008; Reyes-Dominguez et al. 2010). *N. crassa* Hpo was reported to be involved in gene repression or gene silencing by binding H3K9me3 and recruiting DNA methyltransferase DIM-2, to form distinct DNA methylation complexes (Reyes-Dominguez et al. 2010). *P. oxalicum* HepA encodes 241 amino acids, with two conserved domains. One is a 54-amino-acid chromatin organization modifier domain at N-terminal, and the other is a 58-amino-acid chromo shadow domain at C-terminal. The deletion of *hepA* ($\Delta hepA$) led to downregulation of prominent extracellular cellulolytic enzyme genes according to the transcriptome data. Among the top ten extracellular glycoside hydrolases, all five cellulase genes (*cel7A/cbh1*, *cel6A/cbh2*, *cel7B/leg1*, *cel5B/leg2*, and *cel3A/bgl1*) and the cellulose-active LPMO gene (*cel61A*) expression were downregulated, as well as two amylase genes (*amy15A* and *amy13A*). Meanwhile, HepA regulated chromatin structure. In $\Delta hepA$ mutant, the chromatin of all three tested upstream regions for cellobiohydrolase gene *cel7A/cbh1* and endoglucanase gene *cel7B/leg1* opened specifically. However, the open chromatin status did not positively correlate with the activation of these genes (Zhang et al. 2016). In addition, *hepA* overexpression did not change the chromatin status but led to upregulation of the cellulolytic enzyme gene expression. Indeed, most genes (95.0%) were upregulated in $\Delta hepA$ compared with WT, and it was expected as HepA always played a main negative role in transcription (Hiragami and Festenstein 2005). However, HepA is actually a specific positive regulator for cellulolytic enzyme gene expression. HepA is required for chromatin condensation of prominent cellulase genes and could be a promising target for genetic modification to improve cellulolytic enzyme synthesis.

4.3 Redesign the Regulatory Pathway to Improve Cellulolytic Enzyme Synthesis by Combinational Manipulation of Key Regulators

Traditionally, the method of random mutagenesis was used as the main approach to obtain industrial fungal strains for high cellulase production. However, this method is time-consuming and laborious. With the progress of function genome research,

the investigation of regulatory network in *P. oxalicum* led to the identification of many key regulators for cellulolytic enzyme gene expression, including transcription factors such as ClrB, XlnR, CreA, BrlA, and FlbC and other regulators such as Bgl2, LaeA, and HepA (as mentioned above). So, it is possible to improve cellulolytic enzyme synthesis by combinational manipulation of several key regulators rather than only one gene as target. For example, deletion of Cre1 in *N. crassa* $\Delta 3\beta G$ (three β -glucosidases absent) mutant showed higher ability to produce cellulases than $\Delta 3\beta G$ in response to induction with cellobiose (Znameroski et al. 2012).

Simultaneous deletion of *creA* ($\Delta creA$) and overexpression of *clrB* (*OEclrB*) can upregulate cellulase expression. The strong cellulase gene upregulation observed in the *OEclrB* and $\Delta creA$ mutants indicated that these two genes encoded the major transcription factors that oppositely regulate cellulolytic gene expression. *P. oxalicum* mutant with simultaneous deletion of *creA* and overexpression of *clrB* ($\Delta creA OEclrB$) mutant exhibited higher steady-state amounts of cellobiohydrolase gene *cel7A-2*, endoglucanase gene *cel5B*, and xylanase *xyn10A* (Li et al. 2015). As previous work demonstrated that the major intracellular β -glucosidase Bgl2 plays a negative role in cellulases and xylanase induction, the deletion of *bgl2* was introduced to $\Delta creA OEclrB$ to obtain trigenic mutant $\Delta creA OEclrB \Delta bgl2$. The cellulolytic ability of $\Delta creA OEclrB \Delta bgl2$ improved remarkably by simultaneously strengthening induction and relieving repression. The FPA increased by up to over 20-folds than that of the 114-2. In addition, pNPCase, CMCcase, xylanase activities, and extracellular protein level in $\Delta creA OEclrB \Delta bgl2$ increased by 10-, 16-, 5-, and 10-fold compared with those of WT, respectively. Meantime, substantial transcription upregulation of major cellulolytic enzyme genes was observed. Totally, the trigenic mutant exhibited remarkably strong cellulolytic ability compared with WT. The performance of the mutant was even comparable with that of industrial strain Ju-A10-T (Yao et al. 2015).

XlnR/Xlr-1/Xyr1 was another transcription activator which could be used as a genetic target to improve cellulolytic gene expression. In *T. reesei* bearing constitutively active mutants of Xyr1 (Xyr1_{A824V} or Xyr1_{V821F}), higher cellulolytic gene expression was observed (Dernt et al. 2013). In *P. oxalicum*, a chimeric transcription factor which contain the DNA-binding domain of activator ClrB linked to the C-terminal sequences of XlnR_{A871V} (homolog of Xyr1_{A824V} in *T. reesei*) was designed and was introduced to wild type. The mutant with a chimeric transcription factor exhibited dramatically improved cellulase production (7.3-fold increase) compared with that of wild type (Gao et al. 2017).

These findings showed that it is feasible to redesign the regulatory pathway to improve cellulolytic enzyme synthesis by combinational manipulation of key regulators. Given the conservation of the regulation of cellulolytic enzymes among fungi, the strategies could help to engineer other fungal strains for improving cellulolytic enzyme synthesis.

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Chapter 5

A β -glucosidase Hyperproducing Strain, *Penicillium piceum*: Novel Characterization of Lignocellulolytic Enzyme Systems and Its Application in Biomass Bioconversion



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Abstract In this chapter, the β -glucosidase over producing strain *Penicillium piceum* and the β -glucosidase characteristics will be introduced. Through several rounds of dimethyl sulfate mutagenesis, the β -glucosidase activity of *P. piceum* reached 53.12 IU/ml. Two new β -glucosidases, promising bifunctional enzymes for lignocellulosic bioconversion, have been found in the extracellular protein of *P. piceum*. The two new β -glucosidases played an important role in forming multiple soluble cellulose inducers via high transglycosylation activity and novel enzymatic activity. Further, the two new β -glucosidases showed the strong synergism with different cellulases by removing multiple inhibitors for cellulase. The rational computer-aided strategies were devised to enhance the thermostability of the main β -glucosidase (Cel3A) from *Penicillium piceum* H16. *Penicillium piceum*, high-yielding β -glucosidase with high enzymatic activity and good thermostability, may provide a good synergetic effect on *Trichoderma reesei* for improving cellulose hydrolysis of different substrates.

Keywords β -glucosidase · Lignocellulolytic enzyme systems · Biomass bioconversion · *Penicillium piceum*

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1 Introduction

Lignocellulosic biomass is one of the most abundant renewable resources in nature, the biorefinement of which is considered to be an important alternative for the sustainable development of the environment. Thus far, the high cost of lignocellulolytic enzymes for lignocellulosic degradation is one of the most important limiting factors in the biorefinement of lignocellulosic materials (Sanchez 2009). Cellulose hydrolysis requires the synergism of different cellulolytic enzymes, including three cellulolytic enzymes, endoglucanase (EC 3.2.1.4), exo-glucanase (EC 3.2.1.91), and β -glucosidase (EC 3.2.1.21) (Zhang et al. 2006). Filamentous fungi are the major source of the extracellular cellulase, such as species belonging to the genera *Trichoderma*, *Penicillium*, and *Aspergillus*, among which *T. reesei* is still the major producer for lignocellulolytic enzyme systems commercially (Kubicek et al. 2009). In many of the studies examining the hydrolysis of lignocellulosic materials, *T. reesei* has been the first and foremost choice for enzymatic cellulose saccharification (Merino and Cherry 2007). *T. reesei* has a high capacity for cellulase production but is deficient in β -glucosidase, which decreases the hydrolysis efficiency of the lignocellulosic materials (Ryu and Mandels 1980). Low β -glucosidase activity often results in cellobiose accumulation in cellulose hydrolysis and the subsequent product inhibition of cellulase (Mandels and Andreotti 1978). To overcome this limitation, supplementing a native *T. reesei* enzyme system with exogenous β -glucosidase is a feasible way to achieve highly efficient hydrolysis of the lignocellulosic materials.

Many *Penicillium* species have been reported to be potential candidates for cellulase production (Gusakov 2011). Due to its more balanced cellulase system, especially high β -glucosidase in extracellular cellulase systems of *Penicillium* species, the performance of cellulase produced by *Penicillium* species is even better than that of *Trichoderma* species (Qu et al. 2006). In addition, an efficient synergy was observed upon mixing the cellulases from *P. funiculosum* and *T. reesei* (Van Wyk 1999). Thus, exploring novel cellulolytic enzyme producing *Penicillium* species is a feasible direction for the further study of lignocellulosic biomass hydrolysis.

In this chapter, a *Penicillium* strain identified as *P. piceum* 9-3 was isolated from compost sample. It could produce a relatively high level of cellulase activity, especially β -glucosidase activity. Dimethyl sulfate (DES)-induced mutagenesis was performed to obtain a cellulase over producing mutant, based on its potential for cellulase production. Two new β -glucosidases, promising bifunctional enzymes for lignocellulosic bioconversion, have been found in the extracellular protein of *P. piceum*. The characteristics and function of the two new β -glucosidases potentially have been introduced. Finally, rational computer-aided strategies were devised to enhance the thermostability of main β -glucosidase (Cel3A) from *P. piceum* H16. Their application of *P. piceum* after modification to the synergistic effect on the cellulase system from *T. reesei* in hydrolyzing lignocellulosic materials was also investigated.

2 A β -Glucosidase Hyperproducing Strain Identification and Mutagenesis

2.1 Strain Identification

Through the enrichment culture of the compost, three different species of cellulose degrading fungi were isolated. Among the three isolated fungi, fungus 9-3 showed maximum zone of clearance in the cellulase screening medium with Congo red (Fig. 5.1a). The strain was identified through morphology and 18S rDNA gene sequencing. This fungus showed the green colony, smooth conidia, and *Penicillium* conidiospores (Fig. 5.1a, b), typically characteristics of the *Penicillium* genus (Abe 1956). As shown in Fig. 5.1c, the phylogenetic analysis based on the 18S rDNA gene sequence alignment demonstrated that the strain 9-3 (Genbank No. GU477623) belonged to the *Penicillium* genus and clustered with *P. piceum* (99% 18S rDNA gene sequence similarity). Therefore, this strain was named as *P. piceum* 9-3.

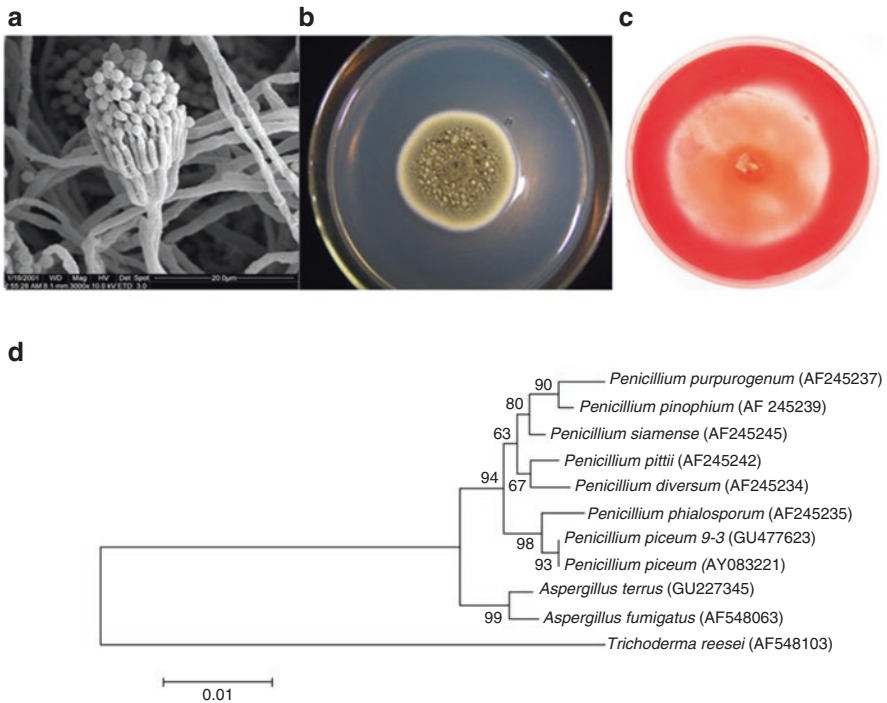


Fig. 5.1 Screening and identification of cellulolytic fungus *Penicillium piceum* 9-3, (a) mycelial morphology, (b) colony morphology of *P. piceum* 9-3, (c) hydrolysis zone of *P. piceum* 9-3 on CMC agar plate after Cargo red flooding, and (d) phylogenetic tree of *P. piceum* 9-3 and relative species using 18S rDNA sequence, based on the neighbor-joining method. Bootstrap probability values (percentages of 1000 tree replications) were indicated at branch points

Table 5.1 Cellulase and hemicellulase production by *Penicillium piceum* 9-3, *Aspergillus niger* 3.316, and *Trichoderma reesei* RUT C30

Strain	Enzyme activities (IU ml ⁻¹) ^a				Protein (mg ml ⁻¹)
	FPase	CMCase	β -glucosidase	Xylanase	
<i>P. piceum</i> 9-3	1.09 $\pm$ 0.08	8.56 $\pm$ 0.75	12.91 $\pm$ 0.20	753.38 $\pm$ 1.32	1.42 $\pm$ 0.13
<i>A. niger</i> 3.316	0.17 $\pm$ 0.01	0.87 $\pm$ 0.26	10.22 $\pm$ 0.08	119.86 $\pm$ 5.30	1.08 $\pm$ 0.05
<i>T. reesei</i> RUT C30	2.13 $\pm$ 0.50	62.59 $\pm$ 0.20	1.14 $\pm$ 0.10	841.53 $\pm$ 2.63	1.29 $\pm$ 0.07

^aAll of the strains were grown in shake flasks at 28 °C for 7 days with microcrystalline cellulose and corn steep liquor as the carbon and nitrogen sources, respectively

2.2 Evaluation of Cellulase Production in *P. piceum* 9-3

To systematically analyze the cellulase production of *P. piceum* 9-3, FPase, CMCase, β -glucosidase, and xylanase activities were determined in flasks. The cellulase production in *Trichoderma reesei* RUT C30 and *Aspergillus niger* 3.316 were also evaluated for comparison. *P. piceum* 9-3 exhibited high β -glucosidase activity (12.91 IU ml⁻¹) compared with *T. reesei* RUT C30 and *A. niger* 3.316 (Table 5.1). The xylanase activity of *P. piceum* 9-3 was 753 IU ml⁻¹, which was comparable with that of *T. reesei* RUT C30 and much higher than that of *A. niger* 3.316. In addition, the FPase activity and CMCase activity were much lower than those of *T. reesei* RUT C30 but much higher than those of *A. niger* 3.316 (Table 5.1).

2.3 Strain Mutagenesis and Screening of Cellulase Over Producing Mutants

The conidia of *P. piceum* 9-3 were treated with DES and then selected on plates containing the modified cellulase screening medium. Fifty-five colonies with hydrolysis halos were obtained and chosen for the next step. To evaluate the cellulase production accurately, each of the mutants was inoculated into the cellulase-producing medium for determination of enzyme activity. As shown in Fig. 5.2, five mutants (C25, F18, G1-16, G22, and H16) with high FPase activity and β -glucosidase activity were screened out. Among these mutants, compared with the parent strain *P. piceum* 9-3, the FPase activity and β -glucosidase activity of H16 exhibited 6.55- and 4.33-times improvement, respectively. The protein secretion of H16 was also significantly higher than that of the parent strain *P. piceum* 9-3, whereas the protein secretion of the other four mutants was comparable with the parent strain. Thus, the mutant H16 with highest cellulase activity was picked out for further study. Figure 5.3a showed the kinetics of the cellulases and the total extracellular protein production during the fermentation in liquid medium. The FPase activity, β -glucosidase activity, and the extracellular protein of H16 were higher than those of 9-3. The maximum FPase and β -glucosidase activity were achieved at 144 h,

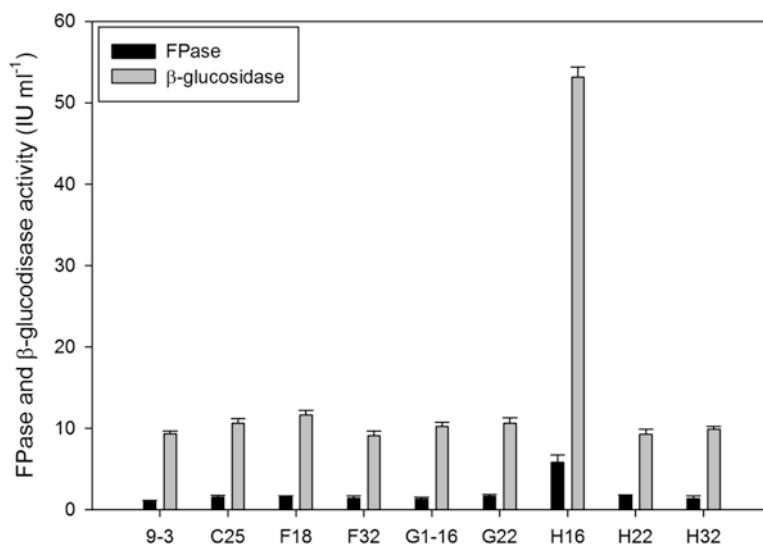


Fig. 5.2 Cellulase activity and protein concentration of *P. piceum* 9-3 and its derived mutants. All strains were inoculated in cellulase-producing medium and cultivated for 7 days. The error bars indicated the standard deviations in three replicate experiments

reaching 5.83 and 53.12 IU ml⁻¹, a 5.34- and 4.43-times improvement from the parent strain *P. piceum* 9-3, respectively. The cellulase activity and secreted protein of H16 were kept stable after eight successive generations (Fig. 5.3b). The production of cellulases in submerged fermentations by *P. piceum* H16 and other mutants from *Penicillium* species was also compared (Table 5.2). The levels of the FPase activity and extracellular protein of *P. piceum* H16 were lower than *P. pinophilum* NTG III/6 and *P. occitanis* Pol6. However, the levels of the β -glucosidase activity were significantly higher than other mutants from *Penicillium* species, demonstrating that *P. piceum* H16 is a promising strain for cellulase production especially for β -glucosidase production.

2.4 Influence of pH and Temperature on Cellulase Activity and Stability

The optimal pH for cellulase activity was pH 5.0, at which the FPase and β -glucosidase activities reached the maximum values (Fig. 5.4a). Regarding the optimal temperature for cellulase activity (Fig. 5.4b), the maximum activities of FPase and β -glucosidase were achieved at different temperature (50 °C for FPase activity and 55 °C for β -glucosidase activity).

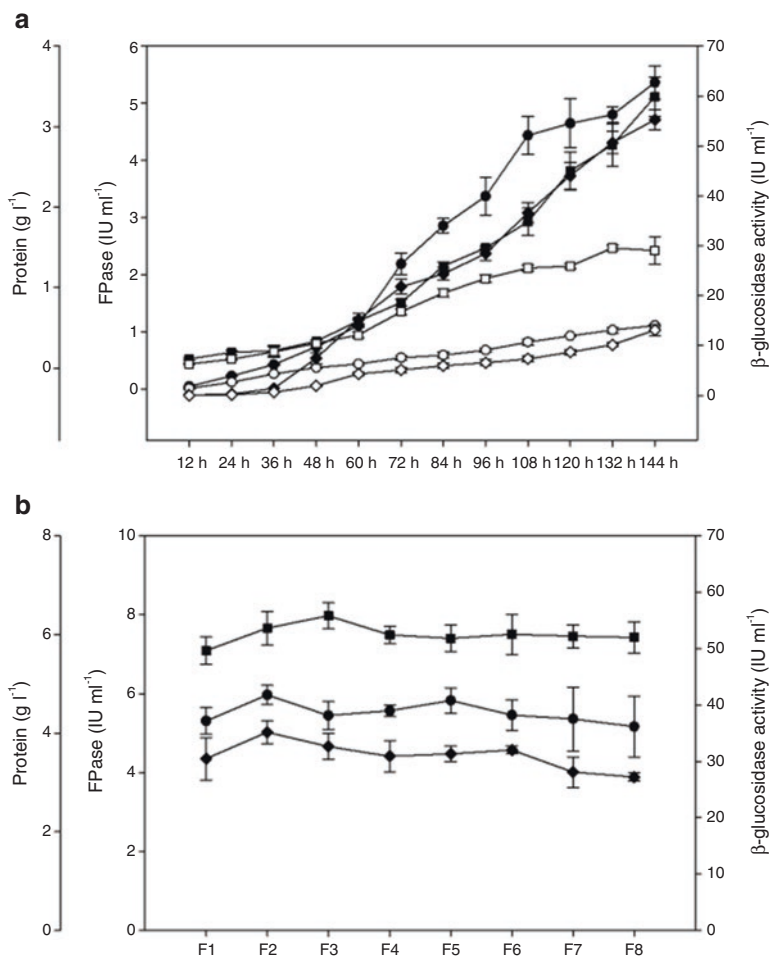


Fig. 5.3 Comparison of cellulase production in *P. piceum* 9-3 and H16 (a), filled symbol for H16 and blank symbol for 9-3 (circles for FPase, squares for protein, and diamonds for β -glucosidase), and stability of cellulase production of H16 after eight successive generations (b). Cellulase activity and protein concentrations were determined every 12 h until 144 h. All the error bars indicated the standard deviations in three replicate experiments

3 Novel β -Glucosidases from *P. piceum*

Theoretically, genome sequencing can provide the sequences of all known kinds of lignocellulolytic enzymes in one organism (Liu et al. 2013). The available genome sequences for an increasing number of microorganisms offer powerful tools for enzyme discovery. Fungal genome sequencing from *T. reesei* based on CAZy

Table 5.2 Cellulase production by mutants from *Penicillium* species

Organism	Substrate	Fermentation time (h)	Protein (g l ⁻¹)	FPA (FPU ml ⁻¹)	BGL (IU ml ⁻¹)	References
<i>P. piceum</i> H16	Avicel (3.3%)	144	3.66	5.83	53.12	This study
<i>P. decumbens</i> JU-A10	Wheat bran	144	0.51	1.96	1.02	Sun et al. (2008)
<i>P. pinophilum</i> NTG III/6	A	72–240	14.5	9.8	38	Brown et al. (1987)
<i>P. echinulatum</i> 9A02S1	Cellulose plus lactose (1% in total)	168	0.27	1.6	0.5	Sehnm et al. (2006)
<i>P. occitanis</i> Pol6	Avicel PH101 (8%)	187	11.4	12.6	17.5	Chaabouni et al. (1994)
<i>P. echinulatum</i> S1 M29	B	144		2	1.5	Dillon et al. (2011)

A, Solka-Floc BW40, milled barley straw, wheat bran, Avicel PH101 (1–6%)
B, Microcrystalline cellulose (1%) plus soy bran (0.2%) plus glucose or sucrose (0.5–1%)
BGL β-glucosidase, FPA filter paper activity, FPU filter paper unit

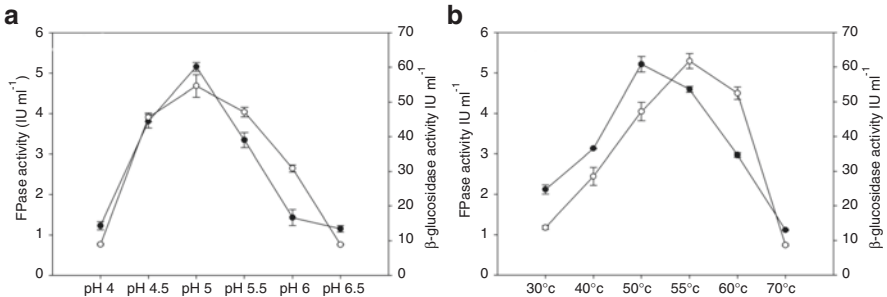


Fig. 5.4 Effects of temperature and pH on activity of cellulase; **(a)** influence of pH on FPase activity and β-glucosidase activity (filled symbol for FPase and blank symbol for β-glucosidase), **(b)** influence of temperature on FPase activity and β-glucosidase activity (filled symbol for FPase and blank symbol for β-glucosidase)

database predictions has revealed the presence of seven β-glucosidases: Cel1A, Cel1B, Cel3A, Cel3B, Cel3C, Cel3D, and Cel3E. Cel3A, Ce3B, and Cel3E, which have signal peptides, are extracellular BGLs that belong to glycosyl hydrolase family 3 based on the sequence similarity. Cel3A, as the main extracellular β-glucosidase, has been studied for many years. However, Cel3B and Cel3E have never been isolated from fungal extracellular proteins. The biological function and characteristics of Cel3B and Cel3E have not been characterized in detail.

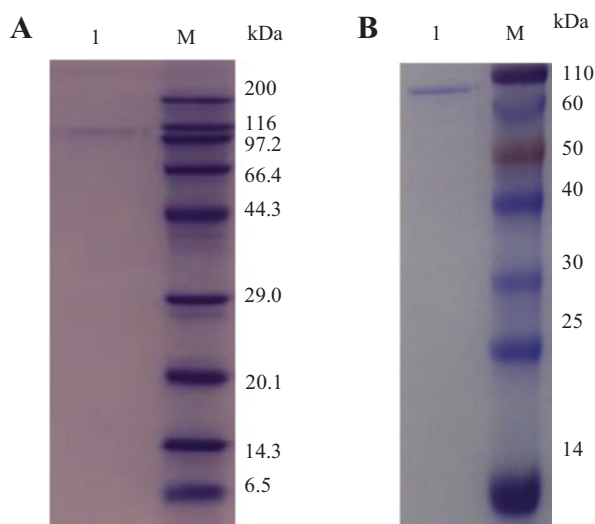
3.1 Purification and Identification of Two Novel β -Glucosidases

Cel3B and Cel3E have been found in *P. piceum* genome (unpublished data) and purified from the extracellular protein from *Penicillium piceum* via a gel filtration chromatography (Sephadex S200) (GE, Sweden). The two β -glucosidases had different molecular weight of 92 kDa and 80 kDa, respectively (Fig. 5.5).

Two β -glucosidases were identified by MALDI-TOF (MS/MS). A peptide with sequence of HYIANEQEHFR was found. The sequence of the protein was blasted using the search protocol blast. The peptide showed 99% similar to cel3b-like protein from *Beauveria bassiana*. The results of MALDI-TOF showed that the protein may be ranged to Cel3B belonging to family 3. The protein was designated as PpCel3B.

One peptide was sequenced as HYIGNEQETNR. The protein sequence was blasted using the search protocol blast. The peptide showed 92.977% similarity to BGL M from *Aspergillus niger* CBS 513.88. The results of the MALDI-TOF indicated that the protein belonging to GH family 3 should be ranged to Cel3E. The protein was designated as PpCel3E. PpCel3E showed 62.92% similarity with Cel3E from *Aspergillus niger*, 49.62% identity with Cel3E from *Trichoderma reesei*, 47.11% identity with Cel3E from *Metarhizium acridum* CQMa 102, and 49.43% identity with Cel3E from *Trichoderma atroviride*. Phylogenetic analysis of the orthologues was listed in Fig. 5.6.

Fig. 5.5 SDS-PAGE analysis of purified PpCel3B (a) and PpCel3E (b) from *P. piceum*. Lane M, protein marker (TransGen Biotech)



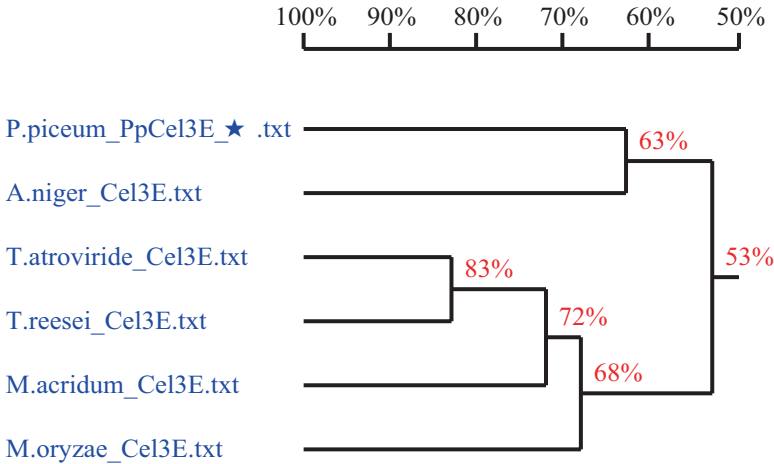


Fig. 5.6 Phylogenetic analysis of Cel3E from different fungus

Table 5.3 Substrate specificity of PpCel3B and PpCel3E

Substrate	Specific activity of PpCel3B (IU/mg)	Specific activity of PpCel3E (IU/mg)
CMC-Na	0	0
Xylan	0	0
pNPC	0	0
Cellobiose	23.4	14.4
pNPG	80	31
Salicin	408	0
Avicel		0
Xylotriose	188	125

3.2 Characteristics of Two Novel β -Glucosidases

Most BGLs could be classified into three groups based on their substrate specificity: (A) those with highly specificity for aryl β -D-glucosides, (B) those that with high specificity for cellobiose and cello-oligosaccharides, and (C) those with high specificity for both types of substrate (Rojas et al. 1995; Harnpicharnchai et al. 2009). PpCel3B and PpCel3E, which belong to family 3 of glycosyl hydrolases as a typical GH3 BGL, hydrolyze the non reducing terminal β -D-glucose residues of both types of substrates and release β -D-glucose (Liu and Yang 2005). PpCel3B, like Cel3A, had high activity of 23.4 IU/mg and 80 IU/mg against cellobiose and pNPG, respectively (Table 5.3). PpCel3E showed lower enzymatic activity of 14.4 IU/mg and 30 IU/mg against cellobiose and pNPG than PpCel3B. PpCel3B showed the highest

activity against salicin with a specific activity of 408 IU/mg. Moreover, PpCel3B and PpCel3E were active on β -1, 4 oligosaccharides consisting of up to seven glucose units. Surprisingly, PpCel3B and PpCel3E were found to be novel bifunctional glycoside hydrolases with both β -glucosidase and β -xylosidase like β -glycoside from *Chrysosporium lucknowense* (Dotsenko et al. 2012). PpCel3B and PpCel3E had new enzymatic activity of 188 IU/mg and 125 IU/mg against xylofuranose (Table 5.3).

PpCel3B and PpCel3E had the similar enzymatic properties. The optimum temperature of the two novel β -glucosidases was observed the same at 60 °C, while the optimum pH of the two novel β -glucosidases was detected at pH 5.0 (Fig. 5.7).

The reaction kinetic parameters of PpCel3B and PpCel3E were determined using double reciprocal Lineweaver–Burk plots. PpCel3B had an apparent K_m value of 0.003 mM and V_{max} value of 3.1 mM/min, while PpCel3E had an apparent K_m of 0.0019 mM and a V_{max} of 0.8 mM/min (Table 5.4) (Gao et al. 2013, 2014). The K_m of the two novel β -glucosidases for pNPG was in the same range as the K_m obtained

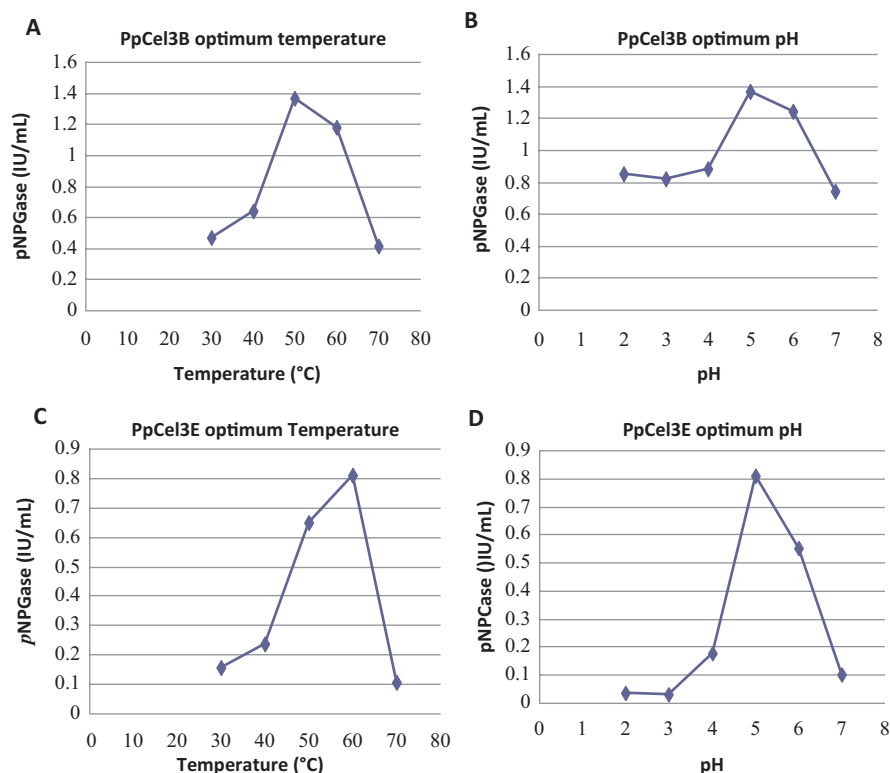


Fig. 5.7 Effect of optimum temperature (a) and optimum pH (b) on the activity of purified PpCel3B. Effect of optimum temperature (c) and optimum pH (d) on the activity of purified PpCel3E

Table 5.4 K_m values toward *p*NPG of native BGLs in fungi

Strains	K_m (mM)	Reference
<i>Penicillium piceum</i> (PpCel3E)	0.0019	Gao et al. (2014)
<i>Penicillium piceum</i> (PpCel3B)	0.0030	Gao et al. (2013)
<i>Penicillium pinophilum</i> (Cel3A)	5.5	Joo et al. (2010)
<i>Trichoderma reesei</i> (Cel3A)	0.14	Chen et al. (1992)
<i>Trichoderma reesei</i> (Cel1A)	0.18	Chen et al. (1992)
<i>Penicillium decumbens</i> (Cel3A)	0.006	Chen et al. (2010)
<i>Penicillium purpurogenum</i> (Cel3A)	5.1	Paavilainen et al. (1993)
<i>Aspergillus niger</i> (Cel3A)	0.57	Saloheimo et al. (2002)

for *P. decumbens* BGLs (0.006 mM) but much lower than those of *Aspergillus* BGLs (0.2–1.6 mM). *P. purpurogenum* BGL exhibited a comparable K_m for *p*NPG (5.1 mM) (Jeya et al. 2010), whereas a *P. pinophilum* BGL exhibited a K_m of 5.5 mM (Table 5.4) (Joo et al. 2010). A low K_m is very important for industrial saccharification because it reduces the product inhibition of cellobiose on other enzymes in the cellulase system (Chen et al. 2010). The K_m of PpCel3E was the lowest for *p*NPG ever reported for an extracellular BGL in fungi. This finding implies that PpCel3E has a high affinity for cellobiose analogues.

3.3 Analyzing the Biological Function of the Two Novel β -Glucosidases

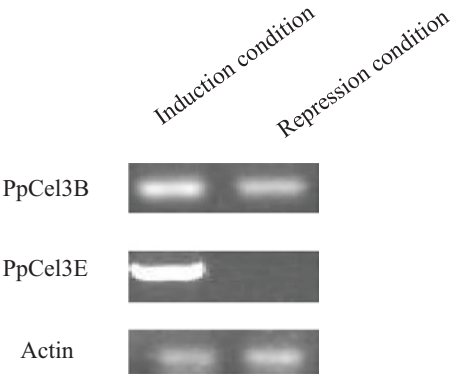
The enzyme BGL has many distinct biological roles in cellulase systems, such as minimizing product inhibition (Takashima et al. 1999; Paavilainen et al. 1993) and transglycosylation (Vaheri et al. 1979), thereby facilitating the formation of soluble inducer compounds for cellulase production.

Various studies have reported that the BGL showing transglycosylation activity is Cel3A. Several studies about transglycosylation of Cel3A have been reported. *Aspergillus oryzae* Cel3A was able to convert glucose to produce 52.48 mg/mL gentiobiose (He et al. 2013). *Fusarium oxysporum* Cel3A synthesizes cellotriose with 15–20% recovery and with initial substrate concentration of 16% cellulose and 40% gentiobiose mixture (Christakopoulos et al. 1994). Disruption of the *Cel3a* gene delays the induction of other cellulose-induced cellulase genes, but not sophorose-induced genes (Fowler and Brown 1992; Kubicek et al. 2009). A *cel3a*-multicopy strain produces larger amounts of cellulases than the parent strain under unsaturated sophorose concentrations (Kubicek et al. 2009). However, the BGL inhibitor nojirimycin strongly inhibits cellulase induction in all strains, including the *cel3a*-disrupted strain, which suggests that cel3A is not the only BGL involved in inducer formation (Kubicek et al. 2009; Stricker et al. 2006). Other protein-forming cellulase inducers among extracellular fungal proteins remain unidentified.

Table 5.5 Concentration of inducer produced by PpCel3B and PpCel3E

Produced inducer	Concentration (g/g PpCel3E)	Concentration (g/g PpCel3B)
Gentibiose	1100	75
Sophorose	142	150
Xylobiose	42	64

Fig. 5.8 Semi quantitative RT-PCR analysis of *Ppcel3b* and *PpCel3e* transcript in *P. piceum* grown on glucose and cellulose. β -actin was used as internal control to verify the RT-PCR reaction



PpCel3B and PpCel3E were firstly found to have high transglycosylation activity during disaccharide synthesis. The two novel β -glucosidases successfully synthesized two important distinct disaccharide derivatives (sophorose and gentiobiose) from glucose. PpCel3B showed high transglycosylation activity of 150 mg sophorose/mg protein and 75 mg gentiobiose/mg protein (Table 5.5). PpCel3E catalyzed gentiobiose production at 1100 mg gentiobiose/mg PpCel3E and sophorose production at 142 mg sophorose/mg PpCel3E (Table 5.5). This result suggests that two novel β -glucosidases may be the key enzymes in the extracellular cellulase system for inducer formation aside from Cel3A.

Besides the inducers by transglycosylation, PpCel3B and PpCel3E still could form other inducers. PpCel3B and PpCel3E had novel enzymatic activity against xylotriase to produce xylobiose and D-xylose (Table 5.5). Xylobiose and D-xylose could activate the regulatory transcription factor, which could improve the cellulase and hemicellulase production.

3.4 Transcript Pattern of Two Novel β -Glucosidases

Although PpCel3B and PpCel3E had the similar enzymatic characteristics, *PpCel3b* and *PpCel3e* had different transcript pattern. Semi quantitative PCR analysis showed that *PpCel3b* could transcript both under repression and induction condition and that *PpCel3e* could transcript only under induction condition (Fig. 5.8).

The transcription level of *PpCel3b* was 2.2 times as much as that produced under repression condition (Fig. 5.8). It implied that *PpCel3b* was a constitutive gene and *PpCel3e* was an induced gene. PpCel3B could hydrolyze the oligosaccharides and xylooligomer, which simultaneously form multiple inducers for cellulase and hemicellulase.

3.5 The Role of Two Novel β -Glucosidases in Boosting Enzymatic Degradation of Delignified Corn Stover

In order to clarify the role of two novel β -glucosidases in boosting the enzymatic degradation of delignified corn stover, PpCel3B and PpCel3E were supplemented, respectively, to different cellulases with a small dosage of 52 and 40 $\mu\text{g/g}$ substrate in total of 50 mL volume, respectively. PpCel3B and PpCel3E both showed apparent synergism with cellulase from *T. reesei* and *P. piceum*. In the supplementation experiment, although the dosage of PpCel3B was small, the glucose yield released from delignified corn stover by the cellulase from *T. reesei* and *P. piceum* increased to 15% and 35%, respectively (Fig. 5.9a). Adding 40 μg PpCel3E/g substrate into the commercial *T. reesei* cellulase increased the glucose concentration by 20%, whereas adding it into commercial *P. piceum* cellulase increased the glucose concentration by 27% (Fig. 5.9b).

Alkali pretreatment of corn stover resulted in a large number of hemicellulose existence. Qing et al. showed that xylanase activities in most commercial enzyme preparations have been shown to be insufficient to completely hydrolyze xylan, resulting in high xylooligomer concentrations remaining in the hydrolysis broth (Qing and Wyman 2011). The accumulation of xylooligomer resulted in the decrease of initial hydrolysis and a lower final glucose yield. A comparison among glucose sugars and xylose sugars also showed that xylooligomers were more powerful inhibitors than well-established glucose and cellobiose (Qing and Wyman 2011). In the saccharification of cellulase from *T. reesei* and *P. piceum*, the main accumulation of xylooligomers was xylotriose (data not shown). PpCel3B and PpCel3E showed a new enzymatic activity with 188 IU/mg and 125 IU/mg against xylotriose, respectively. Due to the addition of the novel β -glucosidases, the accumulation of xylotriose apparently decreased (data not shown). Removing the effect of xylotriose and cellobiose inhibition by applying the novel β -glucosidases could play an important role in improving the lignocelluloses conversion.

The yields of glucose released by *T. reesei* cellulase increased by 19.4% after addition of β -glucosidase (Novozyme 188) with 1.45 mg protein/g glucan (Zhang et al. 2010a, b). The supplementation of commercial β -glucosidase (60 IU/mL) to cellulase from *Penicillium* could improve biomass hydrolysis efficiency by 25.7% (Rajasree et al. 2013). The saccharifying ability of QM9414 cellulase toward corn-cob improved 76.4% by adding purified β -glucosidase (Novozyme NS-50010) at the ratio of FPA and β -glucosidase to 1:1 (Table 5.6) (Chen et al. 2010). However,

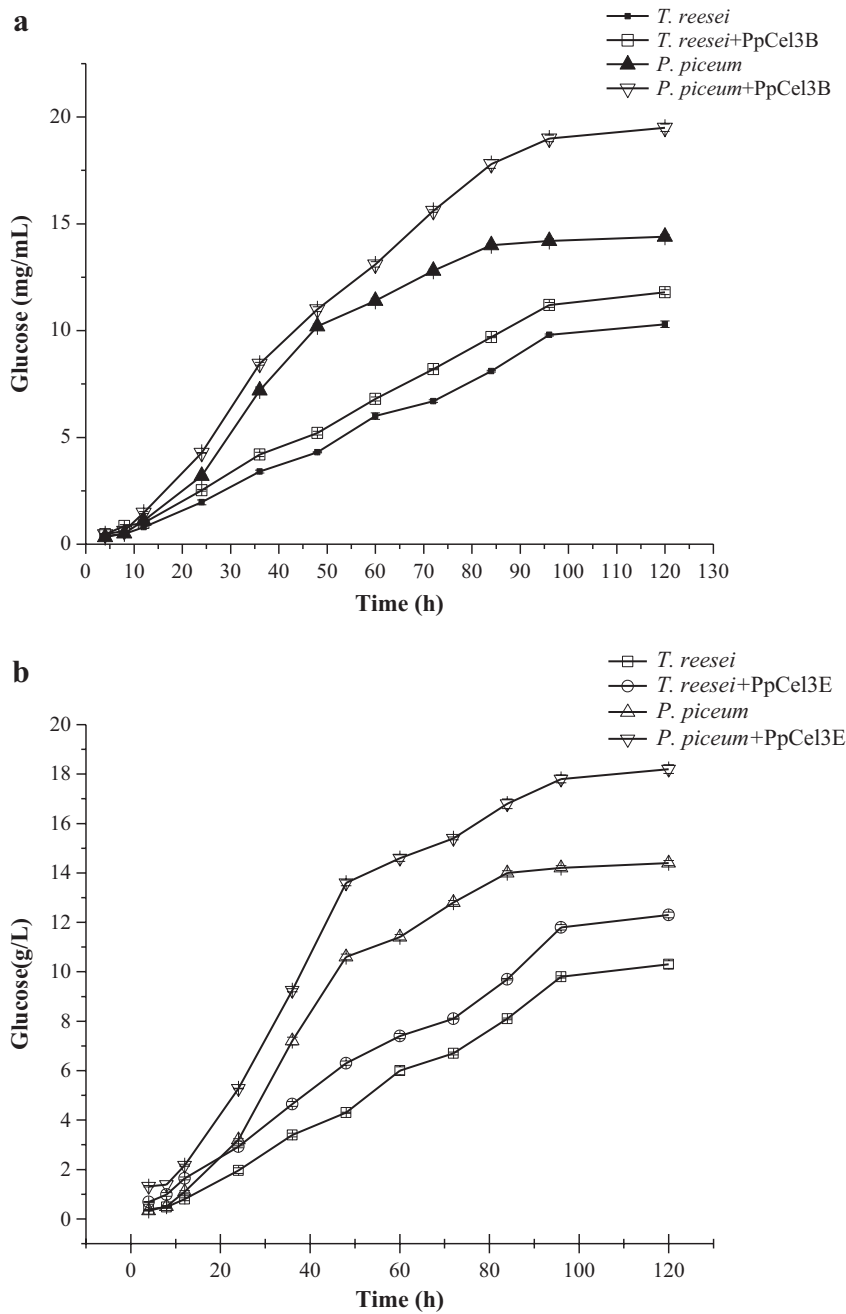


Fig. 5.9 (a) Synergism between PpCel3B and different cellulase, with a supplementation dosage of 52 μg PpCel3B/g substrate. (b) Synergism between PpCel3E and different cellulase, with a supplementation dosage of 40 μg PpCel3E/g substrate

Table 5.6 Improving lignocellulolytic enzymes by different β -glucosidase supplementation

Starting enzyme system	Supplement	Protein concentration	Achievement	References
<i>T. reesei</i> cellulase	β -glucosidase (Novozyme 188)	1.45 mg protein/g glucan	19.4%	Zhang et al. (2010a, b)
Cellulase from <i>Penicillium</i>	Commercial β -glucosidase	(60 IU/mL)	25.7%	Rajasree et al. (2013)
QM9414 cellulase	Purified β -glucosidase (Novozyme NS-50010)	FPA and β -glucosidase to 1:1	76.4%	Chen et al. (2010)
<i>Penicillium piceum</i> cellulase	PpCel3B	52 μ g/g substrate	35%	Gao et al. (2013)
<i>Penicillium piceum</i> cellulase	PpCel3E	40 μ g/g substrate	27%	Gao et al. (2014)

the promoting effect of PpCel3B and PpCel3E was not as remarkable as mentioned by Chen et al. (2010), which could be due to the variations of β -glucosidase supplementation quantity and the different sources of enzyme complex (Chen et al. 2010). Previous studies only demonstrated that commercial β -glucosidase plays an important role in removing cellobiose inhibition. In this paper, the addition of PpCel3B or PpCel3E to different cellulase preparation could help alleviate both effect of inhibition by cellobiose and xylooligomers and thus facilitate cellulose saccharification (Chen et al. 2010).

4 Thermostability Improvement of Cel3A from *P. piceum*

As shown in Fig. 5.10, the β -glucosidase from *Aspergillus niger* had high thermostability, with 95.6% of its activity remaining at 50 °C for 48 h. However, the main β -glucosidase from *P. piceum* H16 (Cel3A from *P. piceum* H16; PpCel3A used in the following study for better understanding) exhibited significantly poor thermostability under the same conditions, only 58.5% of its activity remained. Thus, the 3D structure of PpCel3A was built and used to select the single-point mutants and triple mutations (Fig. 5.10).

Glycine is the only amino acid that lacks a β -carbon, while proline can adapt a few configurations and has the lowest conformational entropy (Schultz et al. 2005; Vieille and Zeikus 2001). A glycine to proline mutation could decrease conformational fluctuation and lead to stabilization. Gly233, which is located close to a fifteen amino acid loop region, and Gly305, which is located close to a four amino acid loop region, were selected as potential mutation sites, and the mutants for G233P and G305P were generated (Fig. 5.11). Both of these mutant sites are far away from the active site, and according to the hypothesis of Cui et al. (2013), the enzymatic activity should not be influenced.

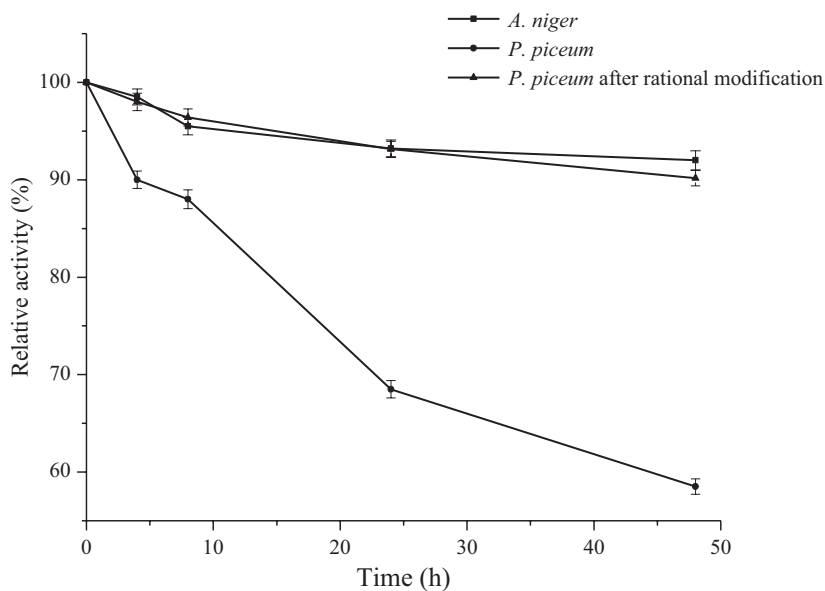


Fig. 5.10 Thermostability test of the β -glucosidase from *Aspergillus niger* and *Penicillium piceum* H16 before and after modification at 50 °C for 48 h. Each point presented is the mean of triplicate assays

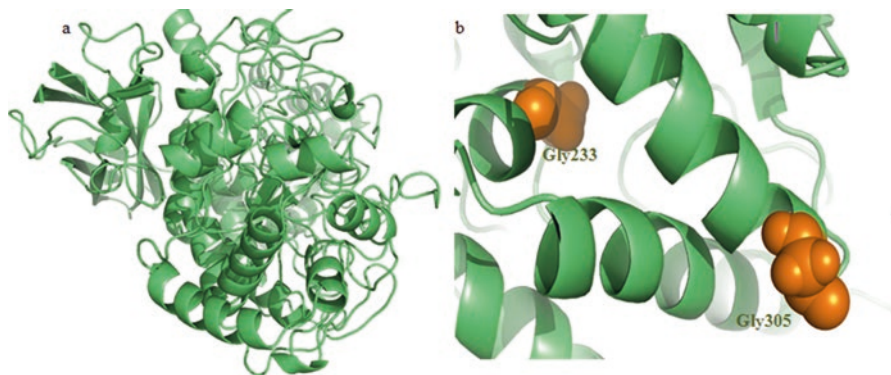


Fig. 5.11 The model of β -glucosidase and single-point mutant selection using proline theory. (a) the model of β -glucosidase from *Penicillium piceum* H16; (b) the mutant sites of Gly233 and Gly305 selected by proline theory

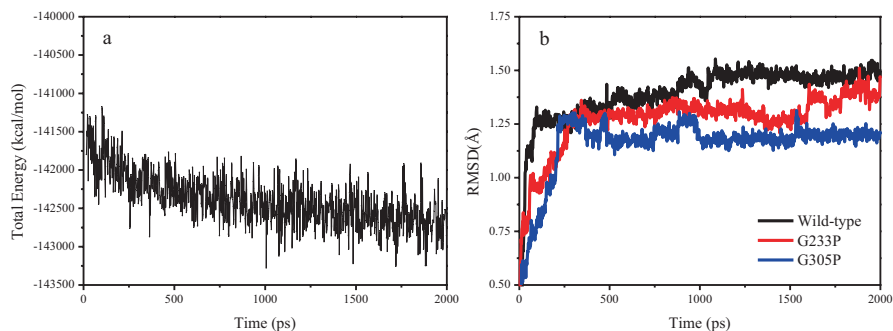


Fig. 5.12 The 2000 ps MD simulation for wild-type β -glucosidase and mutants. (a) The total energy of the wild-type β -glucosidase during MD simulation; (b) the RMSD values for wild-type and mutants

4.1 Molecular Dynamics (MD) Simulation to Predict Mutant Effects on Protein Stability

To decrease cost and increase experimental efficiency, the root mean square deviation (RMSD) value was used to evaluate the performance of the attempted mutants. As shown in Fig. 5.12, the total energy of the wild-type protein indicated that this structure reached equilibrium during the MD simulation after 1500 ps. The RMSD values of the backbone atoms for the wild type and mutants are shown in Fig. 5.12, and the step which was performed immediately before the MD simulation was used as the reference. As the RMSD value reflects fluctuation of the protein structure, a higher RMSD value indicates lower protein stability (Fujiwara et al. 2009). The mutants, G233P and G305P, which have RMSD values lower than that of the wild-type β -glucosidase, indicated that substitution of a proline for a glycine at amino acid 233 or 305 yielded a more stable conformation.

4.2 Computer-Assisted Virtual Saturated Mutation for the Design of a Triple Mutation

The increase of thermostability, which can be achieved with the incorporation of a single mutation into a protein, is limited (Cui et al. 2013). Thus, triple mutations were designed using the *Calculate Mutation Energy/Stability* module of DS 4.0 in an effort to provide increased thermostability beyond what could be obtained by incorporating a single mutation. As shown in Fig. 5.13, the root mean square fluctuation (RMSF) values of all amino acids indicated that a nine amino acid loop region showed the highest conformation fluctuation which, in turn, might have negative effects on the thermostability of PpCel3A. Thus, these nine amino acids were selected as possible sites for mutagenesis and defined as a mutant group.

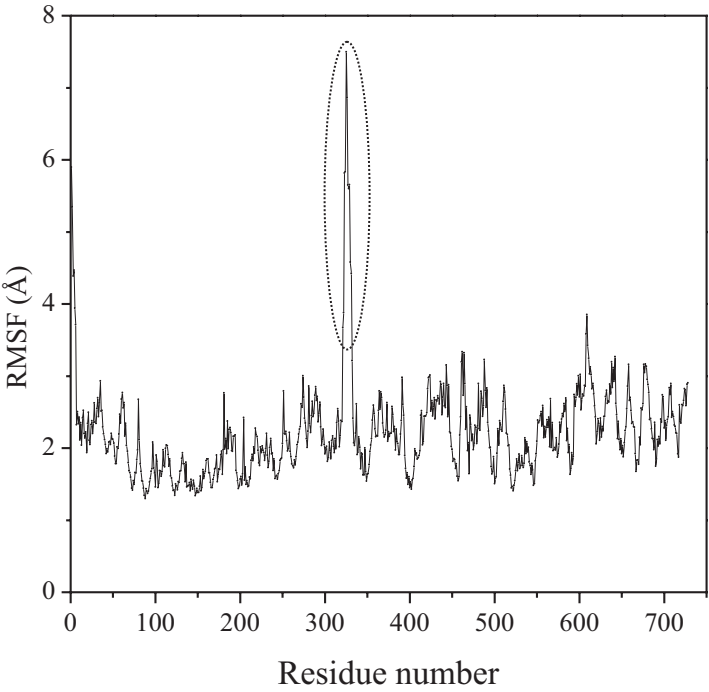


Fig. 5.13 The RMSF values were calculated for all amino acids of wild-type β -glucosidase. The nine amino acid loop region which is marked by the black dotted line showed the highest fluctuation and was selected as the region subjected to virtual mutation

Table 5.7 Single-point mutations generated for wild-type β -glucosidase

Index	Mutation	Mutation energy (kcal/mol)	Effect
1	:GLN512>TRP	−2.23	Stabilizing
2	:SER507>PHE	−2.08	Stabilizing
3	:SER514>TRP	−2.07	Stabilizing
4	:ASN506>TRP	−1.9	Stabilizing
5	:SER514>ARG	−1.82	Stabilizing
221	:SER507>TRP	7.5	Destabilizing
222	:THR513>TYR	9.87	Destabilizing
223	:ASN509>PRO	10.13	Destabilizing
224	:THR513>PRO	16.65	Destabilizing
225	:GLY511>PRO	83.79	Destabilizing

First, computer-assisted virtual saturation mutagenesis was carried out for this mutant group to obtain the optimal single-point mutants using the mutation energy as the unique standard to evaluate mutant stability (Table 5.7). Then, five optimal triple mutations were generated with the *Calculate Mutation Energy/Stability*

Table 5.8 Triple mutations generated for wild-type β -glucosidase

Index	Mutation	Mutation energy (kcal/mol)	Effect
1	:SER507>PHE.:GLN512>TRP.:SER514>TRP	-9.57	Stabilizing
2	:ASN506>TRP.:GLN512>TRP.:SER514>TRP	-7.8	Stabilizing
3	:ASN506>TRP.:SER507>PHE.:GLN512>TRP	-7.53	Stabilizing
4	:ASN506>TRP.:SER507>PHE.:SER514>TRP	-6.75	Stabilizing
5	:ASN506>TRP.:SER507>PHE.:SER514>ARG	-5.8	Stabilizing

Table 5.9 The thermostability of wild-type β -glucosidase and the mutants after 48 h at 50 °C

Mutations	Relative activity (%)
Wild type	58.5 $\pm$ 1.6
G305P	70.2 $\pm$ 3.5
G233P	62.5 $\pm$ 2.2
S507F/Q512W/S514W	85.6 $\pm$ 3.2
N506W/Q512W/S514W	77.6 $\pm$ 2.9
N506W/S507F/Q512W	70.3 $\pm$ 0.9
N506W/S507F/S514W	65.9 $\pm$ 2.8
N506W/S507F/S514R	71.8 $\pm$ 3.1

module by taking into account the above optimal single-points (Table 5.8). Based on the assumption that the lowest mutation energy represented the most stable mutation, the energies for the triple mutations were much lower than the optimal single-point mutants. The triple mutants with the lowest mutation energies were selected for further study and used to improve the thermostability of PpCel3A.

4.3 Improvement of Mutant PpCel3A Thermostability

To evaluate the stabilities of these mutants in vitro, the wild type and mutants were cloned and expressed in *E. coli* and purified. The thermostability of the mutants were determined by measuring the residual activity after incubation at 50 °C for 48 h. The most stable mutants were the single-point mutant G305P and the triple mutations of Ser507Phe/Gln512Trp/Ser514Trp (S507F/Q512W/S514W), which had 70.2% and 85.6% activity remaining, respectively, and showed an improvement in thermostability of about 20.0% and 46.3%, respectively (Table 5.9).

Additionally, the G233P mutant retained 62.5% activity and the triple mutant N506W/Q512W/S514W, N506W/S507F/Q512W, N506W/S507F/S514W, and N506W/S507F/S514R retained from 65.9% to 77.6% activity. In agreement with the predictions from the MD simulation, the experimental results showed that these mutations remarkably enhanced the thermostability of PpCel3A.

Table 5.10 Kinetic parameters for the wild-type β -glucosidase and mutants in the hydrolysis of *p*NPG

Enzyme	k_{cat} (s^{-1})	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ ($\text{s}^{-1} \text{mM}^{-1}$)
Wild type	2.6 ± 0.11	1.2 ± 0.04	2.1 ± 0.04
G305P	3.2 ± 0.18	0.8 ± 0.04	4.0 ± 0.11
G233P	2.9 ± 0.15	1.5 ± 0.06	1.9 ± 0.03
S507F/Q512W/S514W	3.7 ± 0.22	1.7 ± 0.05	2.1 ± 0.09
N506W/Q512W/S514W	3.0 ± 0.13	1.1 ± 0.03	2.7 ± 0.02
N506W/S507F/Q512W	2.6 ± 0.08	0.9 ± 0.04	2.8 ± 0.08
N506W/S507F/S514W	2.8 ± 0.11	1.5 ± 0.08	1.8 ± 0.06
N506W/S507F/S514R	3.2 ± 0.14	1.9 ± 0.07	1.6 ± 0.08

4.4 Kinetic Characterization

Kinetic parameters of wild-type and mutant PpCel3A were measured as described in the Materials and Methods section. Table 5.10 shows that the K_{m} values for the optimal single-point mutant was the lowest. At the same time, most of the mutants showed an increased catalytic turnover frequency (k_{cat}). In case of the mutant S507F/Q512W/S514W, 42.5% increase in k_{cat} were observed for the substrate of *p*NPG.

4.5 Homologous Replacement of Original PpCel3A by Triple-Mutant PpCel3A in *P. piceum* H16

The triple mutation of PpCel3A (S507F/Q512W/S514W) having the good thermostability was verified in vitro. The triple mutation of PpCel3A with homologous arms and resistance screening plasmid was cotransformed into *P. piceum* H16. The modified PpCel3A successfully replaced the original PpCel3A. The extracellular β -glucosidase thermostability of *P. piceum* H16 after modified PpCel3A homologous replacement had 90.16% activity remaining and showed an improvement of thermostability by about 54.12% after 50 °C incubation for 48 h (Fig. 5.10). Directed evolution of enzyme thermostability using computer-assisted rational design was proved useful for improving unique properties of *P. piceum* H16.

4.6 Model and Mechanism Analyses

To investigate the mechanism responsible for the improvements in thermostability, the structures of the optimal single-point and triple mutants were analyzed in detail. It is well accepted that hydrogen bonds and hydrophobic interactions are the dominant structural factors responsible for protein thermostability. The former

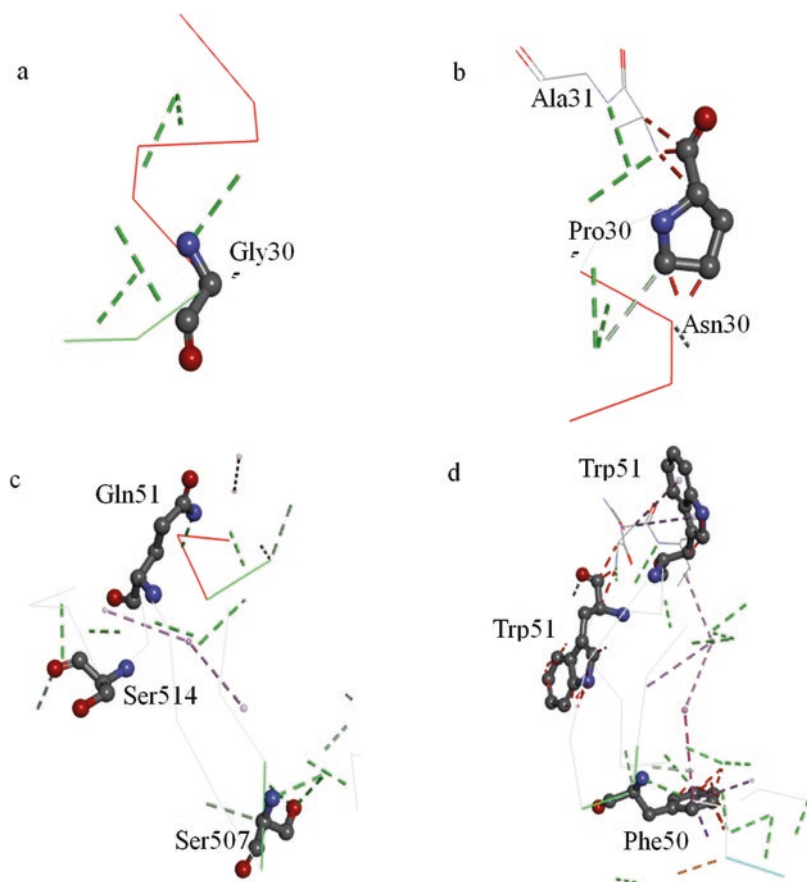


Fig. 5.14 Predicted hydrogen bonds and hydrophobic interactions in the models of wild-type glucosidase (a), G305P glucosidase (b), wild-type glucosidase (c), and S507F/Q512W/S514W glucosidase (d). Hydrogen bonds are represented by green dotted lines. Hydrophobic interactions are shown by red and pink dotted lines

contributes to the stability of the protein, and the latter is thought to provide the energy required for proteins to fold (Vieille and Zeikus 1996, 2001; Zhang et al. 2010a, b).

Five hydrogen bonds were observed in the model of both the wild-type Gly305 and the mutant G305P (Fig. 5.14). Five additional hydrophobic interactions including Pro305: CG- O: Asn301, Pro305: CD- O: Asn301, Pro305: CA- N: Ala312, Pro305: C- N: Ala312, and Pro305: C- CA: Ala312 were apparent after substitution with proline at amino acid 305. This indicated that the improved thermostability gained from the G305P substitution was primarily a result of the additional hydrophobic interactions and the overall improvement of stability generated by incorporating a proline into the protein structure.

As shown in Fig. 5.14, 17 and 19 hydrogen bonds were predicted in the model of the wild-type and the triple mutant S507F/Q512W/S514W β -glucosidase, respectively. The triple mutations also resulted in 42 additional hydrophobic interactions of which 38 of these directly involved the three incorporated amino acids of Phe507, Trp512, and Trp514. This indicated that the improvement in thermostability of the triple mutant was primarily attributed to the large number of additional hydrophobic interactions.

The results showed that the triple mutation, which was selected by the computer assisted virtual saturated mutation approach, gave the most meaningful outcome. Moreover, the virtual saturated mutation, which is based on the reliable computation of three or more combination mutations, was inexpensive, highly efficient, and able to predict the output.

5 Application of the Modified *Penicillium piceum*

The modified *Penicillium piceum*, high-yielding β -glucosidase, had the high β -glucosidase activity of 53.12 IU/ml. The PpCel3A was the main extracellular β -glucosidase and improved the thermostabilities by computer-assisted rational modification by 54.12%. PpCel3B and PpCel3E, novel bifunctional β -glucosidase, could boost the enzymatic degradation of delignified corn stover by removing both effect of inhibitions by cellobiose and xylooligomer. The β -glucosidase produced by *P. piceum* had unique properties of high β -glucosidase activity and good thermostability which were not processed by other fungal strains and thus facilitate cellulose saccharification.

To assess synergism effect of extracellular β -glucosidase produced by *P. piceum* H16 on different substrates, the hydrolytic capacities of cellulase from a single culture of *T. reesei* RUT C30, *P. piceum* H16, and those two enzyme culture mixtures using the same enzyme loading were compared in the hydrolysis of different substrates, including untreated corn stover (UCS), corncob residue (CCR), hot-water pretreated corn stover (HWCS), cassava stillage residue (CSR), and steam-exploded corn stover (SECS). As shown in Fig. 5.15, the glucose concentrations produced by cellulase from a single culture of *P. piceum* H16 were slightly lower than those of a single culture of *T. reesei* RUT C30 on these three substrates. However, glucose concentrations from the mixed enzyme were significantly higher than the treatments with a single cellulase from *T. reesei* RUT C30 or *P. piceum* H16, demonstrating that a good synergistic effect existed between β -glucosidase of *P. piceum* H16 and cellulases of *T. reesei* RUT C30 on hydrolyzing different substrates. The glucose concentrations released from UCS, CCR, and SECS using the mixed enzyme increased by 32.0%, 29.8%, and 26.4% than that using cellulases of *T. reesei* RUT C30, respectively.

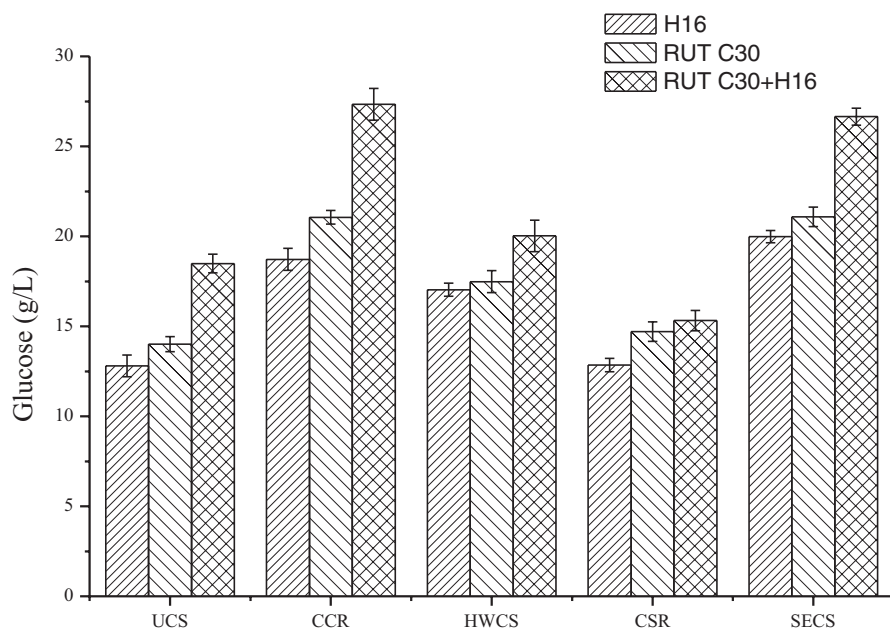


Fig. 5.15 Glucose released from untreated corn stover (UCS), corncob residue (CCR), hot-water pretreated corn stover (HWCS), cassava stillage residue (CSR), and steam-exploded corn stover (SECS) by cellulase from *P. piceum* H16, *T. reesei* RUT C30, and a mixture of both (RUT C30+H16)

6 Conclusion

In conclusion, a fungus belonging to the *Penicillium* genus is attracting increasing attention as an alternative to *T. reesei* for second-generation biofuels. In this chapter, the newly isolated wild strain *P. piceum* 9-3 proved to be a potential strain for cellulase production. Through DES mutagenesis, a cellulase over producing mutant was obtained with significantly improved cellulase productivity, especially β -glucosidase. Two new β -glucosidases (PpCel3B and PpCel3E) were purified in the extracellular proteins from *P. piceum* H16. The two new β -glucosidases were promising bifunctional enzymes for lignocellulosic bioconversion, which could remove multiple inhibitors of cellulose degradation and act as an inducer of cellulase production. Computer-assisted rational design improved thermostability of PpCel3A, in favor of thermostability enhancement of the extracellular *P. piceum* β -glucosidase. *P. piceum* with high β -glucosidase activity and good thermostability had an obvious synergetic effect on *T. reesei* cellulase hydrolysis of different substrates, which suggested that *P. piceum* had unique properties which were not processed by other fungal strains and thus facilitate cellulose saccharification.

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Chapter 6

The Model Filamentous Fungus *Neurospora crassa*: Progress Toward a Systems Understanding of Plant Cell Wall Deconstruction



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Yisong Liu, Duoduo Zhang, Shijie Guo, Qian Liu, Hao Fang, and Yahong Wei

Abstract *Neurospora crassa* colonizes freshly burnt plant biomass and shows robust growth on cellulosic material. This model filamentous fungus has well-developed genetics, biochemistry, and molecular biology. A collection of resources and tools available for this organism makes it an ideal model system for a systems-level elucidation of the mechanisms underlying plant cell wall deconstruction by filamentous fungi. Research on *N. crassa* has contributed to the discovery of a new class of plant cell wall-degrading enzymes called lytic polysaccharide monooxygenases (LPMOs). The availability of a full genome deletion strain set for *N. crassa* has expedited the identification of two essential transcription factors, CLR-1 and CLR-2, which are required for the expression of cellulolytic genes and growth on crystalline cellulose. Recently, a detailed network in *N. crassa* was constructed of biochemical reactions important for the degradation of plant cell wall polysaccharides, providing a scaffold for a systems analysis of plant cell wall deconstruction. In this chapter, we cover recent progress toward elucidation of plant cell wall deconstruction by *N. crassa*. Remarkable recent progress provides new strategies for targeted strain improvement for the production of plant cell wall-degrading enzymes.

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Keywords Plant cell wall deconstruction · Filamentous fungi · *Neurospora crassa*

1 Introduction

Every year, nature produces about 150–170 billion tons of biomass by photosynthesis, of which only 3–4% are used for food and nonfood purposes (Röper 2002; Somerville et al. 2010). Plant cell walls are the major proportion of terrestrial biomass comprises (Somerville 2006). Generally, most of the terrestrial plant biomass contains 35–50% of cellulose, 20–35% of hemicellulose, and 10–25% of lignin (Faik 2013). Plant cell wall polysaccharides are widely viewed as a renewable feedstock for the production of biofuels and other bio-products (Somerville et al. 2010).

Degradation of cellulosic biomass in nature is carried out by microbial communities, in which polysaccharides are consumed as food, while lignin is transferred from plant to soil. There are different mechanisms used by microorganisms to degrade plant cell wall polysaccharides, all of which involve enzymes, such as cellulases and hemicellulases (Wilson 2011). Many aerobic microbes secrete sets of individual cellulases, which act synergistically on crystalline cellulose to hydrolyze the polysaccharide to simple sugars (Wilson 2008). Both ascomycete and basidiomycete filamentous fungi have the capacity to secrete large amounts of plant cell wall-degrading enzymes (Coradetti et al. 2013).

Biofuels production facilities have been constructed around the world to convert cellulosic biomass to ethanol. It is a major technical challenge to overcome the heterogeneity and recalcitrance of plant cell walls in a cost-effective manner (Payne et al. 2015). In particular, enzymatic hydrolysis of cellulosic biomass is a major cost driver due to the high cost of enzyme production (Davis et al. 2013; Humbird et al. 2011; Kleinmarcusamer et al. 2012). Therefore, significant efforts have been made to understand and improve the processes associated with plant cell wall deconstruction and cellulolytic enzyme production, with the aim to decrease the cost of biofuels and bio-products production (Huberman et al. 2016; Glass et al. 2013).

Filamentous fungi are the main source of plant cell wall-degrading enzymes for the production of biofuels and bio-products. The most commonly used filamentous fungus for the production of cellulolytic enzymes in industry is the ascomycete species *Trichoderma reesei* (*Hypocrea jecorina*) (Druzhinina and Kubicek 2017). Other filamentous fungi, such as *Penicillium oxalicum*, are potential alternatives to *T. reesei* (Gusakov 2011; Li et al. 2017). Cellulase hyperproducing mutants of *T. reesei* were obtained by random mutagenesis and screening. Targeted strain improvement requires a systems-level understanding of the mechanisms underlying plant cell wall deconstruction and the production of plant cell wall-degrading enzymes. However, it has been somewhat problematic to unravel the mechanisms of plant cell wall deconstruction and the production of plant cell wall-degrading enzymes in *T. reesei* due to lack of extensive genetic and molecular tools (Gusakov 2011; Znameroski and Glass 2013).

Neurospora and *T. reesei* are phylogenetically related, as both belong to the class of Sordariomycetes (Fitzpatrick et al. 2006; Seibert et al. 2016). By contrast to *T. reesei*, a variety of molecular, genetic, and biochemical techniques have been developed for the model filamentous fungus *Neurospora crassa* (Seibert et al. 2016; McCluskey and Baker 2017). In particular, the availability of a near-complete collection of gene deletion mutants of *N. crassa* has expedited high-throughput screening for mutants that display a cellulolytic phenotype (Glass et al. 2013; Znameroski and Glass 2013). The availability of diverse tools, the rapid growth, and the general accepted safety profile make *N. crassa* an attractive system to study plant cell wall deconstruction (McCluskey and Baker 2017; Colot et al. 2006; Havlik et al. 2017; Perkins and Davis 2000; Allgaier et al. 2009). This chapter covers recent progress toward understanding plant cell wall deconstruction by *N. crassa* and its regulatory mechanisms.

2 Lifestyle of *Neurospora crassa*

N. crassa is proficient at degrading plant cell wall for use as a source of carbon. It first received major attention in 1843, when there was a *Neurospora* infestation in French bakeries (Davis and Perkins 2002). The mycologists Cornelius L. Shear and Bernard O. Dodge assigned this fungus to the genus *Neurospora* in the mid-1920s (Shear and Dodge 1927). The lifestyle of *N. crassa* in nature is still under investigation (Kuo et al. 2014; Gladieux et al. 2015). *N. crassa* is known as a saprotroph, and most of its isolates have been obtained from vegetation or trees after forest fire (Jacobson et al. 2004). It is suggested that heat from forest fire stimulates the germination of ascospores and provides a sterile, nutrient-containing environment that favors growth (Kuo et al. 2014; Jacobson et al. 2004).

Recent evidences suggest that the lifestyles of *N. crassa* and other fungi may change in adapting to changing environments. Qi et al. reported the isolation of *Neurospora* species from *Acer ginnala* (Amur maple) in Northeast China and proposed *Neurospora* an endophyte (Qi et al. 2012). Field observations (Jacobson et al. 2004) and experimental inoculations of pine seedlings (Kuo et al. 2014) suggest that *Neurospora* species may be living as an endophyte or a saprotroph, which is likely controlled by both environmental and host factors. Kuo et al. further reported that *N. crassa* may switch to a pathogenic state when its balanced interaction with the host is disrupted (Kuo et al. 2014). Lifestyle switching has been reported with other fungi as well (Rai and Agarkar 2016), such as *Epichloe festucae* (Tanaka et al. 2006), *Diplodia mutila* (Alvarez-Loayza et al. 2011), *Moniliophthora perniciosa* (Lana et al. 2011), and some *Colletotrichum* species (Redman et al. 2001).

Neurospora and other fungal species can secrete various functional groups of proteins (Glass et al. 2013), including effector proteins as well as groups of enzymes for plant cell wall deconstruction, self-protection, or nutrient acquisition, such as carbohydrate-active enzymes (CAZymes), oxidoreductases, proteases, and lipases, which appear to reflect the lifestyle and ecological niches of individual species

(Girard et al. 2013; Kim et al. 2016). In general, biotrophs encode fewer CAZymes than hemibiotrophs and necrotrophs (Zhao et al. 2013). However, a large-scale screening of hydrolytic activities among 156 species of plant pathogenic and nonpathogenic fungi showed that among moderately and highly active species, plant pathogenic species were found to be more active than nonpathogens on the tested plant cell wall substrates (King et al. 2011).

Epigenetic analysis suggests that epigenetic mechanisms play a role in homeostasis and phenotypic plasticity of *N. crassa* across a range of controlled environments (Kronholm et al. 2016). In particular, histone methylation at H3K36 affected plastic response to high temperatures, H3K4 methylation affected plastic response to pH, but H3K27 methylation had no effect (Kronholm et al. 2016). Epigenetic mechanisms may also facilitate evolutionary adaptation via phenotypic plasticity, as suggested by modeling and experimental analysis (Draghi and Whitlock 2012; Lind et al. 2015). A clear understanding of the mechanisms underlying the lifestyle of *N. crassa* is essential to fully understand how fungi evolve different secretomes to work toward the complexity and diversity of plant cell walls of different species.

3 Plant Cell Wall and Its Deconstruction

3.1 Plant Cell Wall and Its Recalcitrance to Deconstruction

The biomass of plants consists predominately of cell walls. During cell wall biogenesis, massive amounts of atmospheric carbon dioxide are assimilated through photosynthesis and deposited and stored in the cell wall (Rubin 2008). In addition to being the dominant carbon sequestration system on this planet, the cell wall has numerous essential functions in the life cycle of a plant, including providing shape to plant cells needed to form the tissues and organs of a plant and playing important roles in intercellular communication as well as plant-microbe interactions, such as defense responses against potential pathogens (Somerville et al. 2004).

When plant cells are elongating, they are surrounded by a primary wall, which is primarily composed of polysaccharides, including cellulose, hemicelluloses, and pectin. Once cell elongation ceases, a secondary wall is synthesized in some types of cells. Secondary walls consist of cellulose microfibrils and various hemicelluloses as well as the hydrophobic polyphenol lignin (Loque et al. 2015). Cellulose microfibrils are insoluble cable-like structures that are typically composed of multiple hydrogen-bonded β -1,4-linked glucose homopolymer chains (Somerville 2006). Hemicellulose is a heterogeneous, branched polysaccharide primarily made up of a β -1,4-linked polymers including xylan, glucuronoxylan, xyloglucan, glucomannan, and arabinoxylan backbones with heterogeneous side chains (Scheller and Ulvskov 2010; Gírio et al. 2010). Pectin is a complex set of polysaccharide polymers enriched in α -linked galacturonic acid or galacturonic acid and rhamnose monomers (Mohnen 2008; Latarullo et al. 2016). Lignin is a heterogeneous, branched, alkyl aromatic polymer comprising three phenylpropanoid monomers

linked by myriad C-O and C-C bonds that are likely formed through radical coupling reactions during cell wall biogenesis (Ragauskas et al. 2014).

Given the diversity of monosaccharides and the multiple types of glycosidic linkages, polysaccharides in the plant cell wall form the most diverse set of molecules in nature, and their assembly into the cell wall results in a diverse plant cell wall structure and functions (Himmel et al. 2007). Furthermore, plant cell wall compositions and structures are highly dynamic in nature and vary depending on plant species, tissue type, and developmental state of the tissue (Pauly and Keegstra 2010). This complexity and the highly cross-linked nature of the cell wall contribute to biomass recalcitrance to microbial degradation. In particular, the high crystallinity of cellulose fibrils renders the internal surface of cellulose inaccessible to the hydrolyzing enzymes as well as water, and the presence and integration of hemicellulose and lignin further limit access of microbial enzymes to cellulose.

3.2 Cellulolytic Enzymes from *N. crassa*

Microorganisms have evolved a diverse set of enzymatic machinery for plant cell wall deconstruction. Cellulolytic enzymes are primarily composed of various glycoside hydrolases (GHs). They act synergistically to efficiently cleave the glycosidic linkages in plant cell walls (Payne et al. 2015; Wilson 2011). Fungi primarily employ the GHs from families 5, 6, 7, 12, and 45 for cellulose hydrolysis (Table 6.1). Research on *N. crassa* contributed to the discovery of a new class of lytic polysaccharide monooxygenases (LPMOs), which greatly increase synergy in cellulose degradation (Beeson et al. 2011; Phillips et al. 2011a; Li et al. 2012) (Fig. 6.1). LPMOs of Auxiliary Activity Family AA9 (previously GH61) do not employ a hydrolytic mechanism for cellulose deconstruction. They improve the effectiveness of cellulases by oxidatively cleaving glycosidic bonds of polysaccharides in plant cell wall (Beeson et al. 2011; Phillips et al. 2011a; Li et al. 2012).

Many enzymes to degrade insoluble substrates contain a substrate-binding domain, which is linked to a catalytic domain by a linker peptide (Shoseyov et al. 2006; Wilson 2011). In the case of cellulases, the enzyme usually contains a carbohydrate binding module (CBM) joined by a flexible linker peptide to the catalytic domain. Many biomass-degrading fungi commonly employ family 1 CBMs for plant cell wall degradation. The functional roles of CBM in cellulose deconstruction by fungi have been previously reviewed (Payne et al. 2015; Hatakka and Viikari 2014). Fungal cellulolytic enzymes include (1) endoglucanases (endo- β -1,4-glucanases, EC 3.2.1.4) that randomly cleave glycosidic bonds on cellulose, (2) cellobiohydrolases (cellulose β -1,4-cellobiosidases, EC 3.2.1.94) that sequentially release cellobiose from cellulose chain ends, (3) β -glucosidases (β -1,4-glucosidases, EC 3.2.1.21) that catalyze the last step of cellulose hydrolysis as they act on the products (mainly cellobiose) generated by endoglucanase (EG) and cellobiohydrolase (CBH), and (4) newly identified LPMOs that can enhance the conversion of cellulose by oxidative cleavage of glycosidic bonds of polysaccharides in the cell

Table 6.1 Quantification of secreted *N. crassa* proteins during growth on cellulose

Locus	Nomenclature in <i>T. reesei</i>	Function (names)	CAZy family	Weight percent of supernatant	References
NCU07340	CBH1/Cel7A	Exo- β -1,4-glucanase or cellobiohydrolase (CBH-1)	CBM1, GH7	39.5%	Radford (2013) and Samal et al. (2017)
NCU09680	CBH2/Cel6A	Exo- β -1,4-glucanase or cellobiohydrolase (GH6-2 or CBH-2)	CBM1, GH6	13.4%	Tang et al. (2016) and Samal et al. (2017)
NCU00762	EG2/Cel5A	Endo- β -1,4-glucanase (GH5-1)	CBM1, GH5	5.9%	Sun et al. (2011) and Samal et al. (2017)
NCU05057	EG1/Cel7B	Endo- β -1,4-glucanase (GH7-1)	GH7	4.0%	Sun et al. (2011) and Samal et al. (2017)
NCU07190	N/D	Exo-b-1,4-glucanase or cellobiohydrolase (GH6-3)	GH6	3.2%	Sun et al. (2011) and Samal et al. (2017)
NCU04952	Bgl1/Cel3A	Extracellular β -glucosidase (GH3-4 or Bgl-1)	GH3	3.8%	Znameroski et al. (2012), Wu et al. (2013) and Samal et al. (2017)
NCU02240	N/D	Lytic polysaccharide monoxygenase 2 (NcLPMO9A)	CBM1, AA9	3.4%	Phillips et al. (2011a), Li et al. (2012), Vu et al. (2014) and Samal et al. (2017)
NCU07898	N/D	Lytic polysaccharide monoxygenase 3 (NcLPMO9M), oxidized at C1 and C4	AA9	6.6%	Phillips et al. (2011a), Li et al. (2012) and Samal et al. (2017)
NCU08760	N/D	Lytic polysaccharide monoxygenase 1 (NcLPMO9E), oxidized at C1	CBM1, AA9	4.6%	Phillips et al. (2011a), Li et al. (2012) and Samal et al. (2017)
NCU00206	N/D	Cellobiose dehydrogenase (CDH-1)	CBM1, AA3, AA8	2.4%	Phillips et al. (2011a), Zhang et al. (2013) and Samal et al. (2017)
NCU07143	N/D	Extracellular lactonase (LAC-2)	N/A	1.0%	Beeson et al. (2015) and Samal et al. (2017)
NCU09764	N/D	Endo- β -1,4-glucanase and exo- β -1,3/1,6-glucanase	CBM1, GH131	1.6%	Radford (2013) and Samal et al. (2017)
NCU05137	N/D	Non-anchored cell wall protein (NCW-1)	N/A	1.5%	Lin et al. (2017)

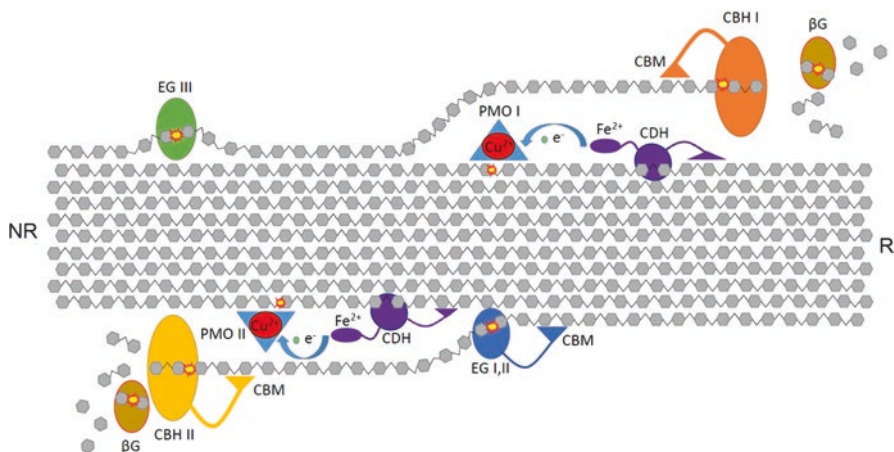


Fig. 6.1 Illustration of cellulose degradation by fungal cellulases and LPMOs. Endoglucanases (EG I, II, III) as well as AA9 LPMOs attack cellulose surface at internal chains, producing action points for exoglucanases CBH I and CBH II to release cellodextrins, particularly cellobiose, from the reducing (R) and nonreducing (NR) ends of cellulose chains, respectively. β -Glycosidase (β G) further releases free glucose from cellobiose (based, among others, on Payne et al. (2015), Kostylev and Wilson (2012), Johansen (2016), and Walton and Davies (2016))

wall (Hemsworth et al. 2015) (Fig. 6.1). A quantitative analysis of *N. crassa* secretome in response to crystalline cellulose showed that cellobiohydrolases (CBH1, GH6-2, and GH6-3) and endoglucanases (GH5-1 and GH7-1) make up 66% of the total secretome (Phillips et al. 2011b) (Table 6.1). After cellulases, the next most abundant group of proteins in the secretome is LPMOs (NcLPMO9A, NcLPMO9E, NcLPMO9M) and cellobiose dehydrogenase (CDH-1). The oxydoreductases comprise 17% of the total secretome.

3.2.1 GH5 Endoglucanase

Based on protein sequence similarity and catalytic domain structure, fungal endoglucanases can be mainly classified into families GH5, GH6, GH7, GH9, GH12, and GH45 (Cantarel et al. 2009). The genome of *N. crassa* encodes five endoglucanases, namely, GH5-1, GH7-1, GH73, and GH45-1. GH5-1 and GH7-1 comprise 5.9% and 4.0% of the total secretome in response to cellulose, respectively (Table 6.1). The observed low percentage of endoglucanases compared to exoglucanases is consistent with that optimum ratios of endocellulases to exocellulases are low for cellulose hydrolysis in many cases (Henrissat et al. 1985; Kostylev and Wilson 2012).

GH5 enzymes exhibit a large variation in specificity and hydrolytic activity, ranging from 1,4-glucanase, 1,6-galactanase, 1,3-mannanase and 1,4-xylanase to xyloglucanase. GH5-1 from *N. crassa* has been purified and characterized for its

endoglucanase activities (Sun et al. 2011). It is a homolog of *T. reesei* endoglucanase Cel5A (formerly EG2) and contains a CBM1 domain in addition to a GH5 catalytic domain. A comparative analysis of 103 representative fungi from *Ascomycota*, *Basidiomycota*, *Chytridiomycota*, and *Zygomycota* demonstrated that GH5 genes are present in all their genomes, while genes from families GH6, GH7, GH9, GH12, GH45, and AA9 are only associated with certain types of fungi (Zhao et al. 2013), suggesting the importance and uniqueness of families GH5 enzymes in plant cell wall deconstruction and/or cellulose hydrolysis. Indeed, TrCel5A is responsible for a significant portion of the endoglucanase action of *T. reesei* (Suominen et al. 1993), and GH5 endoglucanase (HiCel5A) from the saprophytic fungus *Humicola insolens* was shown to be the most active endoglucanase of the families 5, 7, 12, and 45 examined alongside *T. reesei* Cel7B (Karlsson et al. 2002). Therefore, it is not surprising that family 5 endoglucanase is often incorporated as a key component in many industrial biomass conversion cocktails.

3.2.2 GH7 Endoglucanase

N. crassa genome contains five GH7 genes, encoding two endoglucanase I and three cellobiohydrolase I, respectively (Table 6.2). GH7-1 is another endoglucanase identified in the secretome of *N. crassa* in response to cellulose. By contrast to GH5-1, GH7-1 does not have a CBM1 domain. This enzyme is a homolog of the endoglucanase *T. reesei* Cel7B. TrCel7B is a processive endocellulase that attacks the cellulose, chain at random locations (Claeysens and Henrissat 1992). An immuno-EM study suggests that TrCel7B preferentially binds to the amorphous regions of cellulose, while the exoglucanase TrCel7A (CBH1) preferentially binds to the crystalline region (Nieves et al. 1991). Atomic forced microscopy analysis further revealed that hydrolysis dominated the early stage of TrCel7B action, while swelling dominated the later stage (Wang et al. 2012). It is proposed that the combined action of initial hydrolysis followed by swelling softened and swelled the fibers and exposed individual microfibrils or bundles of microfibrils, which, in turn, facilitate the attack by exoglucanases (Wang et al. 2012; Baltierra et al. 2013). This is consistent with the observation that addition of TrCel7A (CBH1) to cellulose fibers pretreated with TrCel7B led to faster rates of cellulose hydrolysis than addition of the enzymes in the opposite order (Baltierra et al. 2013).

3.2.3 GH45 Endoglucanase

Of all GH families of fungal cellulases, GH45 are by far the least well characterized (Payne et al. 2015). In general, GH45 cellulases can degrade cellulose as well as other cell wall polysaccharides. They are relatively small by comparison with other GH families, which may represent an evolutionary advantage, allowing them to penetrate into smaller pores and cavities of plant cell wall and thus gain better access to the substrate (Payne et al. 2015). Another unique feature of GH45 enzymes

Table 6.2 Enzymes and proteins involved in cellulose depolymerization in *N. crassa* and other saprotrophic or plant-pathogenic fungi^a

Lifestyle	Fungi	Host	Cellulases ^b							β-glucosidases ^b		Accessory enzymes ^b		Total cellulolytic activity ^c	Specific cellulolytic activity ^d
			GH5	GH6	GH7	GH12	GH45	GH1	GH3			AA9 ^e	CBM1		
Necrotrophic	<i>Sclerotinia sclerotiorum</i>	D	14	2	3	4	2	3	13			9	19	H	N/D
Necrotrophic	<i>Fusarium oxysporum</i>	M/D/G	22	1	3	4	1	6	32			17	14	H	39
Saprophytic (group 2)	<i>Neurospora crassa</i>	NA	6	3	5	1	1	1	9			14	19	H	100
Facultative pathogenic (group 1)	<i>Aspergillus fumigatus</i>	NA	13	1	4	4	1	5	18			8	17	H	4
Hemibiotrophic	<i>Fusarium graminearum</i>	M	14	1	2	4	1	3	22			14	12	H	104
Facultative pathogenic (group 1)	<i>Aspergillus niger</i>	NA	10	2	2	4	0	3	17			7	8	M	4
	<i>Schizophyllum commune</i>	NA	17	1	2	1	1	3	12			25	5	M	N/D
Necrotrophic	<i>Verticillium dahliae</i>	D	13	4	6	6	2	2	16			29	29	M	N/D
Biotrophic	<i>Ustilago maydis</i>	M	13	0	0	0	3	0	3			0	0	L	0
Necrotrophic	<i>Verticillium albo-atrum</i>	D	13	4	6	5	1	4	21			22	22	L	N/D
Saprophytic (group 2)	<i>Trichoderma reesei</i>	NA	8	1	2	2	1	2	13			6	14	N/D	16

^aThe data from genomic and cellulolytic analysis collected from Reference Zhao et al. (2013) and King et al. (2011), respectively

^bDistribution of the carbohydrate activity enzymes (CAZymes) and cellulose-binding domain (CBM) in *N. crassa* and other saprotrophic or plant-pathogenic fungi

^cRanking of the fungal species for hydrolysis of crystalline cellulose (filter paper) based on Reference King et al. (2011)

^dAbbreviations: CBM cellulose-binding domain, D dicots, G gymnosperms, M monocots, NA not applicable, H ranked as high activities of total cellulases (67–100%); M ranked as medium activities of total cellulases (33–66%), L ranked as low activities of total cellulases (0–32%)

^eA lytic polysaccharide monooxygenase. CAZy family AA9 used to be assigned as GH61

is that they are structurally and evolutionarily related to the domain-1 (D1) of expansin. Kadowaki et al. characterized the structure of *N. crassa* GH45 endoglucanase, NcCel45A (Kadowaki et al. 2015). Unlike other known cellulases, NcCel45A adopts a unique monkey-wrench molecular shape structure in solution. This structure may confer unique properties, such as the observed stability at elevated temperatures up to 70°C and its resistance to a wide range of organic solvents, which may be applied for industrial processes, such as cotton fiber processing and detergent formulations (Kadowaki et al. 2015).

3.2.4 GH7 Exoglucanase

Fungal exoglucanases can be mainly found in families GH6 and GH7 (Cantarel et al. 2009). The genome of *N. crassa* encodes five secreted exoglucanases, namely, GH6-1, GH6-2, GH6-3, CBH1, GH7-2, and GH7-4. CBH1, GH6-2, and GH6-3 comprise 39.5%, 13.4%, and 3.2% of the total secretome in response to cellulose, respectively (Table 6.1). *N. crassa* CBH1 is a homolog of *T. reesei* CBH1 (TrCel7A). Studies with Cel7A provide a convincing argument for a novel synergistic mechanism between endocellulase and exocellulase (Väljamäe et al. 1999; Väljamäe et al. 1998; Igarashi et al. 2009), as reviewed by Kostylev and Wilson (Kostylev and Wilson 2012). It is thought that an optimum mix of cellulases attacks both reducing and nonreducing ends of cellulose chains, as well as regions within the chains, and that hydrolysis of cellulose by endoglucanases in regions within cellulose chains creates new chain ends that are accessible to exoglucanases (Kostylev and Wilson 2012).

While *N. crassa* CBH1 and GH6-2 exoglucanases contain a CBM1 domain, GH6-3 does not. Based on the studies with intact TrCel7A, isolated CBM, and isolated catalytic domain, it is proposed that the catalytic domain is biased toward amorphous regions or chain ends, whereas the CBM is biased toward the crystalline regions, and, therefore, the attachment of the CBM to the catalytic domain would ensure the catalytic domain to have an elevated concentration on the crystalline regions (Ståhlberg et al. 1991). Thus, exoglucanases with and without a CBM may allow them to act on different regions of cellulose fibers. Single-molecule imaging analysis showed that isolated CBM has 12 times higher affinities to the hydrophobic surface than to the hydrophilic surface of cellulose, suggesting that the CBM is necessary to determine the surface specificity of binding (Nakamura et al. 2016). The advantage of CBMs was found to be diminished by reducing the amount of water in the hydrolytic system (Varnai et al. 2013). While CBMs are important in the catalytic performance at low substrate concentration (1% w/w), enzymes lacking CBM outperformed the enzymes carrying the CBM at high substrate concentrations (Varnai et al. 2013; Le Costaouec et al. 2013).

3.2.5 GH6 Exoglucanases

GH6 enzymes are primarily endoglucanases and exocellulases, in which many are thought to display both activities to some extent (Payne et al. 2015). Both GH6 endoglucanases and exoglucanases are capable of endo-initiated attack with specificity toward the nonreducing end of crystalline cellulose. *N. crassa* genome contains three GH6 family genes, and all of them encode cellobiohydrolase II (CBH2). GH6-2 contains a CBM1 domain while GH6-3 does not (Table 6.1). The GH6 family is currently the only known family that has cellulases to act from the nonreducing end of cellulose chains. The most effective cellulase cocktails require exoglucanases that attack both reducing and nonreducing ends of cellulose chains, as well as endoglucanases that attach regions within the chains. The unique role played by GH6 cellulases allows rapid and synergistic degradation of crystalline cellulose. As such, GH6 cellulases are primary components in plant cell wall degradation cocktails.

3.2.6 AA9 LPMO

In 2008, Moser et al. first reported that bacterial LPMOs (family AA10, formerly CBM33) have a cellulase boosting effect (Moser et al. 2008). The family AA9 LPMOs is evolved to enhance cellulase activities in fungi, instead (Beeson et al. 2011; Phillips et al. 2011a; Li et al. 2012). Similar to GH5 family, AA9 family has multiple members in many fungi, and some organisms have over 20 AA9 and/or GH5 genes in their genomes (Zhao et al. 2013), a feature that differentiates them from the other main families of fungal cellulases, particularly GH6, GH7, GH12, and GH45, which have much fewer members per genome. LPMOs and GH5 enzymes are likely evolved to attack polysaccharide bonds that are not accessible to the enzymes of other GH families.

N. crassa genome contains 16 AA9 family genes (Cantarel et al. 2009), and nine of them have been characterized (Cantarel et al. 2009; Hemsworth et al. 2015). NcLPMO9A, NcLPMO9D, NcLPMO9E, NcLPMO9F, NcLPMO9J, and NcLPMO9M, and GH61-2 and GH61-7 can cleave glycosidic bonds of cellulose (Beeson et al. 2011; Li et al. 2012; Phillips et al. 2011a; Zhang et al. 2013; Beeson et al. 2015), while NcLPMO9C demonstrated a relatively broad substrate specificity (Isaksen et al. 2014; Borisova et al. 2015; Agger et al. 2014). The crystal structure of the catalytic domain of NcLPMO9C revealed an extended, highly polar substrate-binding surface well suited to interact with a variety of sugar substrates (Borisova et al. 2015). Indeed, NcLPMO9C is able to degrade various hemicelluloses, in particular xyloglucan. This unique feature may contribute to synergistic cellulose degradation as the attachment of xyloglucan to cellulose hampers depolymerization of cellulose chains by cellulases (Agger et al. 2014).

The presence of LPMO greatly enhances the efficiency of commercial cellulase cocktails in cellulose degradation (Harris et al. 2010; Quinlan et al. 2011; Vaaje-

Kolstad et al. 2010). Neutron crystallography and small-angle neutron scattering analysis of NcLPMO9D and NcCDHIIA suggest that LPMOs can receive electrons from CDHs to activate molecular dioxygen for the oxidation of cellulose and thus cleave cellulose chains and disrupt local crystallinity (Bodenheimer et al. 2017). Atomic forced microscopy analysis of NcLPMO9F demonstrates that NcLPMO9F can degrade cellulose microfibrils or microfibril bundles exposed on the surface into shorter and thinner insoluble fragments (Eibinger et al. 2014). This enables cellulases to attack otherwise highly resistant crystalline substrate areas and promotes an overall faster and more complete cellulose surface degradation.

Recent studies suggest that AA9 LPMOs from *N. crassa* interact with CDH and receive electrons from the action of CDHs for oxidative cleavage of glucan chains (Beeson et al. 2011; Vu et al. 2014; Lin et al. 2017). CDH-I only makes up 2.4% of the *N. crassa* secretome (Table 6.1), but deletion of this gene reduced the total cellulase activity secreted by the fungus by nearly half (Zhang et al. 2013). Structural analysis of NcCDHIIA shows that it contains a haem-binding cytochrome (CYT) connected to a flavin-dependent dehydrogenase (DH) (Zamocky et al. 2006). Electrons are generated from cellobiose oxidation catalyzed by DH and shuttled via CYT to LPMO (Tan et al. 2015). CYT reduces LPMO to initiate oxygen activation at the copper center and subsequent cellulose depolymerization (Tan et al. 2015).

3.2.7 Expansin/Swollenin

Expansins are small proteins that loosen plant cell walls and cellulosic materials without lytic activity (Cosgrove 2017). They were first discovered in plants and later in numerous bacteria and fungi. A recent survey of expansin distribution found its homologs in 3% of the bacterial and 5% of the fungal (Nikolaidis et al. 2014). Blast analysis did retrieve a sequence from the *N. crassa* that showed 59% similarity to the expansin-like protein LOOS1 of the Basidiomycete *Bjerkandera adusta* (Quiroz-Castañeda et al. 2011; Aranzazu et al. 2015). Expansin or expansin-like proteins can cooperate with cellulases to improve cellulose hydrolysis (Cosgrove 2017; Liu et al. 2015).

3.3 Glycosylation of Cellulolytic Enzymes from *N. crassa*

Glycosylation of cellulases is important for their activities. Three N-glycosylation sites have been identified on the catalytic domain of TrCel7A (Klarskov et al. 1997; Maras et al. 1997). Site-directed mutagenesis of the individual sites showed that removal of glycosylation exhibited improved cellulose depolymerization. In particular, the N384A mutation increased the activity of TrCel7A by 70% (Adney et al. 2009). On the other hand, introducing a new glycosylation site resulted in an

increase in activity by A196S mutation on PfCel7A (Adney et al. 2009). In addition to catalytic domain, glycosylation sites were also found on CBMs and linkers of cellulases (Payne et al. 2015). A solid-state glycopeptide synthesis approach demonstrated that glycosylation affects CBM binding affinities (Taylor et al. 2012). O-glycosylation was found to be approximately uniformly distributed across the length of linkers (Sammond et al. 2012), which may help protect the linkers against proteolysis (Payne et al. 2015). Single-molecule imaging analysis suggests that the glycosylated linker region largely contributes to initial binding of cellulases on crystalline cellulose (Nakamura et al. 2016).

Recent analysis of *N. crassa* CBH1, a TrCel7A homolog, revealed that it is O-glycosylated with six types of linear and three types of branched O-glycans, and the glycans were found to contain approximately equal amounts of mannose and galactose (Tang et al. 2016). Genetic resources available for *N. crassa* will enable the future evaluation of the role of glycosylation on cellulase structure and functions, e.g., hydrolytic activity and thermal stability. Modifying the glycosylation of cellulases may increase the hydrophilicity of some regions, leading to reduced non-specific binding of cellulases to lignin and thus cellulase loading for plant cell wall deconstruction (Tang et al. 2016).

4 Regulation of the Cellulolytic Response

Regulation of the cellulolytic response by filamentous fungi has been reviewed recently by Benocci et al. (2017) and Huberman et al. (2016). A number of pathways have been identified to regulate cellulolytic response, including direct transcriptional regulation, upstream regulation by nutrient sensing pathways, and regulatory feedback from the secretory pathway (Huberman et al. 2016). In *N. crassa*, cellulase gene transcription is regulated directly by CLR-1 and CLR-2. CLR-1 activates transcription of *clr-2* as well as some cellulolytic genes (Coradetti et al. 2012). Transcription of cellulases is also subjected to regulation by upstream nutrient sensing pathways, particularly carbon catabolite repression (CCR). It is well known that CCR involves the transcription factor CRE-1, which is regulated in part by VIB-1 in response to preferred carbon sources (Xiong et al. 2014b; Huberman et al. 2016). Activated CRE-1 represses transcription of cellulolytic genes as well as their direct regulators (Sun and Glass 2011). Once plant cell wall-degrading enzymes are sent to the endoplasmic reticulum (ER), it increases demanding for protein folding, disulfide bond formation, glycosylation, and sorting (Qin et al. 2017; Reilly et al. 2015). The resulting secretion stress or ER stress appears to modulate transcription of cellulolytic genes by activating IRE1, which, in turn, activates the transcription factor HAC1 by cleaving a noncanonical intron from *hac1* (Sidrauski and Walter 1997). In *N. crassa*, cellulose is known to upregulate the expression of *ire-1*, *hac-1*, and other genes associated with the pathways such as the unfolded protein response (UPR) (Benz et al. 2014). However, mechanisms

underlying transcriptional regulation of the cellulolytic response to secretion stress are still poorly understood.

4.1 *Regulatory Feedback from the Secretory Pathway*

By screening strains carrying deletions in genes predicted to function in the protein secretory pathway, mutants with enhanced cellulase production were identified. Two such mutants, *dsc-2* and *tul-1*, are homologs of the components of the Golgi apparatus E3 ligase complex in *Schizosaccharomyces pombe* and *Aspergillus fumigatus* (Reilly et al. 2015). In *S. pombe* and *A. fumigatus*, a Golgi apparatus E3 ligase complex is employed for proteolytic activation of the sterol regulatory element-binding protein (SREBP) (Stewart et al. 2011; Willger et al. 2012; Bien et al. 2009), suggesting a functional relationship between the SREBP pathway and cellulase hyperproduction. Indeed, a *N. crassa* strain carrying a deletion of the SREBP homolog *sah-2* showed cellulase hyperproduction as well (Reilly et al. 2015).

The role of the SREBP pathway in the secretion of cellulolytic enzymes was further confirmed by strains carrying mutations in the homologs of SREBP cleavage-activating protein-encoding gene *scp-1* and rhomboid protease-encoding gene *rbd-2*, which also showed a cellulase hyperproduction phenotype (Qin et al. 2017). Global transcriptional profiling further revealed that the function of the SREBP pathway was highly activated in response to cellulolytic conditions, leading to reduced expression of LPMOs as well as a set of genes predicted to be involved in the ER stress response (Qin et al. 2017). The mutations in the SREBP pathway were also found to suppress the inability of a *hac-1* mutant to efficiently produce cellulases, suggesting a linkage between SREBP pathway functions and the UPR (Qin et al. 2017). Further analysis of the regulatory network of the UPR and SREBP pathways will help understand cellulase trafficking and plant cell wall deconstruction and improve the production of plant cell wall-degrading enzymes by filamentous fungi.

4.2 *Direct Transcriptional Regulation*

β -glucosidases have been shown to be involved in the induction of cellulase expression by cellobiose. Deletion of three major β -glucosidases in *N. crassa* led to rapid and efficient induction of cellulase on cellobiose (Znameroski et al. 2012). Two cellobiose transporters, CDT-1 and CDT-2, were found to be involved in cellobiose uptake and cellulase inducer sensing (Znameroski et al. 2014). Deletion of *cdt-2* in *N. crassa* led to reduced expression of not only major cellulase but also hemicellulase genes and caused significant growth defects on both cellulose and xylan (Cai et al. 2014). Expression of CDT-1 or CDT-2 along with a β -glucosidase in *Saccharomyces cerevisiae* promotes its efficient growth on cellobioses for

improved biofuel production (Galazka et al. 2010). In addition to CDT-1 and CDT-2, the transporter CBT-1/CLP-1 is involved in transporting cellobionic acid (Xiong et al. 2014a), a product from the oxidative cleavage of cellulose by LPMOs. CBT-1/CLP-1 also plays an important role in cellulase induction, as reflected by that deletion of *cbt-1/clp-1* in the major β -glucosidase gene knockout background led to upregulation of both CDT-1 and cellulases upon induction by cellobiose (Cai et al. 2015). For detailed review on *N. crassa* sugar transporters, the readers may refer to Seibert et al. (2016).

In addition to transporting sugars, sugar transport systems may have additional functions in signal transduction. For instance, CDT-1 and CDT-2 were suggested to act as both a cellobiose transporter and a transceptor (Znameroski et al. 2014). Signal transduction via transceptors has been reported with various nutrient transport systems, including the glucose transporter RCO-3 of *N. crassa* (Madi et al. 1997) and a lactose permease that is essential for cellulase induction in *T. reesei* (Ivanova et al. 2013). Recent studies provide more evidences for dual functions of sugar transporters. For instance, HGT-1/-2 in *N. crassa* is a high-affinity glucose transporter (Wang et al. 2017). It is found that replacing a conserved arginine with lysine in HGT-1/-2 resulted in dysfunction of glucose transport but no change in CCR signal transduction, suggesting that similar to RCO-3, HGT-1/ HGT-2 acts as both the sugar transporter and transceptor (Wang et al. 2017).

Cell wall is the first contact site between fungi and their environment. Cell wall may also be involved in the regulation of cellulase inducer sensing. A recent study with a non-anchored cell wall protein, NCW-1, demonstrated that deletion of *nsw-1* along with three major β -glucosidase genes enhanced expression of CDT-1 and CDT-2 as well as plant cell wall-degrading enzymes upon induction by cellobiose (Lin et al. 2017). However, the underlying mechanism is not clear. Clearly, future studies are required to understand how fungal sugar transporters and cell wall are involved in the cellulase induction pathway.

4.3 Upstream Regulation by Nutrient Sensing Pathways

In addition to direct transcriptional induction, many competing signals are also involved in regulating the expression of cellulolytic enzymes. Carbon catabolite repression (CCR) is well known to downregulate the expression of cellulolytic enzymes when a more preferred carbon source is available. In *N. crassa*, CCR is thought to regulate the expression of both major cellulase transcription factors, such as CLR-2, and cellulolytic enzymes (Coradetti et al. 2012; Sun and Glass 2011; Xiong et al. 2014a). CCR itself is regulated by the zinc binuclear cluster transcription regulator CRE-1 in *N. crassa*. Deletion of *cre-1* causes an increase in cellulase production in response to crystalline cellulose conditions (Sun and Glass 2011). Besides cellulolytic enzymes, CRE-1 was also found to regulate the expression of some hemicellulases.

External signals, such as nutrient availability, can be sensed by G-protein coupled receptors (GPCR) in fungi (Xue et al. 2008). In *N. crassa*, GPR-4 regulates the response to carbon sources through a cAMP signaling pathway. Deletion of GPR-4 resulted in reduced mass accumulation when the mutants were cultured on different carbon sources, and exogenous cAMP can partially remediate the growth defects (Li and Borkovich 2006). The cAMP signal induces a protein phosphorylation cascade which is mediated, in part, by the activation of cAMP-dependent protein kinase A (PKA) (D'Souza and Heitman 2001). In *A. nidulans*, a PKA catalytic subunit, PkaA, affects CCR and cellulase production. Deletion of *pkaA* resulted in upregulation of the expression of cellulolytic genes and their upstream regulators *xlnR* and *clrB* earlier in the cellulose response, as well as cellulase production under conditions where CCR is active in wild-type cells (de Assis et al. 2015). In *N. crassa*, a genetic analysis of CRE-1 and the *mcb* mutant, which has a defect in the regulatory subunit of PKA, demonstrates that there are regulatory interactions between PKA and CRE-1 that affect cell polarity of *N. crassa* (Ziv et al. 2008). As the control of morphology is critical for the biotechnology applications of filamentous fungi, particularly submerged bioprocesses (Krull et al. 2013), it is important in future studies to further understand the regulatory network between nutrient sensing, morphogenesis, and cellulase production.

The transcription factor VIB-1 is another actor in the glucose response and appears to be an upstream regulator of genes involved in CCR, including CRE-1 (Xiong et al. 2014b; Huberman et al. 2016). VIB-1 is involved in the regulation of extracellular proteases in response to carbon and nitrogen starvation and may be regulated by PKA (de Assis et al. 2015; Huberman et al. 2016). In *N. crassa*, a *vib-1* deletion mutant showed severe growth defects on cellulose as well as a lack of cellulolytic enzyme activity, reflecting its critical role for the induction of cellulolytic enzymes (Xiong et al. 2014b). In addition, VIB-1 regulates the expression of *col-26* (Xiong et al. 2014b), a homolog of *T. reesei* *bglR* that positively regulates cellulolytic enzyme expression (Nitta et al. 2012). Additional elucidation is required for the mechanism of action of *col-26/bglR* and to identify other actors involved in CCR.

5 Systems Analysis of Plant Cell Wall Deconstruction

To be able to rationally engineer filamentous fungi for biotechnological applications, particularly conversion of plant cell wall polysaccharides to fermentable sugars for the production of biofuels and bio-products, it is critical to approach at systems levels the mechanisms underlying plant cell wall deconstruction.

5.1 Transcriptomics Analysis

To examine the cellulolytic machinery of *N. crassa*, early studies by Tian et al. identified 187 or 231 upregulated genes in response to crystalline cellulose or *Miscanthus* cell wall, respectively (Tian et al. 2009). The difference in gene expression is thought to be due to the additional activation of hemicellulases and pectinases on *Miscanthus*. Sun et al. (2012) and Benz et al. (2014) further examined transcriptional expression of genes in response to hemicelluloses, including beechwood xylan and its major hydrolytic degradation product xylose. The transcriptome analyses revealed that 353 genes are significantly induced by beechwood xylan, including those involved in xylose metabolism. However, cellulolytic genes were not induced by beechwood xylan. Xylose appears not to be the hemicellulase inducer as observed with *A. niger* (Gielkens et al. 1999) and *T. reesei* (Mach-Aigner et al. 2010). In *N. crassa*, only 30 genes showed increased expression under 2% xylose conditions (Sun et al. 2012). Li et al. further compared the transcriptomes of *N. crassa* grown on L-arabinose and D-xylose and found that gene expression profiles on L-arabinose were dramatically different from those on D-xylose (Li et al. 2014). The uptake of L-arabinose is prevented until D-xylose and/or D-glucose are completely depleted (Li et al. 2014). This study also identified three novel sugar transporters as well as a transcription factor associated with the regulation of hemicellulases (Li et al. 2014). Benz et al. further analyzed the transcriptome of *N. crassa* in response to pectin and revealed that all predicted pectinase genes, along with several genes encoding enzymes with the potential to degrade pectin side chains, were strongly upregulated (Benz et al. 2014).

5.2 Proteomics Analysis

Phillips et al. quantified 13 secreted proteins of *N. crassa* when grown on crystalline cellulose (Table 6.1) (Phillips et al. 2011b), the first quantitative proteomics approach for *N. crassa* secretome. Benz et al. further compared *N. crassa* secretomes on pectin, xylan, and crystalline cellulose (Benz et al. 2014). A total of 80 proteins were identified in the secretome on pectin, and 55 of them were uniquely identified on pectin (Benz et al. 2014). The results suggest that the response of *N. crassa* to pectin is largely independent of its response to cellulose with some overlap to hemicelluloses (Benz et al. 2014).

Xiong et al. obtained global proteomic and phosphoproteomic profiles of the *N. crassa* grown on different carbon sources, including sucrose, no carbon, and cellulose, by performing isobaric tags for relative and absolute quantification analyses (Xiong et al. 2014a). This analysis showed extensive posttranscriptional regulation in response to exposure to cellulose and several hundred amino acid residues with differential phosphorylation levels on crystalline cellulose (Avicel) or carbon-free

medium vs sucrose medium, including phosphorylation sites in CLR1 and CBT1 (Xiong et al. 2014a).

In summary, transcriptomics and proteomics analyses suggest that *N. crassa* is capable of producing all major plant cell wall-degrading enzymes, and their production appears to be regulated at both transcriptional and posttranscriptional levels. While the cellulolytic and pectinolytic enzymes are more specifically induced, hemicellulases appear to be induced more constitutively, probably due to the fact that hemicelluloses are distributed more evenly throughout the plant cell wall layers (Seibert et al. 2016). Furthermore, many plant cell wall-degrading enzymes appear to be differentially expressed over time when *N. crassa* is grown on plant cell wall substrates, reflecting the complexity of plant cell wall and a possible cascade-like action of different enzymes for plant cell wall deconstruction (Benz et al. 2014). Future studies are required to further understand how the cascade-like action of various enzymes is regulated at both transcriptional and posttranscriptional levels.

5.3 The Plant Cell Wall Degradation Network (PCWDN)

Recently, a detailed plant cell wall degradation network (PCWDN) of *N. crassa* has been built by Samal et al. (2017). This analysis involves the integration of five heterogeneous data types: functional genomics, transcriptomics, proteomics, genetics, and biochemical information, along with extensive manual curation based on more than 130 research articles. The network reconstruction analysis revealed that *N. crassa* PCWDN consists of 202 biochemical reactions and 168 associated genes (Samal et al. 2017). The 202 reactions are further subdivided into 101 extracellular reactions, 35 transport reactions, and 66 intracellular reactions. The regulons of cellulose, xylan, xyloglucan, mannan, mixed-linkage glucan, pectin, and starch are determined to contain 153, 180, 138, 112, 188, 323, and 363 genes, respectively (Samal et al. 2017). Chromatin Immunoprecipitation-sequencing (ChIP-seq) analysis is further used to map the genome-wide binding sites of CLR-1, CLR-2, and XLR-1 under sucrose, cellulose, xylan, and no carbon conditions (Craig et al. 2015). It is found that CLR-1, CLR-2, and XLR-1 bind promoter regions of 27, 37, and 20 PCWDN genes, respectively (Samal et al. 2017) (Fig. 6.2). Of the 37 PCWDN genes regulated by CLR-2, only 23 are involved in cellulose utilization, while 12 other PCWDN genes are involved in xylan or mannan utilization (Fig. 6.2). Further analysis confirmed that CLR-2 regulates not only cellulose utilization but also mannan degradation (Samal et al. 2017).

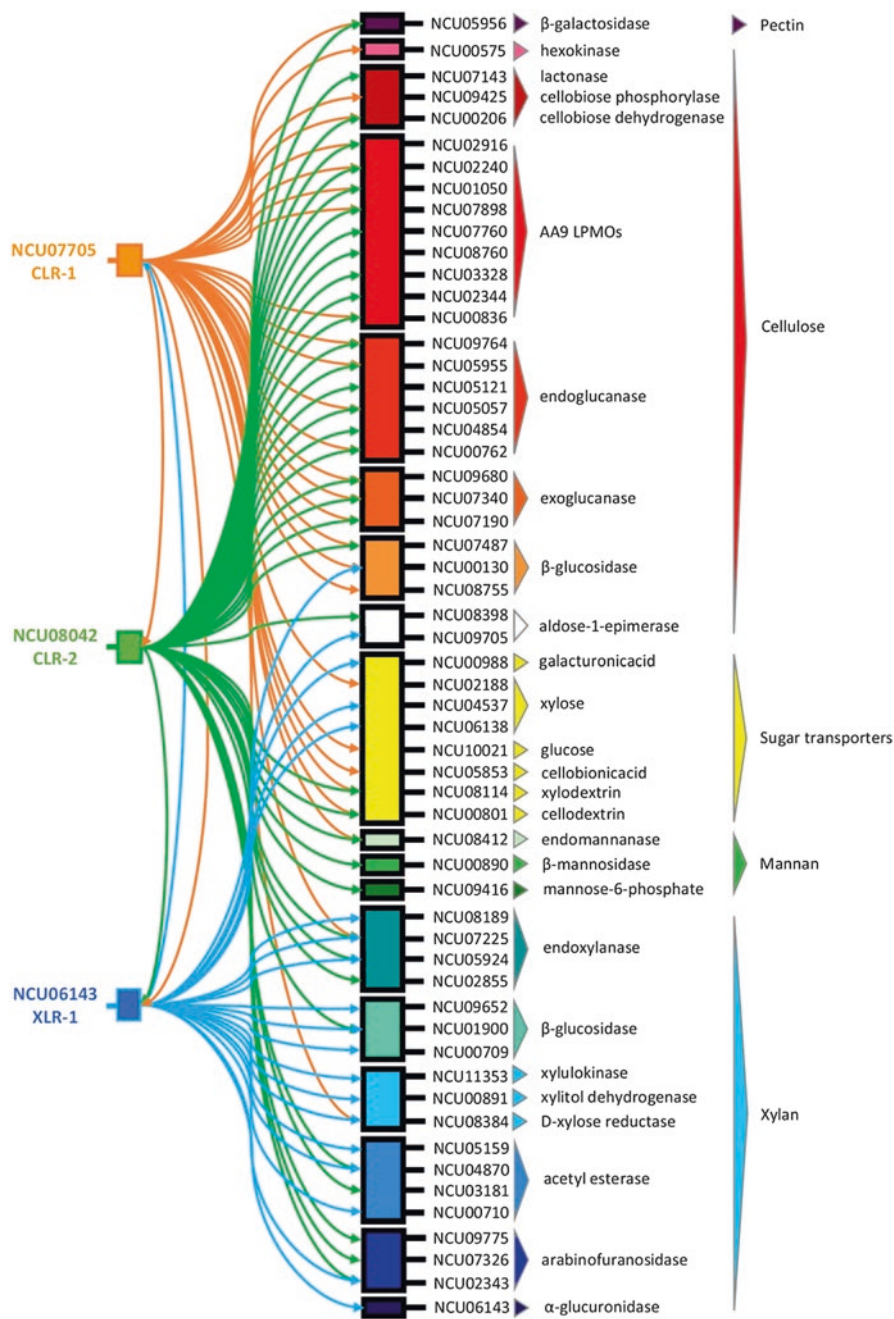


Fig. 6.2 The plant cell wall degradation genes regulated directly by CLR-1, CLR-2, and XLR-1 (based on Samal et al. 2017). CLR-1 and XLR-1 mainly regulate the genes involved in cellulose and xylan degradation, respectively, as well as product transport, while CLR-2 is involved in the regulation of both cellulolytic and hemicellulolytic genes

6 Future Perspectives

Building a systems-level understanding of plant cell wall deconstruction by filamentous fungi requires learning about and integrating various knowledge sources, including how fungi evolve different secretomes to work toward the complexity and diversity of plant cell walls of different species, how different components of the secretomes act synergistically on plant cell wall deconstruction, and how the expression, secretion, and activities of the plant cell wall-degrading enzymes are regulated. Funded by the Energy Biosciences Institute (EBI), the transcriptomics, proteomics, and network reconstruction analysis of plant cell wall degradation by *N. crassa*, along with high-throughput screening of mutant libraries, provides a further step toward a systems level understanding of the regulatory design principles underlying plant cell wall deconstruction by filamentous fungi (Znameroski et al. 2012; Tian et al. 2009; Craig et al. 2015; Coradetti et al. 2012; Benz et al. 2014; Samal et al. 2017; Qin et al. 2017). Such systems-level approaches also include the projects selected for the 2013 Community Sequencing Program (CSP) portfolio of the U.S. Department of Energy Joint Genome Institute (DOE JGI). For instance, the Fungal Nutritional ENCODE project, led by N. Louise Glass of University of California, Berkeley, aims to comprehensively map out the nutritional and metabolic regulatory networks of *N. crassa*. The Mycorrhizal Genomics Initiative, led by Francis Martin of the French National Institute for Agricultural Research (INRA), aims to study the mechanisms of symbiotic interactions between fungi and plants. In China, Yinbo Qu of Shandong University led the “Biodegradation and Bioconversion of Lignocellulosic Resources” project to investigate the mechanisms at both molecular and systems levels of plant cell wall deconstruction by bacteria and fungi, which has been funded by the National Key Basic Research Development Program (Li et al. 2017; Liu et al. 2013). These global efforts not only provide new insights into plant cell wall deconstruction by filamentous fungi but also offer a platform to identify new rational engineering strategies to improve the expression and secretion of plant cell wall-degrading enzymes and, furthermore, the cost-effective production of cellulosic biofuels and bio-products.

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Chapter 7

Strain Improvement for Industrial Production of Lignocellulolytic Enzyme by *Talaromyces cellulolyticus*



Tatsuya Fujii, Hiroyuki Inoue, Shinichi Yano, and Shigeki Sawayama

Abstract *Talaromyces cellulolyticus* (formerly known as *Acremonium cellulolyticus*) is a commercial fungal source used for industrial enzyme production for silage preparation. In this chapter, the development of *T. cellulolyticus* strains to produce lignocellulolytic enzymes suitable for the hydrolysis of target biomass is reviewed. High-yield production and composition improvements of lignocellulolytic enzymes in *T. cellulolyticus* have been succeeded by mutagenesis, genetic engineering, and enzyme preparation up to the present date. Recent developments of *T. cellulolyticus* genetic tools including whole genome sequencing, homologous recombination, marker recycling, RNA interference, genome editing and transcriptional regulation, a concept of core and accessory enzymes, and their utilization for strain improvements will be discussed.

Keywords Lignocellulolytic enzyme · Industrial production · *Talaromyces cellulolyticus*

1 Introduction

Productions of ethanol and other chemicals from lignocellulosic biomass have been attracted much attention to reduce fossil fuel utilization and mitigate global warming, without affecting food supply. The major obstacle for commercialization is, however, its high production costs, especially enzyme cost for saccharification of cellulose, the major component of lignocellulosic biomass. Although appropriate

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pretreatment can lower the amount of necessary enzyme for saccharification, relatively considerable amounts of enzyme are still required, and the commercial cellulase enzymes are more expensive than other enzymes, for example, amylase for the saccharification of starch. One promising solution for the problem is on-site enzyme production; manufacturers produce enzyme by themselves on the site of chemical production instead of buying commercial enzymes. The efficacy of on-site enzyme production for cost reduction has been estimated (Barta et al. 2015; Liu et al. 2016). But for that purpose, the manufacturers need to have a good enzyme-producing microorganism.

The cellulase-producing fungal strain Y-94 was isolated from soil in northeastern district in Japan in 1982 (Yamanobe et al. 1987). Continuously, several research papers on properties of enzymes produced by Y-94 were published (Mitsuishi et al. 1987; Yamanobe et al. 1988, Yamanobe and Mitsuishi 1989; 1990). Although the classification of this strain was obscure because of its unique morphological properties, it was described as *Acremonium cellulolyticus* nomen nudum in Japanese and US patents (Yamanobe et al. 1984, 1985). Cellulase produced with this fungal strain is commercialized by Meiji Seika Pharma Co. Ltd. as *Acremonium* cellulase and has a good reputation, especially for the improvement of silage quality (Tomoda et al. 1996).

Cellulase is not a single enzyme but a mixture of the following three component enzymes: endoglucanase which forms a nick in the amorphous region of cellulose molecules; cellobiohydrolase which cleaves cellobiose monomer units from the both ends of the cellulose polymer molecules; and β -glucosidase which hydrolyzes cellobiose or cellooligosaccharides to produce glucose. Cellulase produced from this fungal strain is characterized by its high β -glucosidase activity compared with conventional cellulase produced from *Hypocrea jecorina* (*Trichoderma reesei*) (Yamanobe et al. 1987; Fujii et al. 2009). This property is especially important for ethanol production from lignocellulosic biomass, because the conventional yeast species used for ethanol production, *Saccharomyces cerevisiae*, cannot utilize cellobiose, so that the cellulose must be completely hydrolyzed into glucose. Therefore, cellulase from this fungus is considered to be very suitable for ethanol production from lignocellulosic biomass. From this property, much attention has been focused on this fungus as a cellulase producer, especially in 2000s when the expectation for cellulosic ethanol commercial production has increased.

On the other hand, recent phylogenetic analyses using ITS1-5.8S-ITS2 and RNA polymerase II subunit gene sequences have revealed that this fungus is closely related to genus *Talaromyces* rather than genus *Acremonium*. Therefore, this species should be described as *Talaromyces cellulolyticus* (Fujii et al. 2014a), and this scientific name is used hereafter. Ex-type culture of *T. cellulolyticus* was deposited in the Central Bureau of Fungal Cultures as CBS 136886 and National Institute of Technology and Evaluation in Japan as FERM BP-5826.

2 Mutagenesis of *Talaromyces cellulolyticus*

At the present time, the most common microorganism used for cellulase production is *H. jecorina*. In the first place, it is recognized as a noxious fungus which promotes decay of cotton clothing or tents during the World War II. Thereafter, extensive research was conducted at the U S Army Natick Laboratories, and the strain QM6a, which has an outstanding ability to degrade crystalline cellulose, was isolated and gave a game-changing impact on cellulose hydrolysis (Bischof et al. 2016). By irradiating conidia of QM6a strain with a linear accelerator, a new strain QM9123, which has greater cellulase-producing ability, was isolated (Mandel et al. 1971). After that, continuous attempts to obtain cellulase-hyperproducing mutant strains were conducted, and several well-known mutants such as QM9414 and RUT C-30 were isolated (Montenecourt 1983; Peterson and Nevalainen 2012).

The mutation strategy to obtain cellulase-hyperproducing strains was also applied to *T. cellulolyticus*. Firstly, strain TN was obtained as a tolnaftate-resisitant mutant from Y-94 (Yamanobe et al. 1986). The strain TN produced three times the amount of Avicelase or CMCase and two times the amount of β -glucosidase, respectively, compared with the parental strain Y-94. Then another mutant strain C-1 was isolated by the method of UV irradiation of strain TN (Yamanobe et al. 2001). The strain C-1 produced 2.1 times the amount of cellulase expressed by the filter paper unit (FPU, Ghose 1987) than the parental strain TN.

As mentioned in the Introduction, the expectation for cellulosic ethanol production has increased in the 2000s, and cellulase from *T. cellulolyticus* was considered as a suitable enzyme mixture for ethanol production from cellulosic feedstock. Then further attempt was tried to obtain more efficient cellulase producer strain by mutation (Fang et al. 2009). The major reason for the classical mutation strategy was that neither genetic information nor transformation technology was available for this species at that time. The parental strain C-1 was cultured on the PDA plate containing 0.1% AZCL-HE-Cellulose (Megazyme, Ireland) and UV-irradiated for 30 s and re-incubated, and then 30 colonies were chosen according to haloes by the hydrolysis of the pigmented substrate. Those colonies were cultured in liquid medium, and their cellulase activities (FPU) were determined. Among them, the strain named CF-26 showed the highest cellulase activity. This strain was treated with 0.05% of N-methyl-N'-nitro-N-nitrosoguanidine (NTG, known as a mutagen) for 30 min. Among the survived colonies, the strain CF-2612 was selected as the best cellulase producer. The cellulase productivity of strain CF-2612 is greater than that of the parental strain C-1, and the enzyme activity was 17.8 FPU per milliliter of the culture (Table 7.1); that is more than three times greater than that of the wild-type strain Y-94 (Yamanobe et al. 1987). Hence, CF-2612 is considered to be the best strain of *T. cellulolyticus* in terms of cellulase productivity. Actually, the cellulase productivity of strain CF-2612 was comparable to those of some practical strain of *H. jecorina*, retaining the higher β -glucosidase activity (Fang et al. 2009).

Table 7.1 Comparisons between strains C-1 and CF-2612 for soluble protein content and cellulase and β -glucosidase activity (Fang et al. 2009)

	Strain C-1	Strain CF-2612
Soluble protein content (mg/ml)	14.6 $\pm$ 0.4	18.0 $\pm$ 0.3
Cellulase activity (FPU/ml)	12.3 $\pm$ 0.5	17.8 $\pm$ 0.5
Cellulase-specific activity (FPU/mg protein)	0.84	0.99
β -Glucosidase activity (IU/ml)	25.2 $\pm$ 1.0	40.3 $\pm$ 1.1
β -Glucosidase-specific activity (IU/mg protein)	1.7	2.2

3 Whole Genome Sequencing

Whole genome sequencing data are essential for genetic engineering to improve cellulase and hemicellulase production by *T. cellulolyticus*. Our group has developed whole genome sequence database of *T. cellulolyticus* Y-94 and registered the all sequencing data with DDBJ/EMBL/GenBank in 2015 (Fujii et al. 2015a).

For sequencing of *T. cellulolyticus* Y-94 genomic DNA, 454/Roche (FLX Titanium) and Illumina Genome Analyzer II sequencers were used. We obtained the sequencing data which were 19-fold coverage of the genome and assembled as 60 scaffolds. The predicted genome size and ORF number were 36.4 Mbp and 10,980 ORFs, respectively. Among the ORFs, at least 249 ORFs were annotated as glycoside hydrolase (GH) family proteins, which include 133 potentially secreted proteins based on a SignalP-v4.1 analysis and our secretome data. The total GH family gene number of 249 in *T. cellulolyticus* was comparable to those of other filamentous fungi: 247 genes in *Aspergillus nidulans*, 285 genes in *A. oryzae*, and 200 genes in *H. jecorina* (Martinez et al. 2008). Analysis of the GH family genes on the CAZy database (Cantarel et al. 2009) revealed that 105 gene products were deduced as carbohydrate-active enzymes related to the hydrolysis of lignocellulosic biomass: 22 cellulases [12 GH5s (including hemicellulases such as mannanase), 1 GH6, 2 GH7s, 4 GH12s, 1 GH61, and 2 GH45s], 37 hemicellulases (22 GH43s, 1 GH10, 7 GH11s, 1 GH74, 1 GH62, 1 GH53, 1 GH54, 2 GH67s, 1 GH26), 38 pectinases (16 GH28s, 12 GH78s, 4 PL1s, 2 PL4s, 2 CE8s, and 2 CE12s), and 8 amylases (5 GH13s and 3 GH15s). These results are consistent with the ability of *T. cellulolyticus* to degrade various types of biomass (Yamanobe et al. 1987; Fujii et al. 2009; Inoue et al. 2013; Gao et al. 2012). These genome sequence data have been used for our molecular study related to *T. cellulolyticus*, and various gene functions have been investigated and identified.

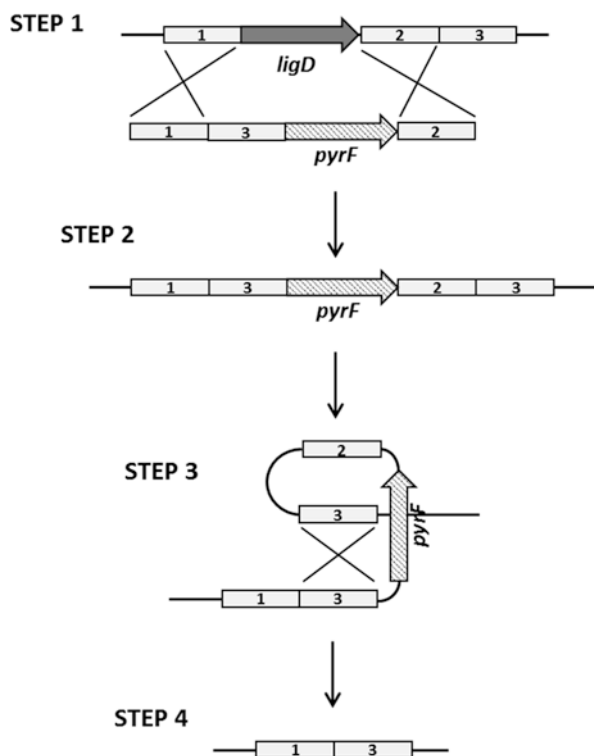
4 Transformation, Gene Targeting, and Marker Recycle

Genetic engineering is a powerful and effective method to improve the strain for enzymatic production and is strongly effective with whole genome sequencing data as mentioned above. However, in general, genetic engineering of filamentous fungi could have some technical problems. In the case of *T. cellulolyticus*, we had to solve some problems of transformation, gene targeting, and marker recycle.

The first example of transformation in *T. cellulolyticus* was performed by Midou et al. (2001). They used an antibiotic-resistant gene as a selection marker and over-expressed cellulase and hemicellulase genes in *T. cellulolyticus*. Our group also succeeded in transformation of *T. cellulolyticus* using a hygromycin-resistant gene (*hph*) (Kanna et al. 2011). Furthermore, we developed an efficient transformation method by using *pyrF* marker gene, encoding orotate phosphoribosyl transferase (Fujii et al. 2012). The transformation efficiency by using *pyrF* was over 50-fold higher than that by using *hph* (our in-house data). But, we have only two available selection marker genes, *pyrF* and *hph*. The transformation system using other marker genes has not been developed because *T. cellulolyticus* has relatively high tolerance to antibiotics and counter selective analogues used to screen for auxotroph strains (our unpublished data). In addition, another problem for genetic engineering was low frequency of homologous recombination in *T. cellulolyticus*. The gene-disrupted strains were not isolated by introducing pDCre1000, which carries the 5' and 3' regions of the *creA* gene at the upstream and downstream regions (each 1000 bp length) of the *pyrF* gene (Fujii et al. 2013). On the other hand, when the plasmid pDCre2500, the homologous region length set to 2500 bp, was introduced, the gene disruption occurred at 27% frequency (Fujii et al. 2013). These data suggest that the long homologous regions are required for the gene targeting (knockout) of *T. cellulolyticus*.

Moreover we constructed a marker recycle system, which had been shown as a useful tool for multiple gene integration or disruption in *Aspergillus aculeatus* (Tani et al. 2013). The introduced *pyrF* to disrupt *ligD* was deleted in the marker recycle system (Fig. 7.1) (Fujii et al. 2017). *ligD* encodes DNA ligase IV and is an essential gene for non homologous end joining. The resultant strain without *ligD* showed uracil auxotrophy and high homologous recombination efficiency. These results indicate that the gene targeting in *T. cellulolyticus* is easily available in this marker recycle system. In addition, the high-efficiency gene-targeting without *ligD* indicates a possibility of further multiple auxotrophic mutant constructions. So far, the auxotrophic mutants have not been isolated because *T. cellulolyticus* has high tolerance to various analogues. The improved gene-targeting frequency in the marker recycle system will lead to easy isolation of auxotrophic mutants by homologous recombination. Disruptions of several marker gene candidates are in progress.

Fig. 7.1 A marker gene recycling system in *T. cellulolyticus* (Fujii et al. 2017). Step1: Disruption of *ligD* gene by homologous recombination. Step2: Selection of *ligD*-disruptant by using *pyrF* marker gene. Step3: Deletion of the introducing *pyrF* gene by self-homologous recombination. Step4: Selection of *pyrF* deleted strain by uracil auxotrophy



5 RNA Interference and Genome Editing

RNA interference (RNAi) is a natural phenomenon occurring in most part of eukaryote and causes gene silencing by degradation of specific mRNA. Relatively long double-strand-type or hairpin-type RNAs are broken down to small interfering RNA (siRNA) of 21–23 nt by Dicer (RNase III), and RNA-induced silencing complex (RISC) composed from siRNA, Argonaute, and other proteins degrades sequence-specific mRNA. Vector expression or direct transfer into cells of hairpin-type RNA reduces transcriptional levels of target genes. RNAi is a powerful genetic knock-down tool for analysis of functionally unknown genes, especially when targeting genes are lethal. RNAi has been found in nematode *Caenorhabditis elegans* and applied for various fungi (Fire et al. 1998; Salame et al. 2011).

As far as we know, there is no RNAi application for direct enhancement of cellulase production in genus *Talaromyces* or *Penicillium*. RNAi of cellulase suppressor genes could lead to enhance fungal cellulase production in genus *Talaromyces*. RNAi applications for potent sugar sensor and DNA ligase IV (*ligD*) genes have been reported in cellulase-producing *Talaromyces cellulolyticus* (Asada et al. 2014; Hayata et al. 2014). In both studies, vectors of the hairpin-type RNAi constructs were transformed for knockdown of these fungal genes. The transcriptional level of the potent sugar sensor gene in the transformant carrying RNAi construct was less

than 10% compared with the parental strain. The transcriptional level of *ligD* in the transformant carrying the RNAi construct was 3.7% of the parental strain. These results indicated that vector transformation method for the hairpin-type RNA expression could be useful for knockdown of genes in *T. cellulolyticus*. As for RNAi of the potent sugar sensor gene, the hyphal branching ratio of the transformant strain was higher than that of the parental strain, and changes in cellulase productivity and protein secretion were not observed. As shown in the previous section, homologous recombination efficiency in *T. cellulolyticus* is very low. RNAi of *ligD* was attempted to suppress a non homologous end-joining system in *T. cellulolyticus* and improve gene targeting efficiency. The knockdown transformant of *ligD* led to higher double crossover gene targeting efficiency of 23.1% than the parental strain. RNAi of cellulase-related genes could bring us further understanding for molecular mechanism of high-speed cellulase secretion system in *T. cellulolyticus*.

In genus *Penicillium*, RNAi applications have been reported in *Penicillium chrysogenum* and *Penicillium marneffeii*. A RNAi vector containing the double-stranded RNA expression cassette targeting for *pcbC* and *cefEF* in the β -lactam pathway was constructed, and 15–20% of the selected transformants produce relatively low amounts of penicillin or cephalosporin in *P. chrysogenum* (Ullán et al. 2008). RNAi for endogenous *PcbrA* morphogene in *P. chrysogenum* caused the reduction of cinidiospore formation in 47% of the transformants (Janus et al. 2009). The class III chitin synthase gene of *chs4* was silenced by RNAi, and mutants had a slow growth rate and shorter but highly branched hyphae (Liu et al. 2013a, b). All mutants formed fewer conidia than their parental strain. The siRNA of isocitrate lyase gene (*acuD*) reduced the *acuD* gene's mRNA level and protein expression to 4.7% and 29%, respectively (Sun et al. 2014). RNAi of *acuD* attenuated the virulence of *Penicillium marneffeii* against macrophages and nude mice. The RNAi applications in the genus *Penicillium* have been useful to understand the functions of target genes.

Gene disruption using homologous recombination occurred in very low probability in *T. cellulolyticus*; therefore, a simple tool for gene targeting has been eagerly demanded for reverse genetic analyses. There are mainly three genome editing methods, zinc finger nuclease (ZFN), transcription activator-like effector-based nuclease (TALEN), and the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system. The CRISPR-Cas9 system is a relatively easy and popular genome editing method. Vector transformation or transfection of Cas9 nuclease and sgRNA creates site-specific double-strand breaks (DSBs) and leads to knockout of specific gene by mainly frame shift. The CRISPR-Cas9 system enables us to create knockout mutants of target genes without using double crossover based on homologous recombination.

CRISPR-Cas9-based genome editing was reported to identify a new gene in *Talaromyces atroroseus* (Nielsen et al. 2017). The green pigment gene was deleted by co-transformation of two vectors. One includes hygromycin-resistant gene of *hph* and up- and downstream sequence of the target gene, and the other includes Cas9- and sgRNA-encoding genes. Double-strand breaks caused by Cas9-sgRNA ribonucleoprotein in the target gene sequence promote repair by homologous

recombination. White colonies were obtained on the hygromycin-containing plates; therefore, this CRISPR-Cas9-based genome editing enables to delete target genes in *T. atrovirens*. The Cas9 protein and sgRNA can be either delivered during transformation, as preassembled CRISPR-Cas9 ribonucleoproteins (RNPs), or expressed from an AMA1-based plasmid within the cell in *P. chrysogenum* (Pohl et al. 2016). The direct delivery of the Cas9 protein with in vitro synthesized sgRNA to the cells allows for a transient method for genome engineering that may rapidly be applicable for other filamentous fungi. The expression of Cas9 from an AMA1-based vector was shown to be highly efficient for marker-free gene deletions. The CRISPR-Cas9 system could strongly enhance further molecular breeding for cellulase production in genus *Talaromyces*.

6 Transcriptional Regulation of Cellulase and Hemicellulase Genes

A number of transcriptional factors have been isolated as regulators of cellulase and hemicellulase gene expressions in other filamentous fungi: XlnR/Xyr1, inducers of cellulase and hemicellulase genes in *A. niger* and *H. jecorina* (Stricker et al. 2006; van Peij et al. 1998); Ace1, repressor of cellulase genes in *H. jecorina* (Aro et al. 2003); CreA, carbon catabolite repressor in various filamentous fungi (Dowzer and Kelly 1989; Ilmen et al. 1996; Wen et al. 2005); Ctf1B, inducer of cutinase genes in the genus *Fusarium* (Li et al. 2002); Hap B/C/E complex, regulator of various genes including cellulase and hemicellulase genes in *Aspergillus* genus (Brakhage et al. 1999); and so on.

Among these regulatory proteins, several gene products have been identified as regulators of cellulase and xylanase gene expressions in *T. cellulolyticus* (Table 7.2). First, CreA, a carbon catabolite repressor protein, represses cellulase and xylanase gene expressions (Fujii et al. 2013). The disruption of *creA* is effective for enhancing enzyme production by *T. cellulolyticus*. Second, XlnR regulates cellulase and xylanase gene expressions. A *xlnR* disruptant derived from strain Y-94 (wild-type strain) produced similar cellulase activity with a control strain (Fujii et al. 2014b). However, interestingly, cellulase productivity of an *xlnR* disruptant derived from strain C-1 (cellulase-hyperproducing mutant) was clearly lower than that of C-1 (Okuda et al. 2016). These data suggest that the regulation of cellulase production by *xlnR* differs between Y-94 and C-1. Third, TacA and TctA, homologues of Ace1 and Ctf1B, respectively (Fujii et al. 2015b), regulate cellulase and xylanase genes expression. Cellulase and xylanase production was decreased by disruption of *tacA* and *tctA*, indicating that these gene products have positive roles in cellulase and xylanase production of *T. cellulolyticus*. Finally, Hap complex could regulate cellulase production. The homologue gene of *hapB* (one subunit of Hap complex) disruptant showed clearly lower cellulase productivity than a control strain (our unpublished data). Furthermore, the deletion analysis of the promoter region of the GH7 endoglucanase gene revealed that the potential binding sequences of Hap com-

Table 7.2 Transcriptional factors related with cellulase and xylanase production in *T. cellulolyticus*

Transcriptional factor name	Function in <i>T. cellulolyticus</i>		References
	For cellulase production	For xylanase production	
CreA	Represses cellulase genes expression in the presence of glucose. <i>creA</i> disrupted strain shows higher cellulase production	Represses xylanase genes expression in the presence of glucose. <i>creA</i> disrupted strain shows higher xylanase production	Fujii et al. (2013)
XlnR	Induces cellulase gene expression only in cellulase-hyperproducing mutant (C-1). <i>xlnR</i> disrupted and overexpressed strains show lower and higher cellulase production, respectively. In wild-type strain (Y-94), <i>xlnR</i> disruption had no effect	Induces xylanase genes expression. <i>xlnR</i> disrupted strains show lower xylanase production	Fujii et al. (2014b)
TacA	Induces cellulase genes expression. <i>tacA</i> disrupted strains show lower cellulase production	Induces xylanase genes expression. <i>tacA</i> disrupted strains show lower xylanase production	Fujii et al. (2015b)
TctA	Induces cellulase gene expression. <i>tctA</i> disrupted strains show lower cellulase production	Induces xylanase genes expression. <i>tctA</i> disrupted strains show lower xylanase production	Fujii et al. (2015b)
Hap B/C/E complex	Induces cellulase gene expression. <i>hapB</i> disrupted strains show lower cellulase production. The potential binding sequences of Hap complex have important role for endoglucanase gene expression	Unknown	Fujii et al. (2017), our unpublished data

plex had important role for gene expression (Fujii et al. 2017). These data suggest that Hap complex works as a cellulase gene regulator. Further exploration and characterization of candidate transcriptional factors found in the *T. cellulolyticus* genome sequence are promising research directions in an effort to the increases in C-1 and CF-2612 based-cellulase productivities.

7 Development of Cellulolytic Enzyme Production Technology

T. cellulolyticus C-1 and CF-2612, hyper-cellulase-producing mutants have been considered as promising fungal strains to supply cellulolytic enzymes for the hydrolysis of lignocellulosic biomass. Two strategies have been employed to reduce the

cost of *T. cellulolyticus* cellulolytic enzymes. One is the quantitative way aimed at reducing enzyme supply cost with the improvement of enzyme production technology, and the other is the qualitative way aimed at reducing enzyme-loading cost with the improvement of cellulase system in *T. cellulolyticus*. The development of new *T. cellulolyticus* strains with the combination of these ways is rapidly progressing by using recombinant techniques and genome information.

The production of cellulolytic enzymes in *T. cellulolyticus* has been examined using various carbon sources in flask and fermenter cultivations (Table 7.3). Ikeda et al. established efficient production conditions of cellulase in a culture of *T. cellulolyticus* C-1 using an industrial cellulose powder Solka-Floc (SF, International Fiber Co., North Tonawanda, NY) as a carbon source (Ikeda et al. 2007). In the optimization of the medium components, urea was an essential nitrogen for efficient cellulase production source, and the C/N ratio significantly affected the cellulase production. As a consequence, the culture of C-1 showed 15.5 FPU/mL of cellulase activity in shake flask culture, and the cellulase production on small scale was successfully reproduced in 50 L pilot-scale fermenter (Table 7.3). Prasetyo et al. have reported that cellulase production by C-1 was the highest at pH 6.0 in pH-controlled fermenter cultures that some buffer components in the medium were reduced (Prasetyo et al. 2010). Cellulase production has been also improved by the addition of lactose in C-1 culture (Fang et al. 2008). On the other hand, cellulase production using strain CF-2612 has been evaluated by Fang et al. (2009). Cellulase activity and yield in a batch culture with 5% SF reached 18.0 U/ml and 360.0 FPU/g carbohydrate, respectively; when fed-batch culture was used, these values reached 34.6 U/ml and 346.0 FPU/g carbohydrate, respectively. It should be noted that the production of cellulolytic enzymes by C-1 and CF-2612 strains were comparable with those by industrial *H. jecorina* mutant strains (Ryu and Mandels 1980; Ikeda et al. 2007; Fang et al. 2009). In addition, C-1 and CF-2612 enzymes produced using SF have been demonstrated to have better performance in the hydrolysis of lignocellulosic substrate than commercial enzymes from *H. jecorina*.

Waste paper and paper sludge have been examined as alternative cheap carbon sources for cellulase production. The cellulase activity of C-1 cultured in cellulase-pretreated waste paper was 16 FPU/ml in a 3 L fermenter and was comparable to that cultured in SF (Park et al. 2011). Cellulase production from paper sludge was approximately 60% of that from SF (Prasetyo et al. 2011). On-site production that lignocellulosic biomass itself could be used as the raw material for enzyme production is proposed as a promising way to the significant reduction of enzyme cost from the viewpoint of the simplified logistics and enzyme preparation process, as well as the potential cheap carbon source utilization (Ellila et al. 2017). Hideno et al. have reported that wet disc-milling-treated rice straw is a promising carbon source for the production of cellulases by CF-2612 (Hideno et al. 2011). The enzyme preparation produced using the pretreated rice straw exhibited higher hemicellulase activity than that using SF and was more suitable for the hydrolysis of pretreated rice straw. Strain improvements for utilization of specific carbon source in commercial enzyme production are still necessary for efficient enzyme production.

Table 7.3 Comparison of cellulase production by combination of *T. cellulolyticus* strains and carbon source

Strain	Carbon source	Culture condition	Protein (g/L)	FPU (U/mL)	References
Y-94	40 g/L cellulose powder	Shake flask	5.0	5.00	Yamanobe et al. (1987)
Y-94	50 g/L potato pulp	Shake flask	–	1.12	Gao et al. (2014)
TN	50 g/L potato pulp	Shake flask	–	2.34	Gao et al. (2014)
C-1	50 g/L SF ^a	Shake flask	–	15.5	Ikeda et al. (2007)
C-1	50 g/L SF	7 L fermenter	–	17.4	Ikeda et al. (2007)
C-1	50 g/L SF	50 L fermenter	–	13.1	Ikeda et al. (2007)
C-1	50 g/L SF	3 L fermenter (pH-controlled)	16.2	13.8	Prasetyo et al. (2010)
C-1	40 g/L SF + 10 g/L lactose	Shake flask	19.4	16.7	Fang et al. (2008)
C-1	100 g/L SF	2 L fermenter (fed-batch)	–	21.9	Fang et al. (2009)
C-1	50 g/L pretreated milk pack	3 L fermenter	23.9	16.0	Park et al. (2011)
C-1	75 g/L paper sludge cellulose	3 L fermenter	17.4	10.96	Prasetyo et al. (2011)
C-1	50 g/L potato pulp	Shake flask	–	2.03	Gao et al. (2014)
CF-2612	50 g/L SF	2 L fermenter	18.1	18.0	Fang et al. (2009)
CF-2612	100 g/L SF	2 L fermenter (fed-batch)	–	34.6	Fang et al. (2009)
CF-2612	100 g/L rice straw (<3 mm)	Shake flask	10.2	4.28	Hideno et al. (2011)
CF-2612	100 g/L dry-ball-milled rice straw	Shake flask	18.9	10.8	Hideno et al. (2011)
CF-2612	100 g/L wet-disc-milled rice straw	Shake flask	18.0	10.5	Hideno et al. (2011)
CF-2612	50 g/L potato pulp	Shake flask	–	2.69	Gao et al. (2014)

^aSF, Solka-Floc

Gao et al. have found that potato pulp, which is a by-product from starch manufacturing process, is a suitable substrate to simultaneously produce cellulase, α -amylase, pectinase, and α -galactosidase in the culture of *T. cellulolyticus* (Gao et al. 2014). The potato pulp is mainly consists of starch, cellulose, and pectin, and its hydrolysate could be used as good nitrogen and carbon sources for ethanol

fermentation (Meyer et al. 2009; Gao et al. 2012). The CF-2612 enzyme preparation produced using potato pulp achieved 79% yield for glucose and 94% yield for galactose in the hydrolysis of hydrothermally treated potato pulp. Thus, *T. cellulolyticus* is proposed to be a promising source of on-site enzyme used in practical bioconversion process of potato pulp.

8 Further Improving Strategy of Cellulase System

Various kinds of cellulolytic enzymes from *T. cellulolyticus* have been purified and characterized to date (Table 7.4). In an earlier study, Yamanobe and Mitsuishi purified 13 endoglucanases (EGs) detected in Y-94 culture by column chromatography and gel slicing after polyacrylamide gel electrophoresis, although their N-terminal sequences have not been identified (Yamanobe and Mitsuishi 1990). Okada and coworkers have purified 12 EGs and 4 β -glucosidases from commercial *T. cellulolyticus* enzyme, and the N-terminal amino acids of major 4 proteins were determined (Kansarn et al. 2000a, b; Nihira et al. 2003, 2004); these have been subsequently identified as cellobiohydrolase II (Cel6A) and three EGs belonging to GH5 (Cel5A and Cel5B) and GH7 (Cel7B) with *T. cellulolyticus* genome sequence data (Inoue et al. 2014). Two-dimensional electrophoresis of CF-2612 culture supernatant revealed that CF-2612 grown on SF secretes cellobiohydrolase I (Cel7A) and Cel6A as the major proteins (Fig. 7.2). These proteins as well as some minor cellulolytic enzymes were concentrated in range of pI 4–5 as multiple isoforms. This may explain the presence of multiple cellulolytic enzymes observed in the complicate purification process from the earlier reports. The presence of isoforms of cellulolytic enzymes has also been commonly observed in other cellulase-producing fungal cultures, more likely by heterogeneous post translational modifications (Liu et al. 2013a, b; Saloheimo and Pakula 2012).

A starch-inducible homologous expression system has been constructed for the specific production of target cellulolytic enzymes from *T. cellulolyticus* (Inoue et al. 2013). The expression of a cellulolytic enzyme gene was controlled under glucoamylase promoter using starch as carbon source, and the produced recombinant enzyme allowed for much easier detection and purification due to the clean cellulase and xylanase background in the *T. cellulolyticus* starch culture. The recombinant Cel7A and GH10 xylanase (Xyl10A) purified from Y-94-based recombinant strains have been found to have the same physicochemical and enzymatic properties without dramatic hyper-glycosylation and altered characteristics which occur in heterologous expression using other eukaryote host such as yeast (Inoue et al. 2013; Kishishita et al. 2014). The starch-inducible homologous expression system has successfully used to produce and characterize all six GH11 xylanases on the *T. cellulolyticus* genome as well (Watanabe et al. 2014). Additionally, this expression system has been reported to be available for a heterologous expression of GH5 and GH12 hyperthermophilic cellulases from archaea *Pyrococcus* sp. (Kishishita et al. 2015).

Table 7.4 Cellulolytic enzymes isolated from *T. cellulolyticus* culture

Enzyme	Type of action	CAZy module	Molecular weight (SDS-PAGE, kDa)	Optimum pH	Thermostability (T _m , °C)	Source	References
Bgl3A	β-glucosidase	GH3-CBM1 at C-terminus	82.1	5.0	66.0	CF-2612 grown on SF ^a	Inoue et al. (2014)
β-Glucosidase A	β-glucosidase	GH3	108	3.5–4.0	–	Y-94 grown on cellulose	Fukasawa et al. (2007)
Cellulase III-B/Cel5A	Endoglucanase	GH5-CBM1 at N-terminus	49–50.4	5.5	77.0	CE ^b /CF-2612 grown on SF	Kansam et al. (2000b) and Inoue et al. (2014)
Cellulase IV	Endoglucanase	GH5	38	4.0	50 for 24 h	CE	Kansam et al. (2000a)
Cellulase II/ Cel6A	Cellobiohydrolase	GH6-CBM1 at N-terminus	5.0	5.00	63.0	CE/CF-2612 grown on SF	Nihira et al. (2003) and Inoue et al. (2014)
Cel7A	Cellobiohydrolase	GH7-CBM1 at C-terminus	60	4.5	67.0	CF-2612 grown on SF, HoE ^c	Inoue et al. (2013)
Cellulase III-A/Cel7B	Endoglucanase	GH7-CBM1 at C-terminus	58–60.9	5.5	64.0	CE/CF-2612 grown on SF	Kansam et al. (2000b) and Inoue et al. (2014)
Xyl10A	Xylanase	GH10-CBM1 at C-terminus	51–53	5.0	80.5	CF-2612 grown on SF, HoE	Kishishita et al. (2014)
Xyl11A	Xylanase	GH11	22.7	4.5	59.0	HoE	Watanabe et al. (2014)
Xylanase III/ Xyl11B	Xylanase	GH11-CBM1 at C-terminus	34.0	3.5–4.5	66.0	CE/HoE	Watanabe et al. (2014)

(continued)

Table 7.4 (continued)

Enzyme	Type of action	CAZy module	Molecular weight (SDS-PAGE, kDa)	Optimum pH	Thermostability (T _m , °C)	Source	References
Xyl11C	Xylanase	GH11	26.4	4.0	61.5	HoE, heterologous expression in <i>E. coli</i>	Watanabe et al. (2014) and Kataoka et al. (2014)
Xyl11E	Xylanase	GH11	24.9	4.0	65.5	HoE	Watanabe et al. (2014)
Xyl11F	Xylanase	GH11-CBM1 at C-terminus	41.8	4.0	60.0	HoE	Watanabe et al. (2014)
Xyl11G	Xylanase	GH11	22.7	4.0	64.5	HoE	Watanabe et al. (2014)
α-L-Arabinofuranosidase	α-L-Arabinofuranosidase	GH54-CBM42 at C-terminus	57	3–4	50 for 24 h	TN grown on cellulose	Nojiri et al. (2011)
FaeB	Feruloyl esterase	CE1-CBM1 at C-terminus	41.3	4.5–6.5	70	HoE	Watanabe et al. (2015a)
AE	Acetyl esterase	CE3-CBM1 at C-terminus	–	6.0	78	Heterologous expression of catalytic domain in <i>E. coli</i>	Watanabe et al. (2015b)

^aSF, Solka-Floc

^bCE, commercial enzyme

^cHoE, starch-inducible homologous expression in Y-94-based recombinant strain

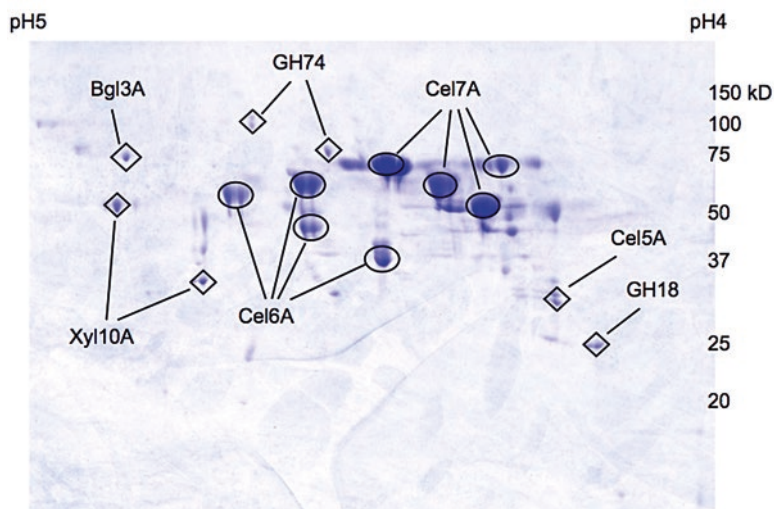


Fig. 7.2 2D gel electrophoresis analysis of *T. cellulolyticus* CF-2612 culture grown on Solka-Floc

To understand the strong hydrolysis of lignocellulosic biomass by *T. cellulolyticus* CF-2612 cellulolytic enzyme preparation, cellulolytic enzymes critical for the hydrolysis of crystalline cellulose were purified and identified from CF-2612 culture supernatant using an enzyme assay based on synergistic hydrolysis of Avicel (Inoue et al. 2014). The purified six enzymes were identified as GH3 β -glucosidase (Bgl3A), Cel5A, Cel6A, Cel7A, Cel7B, and Xyl10A. A mixture of the purified enzymes synergistically hydrolyzed a pretreated lignocellulosic substrate. Furthermore, these enzymes have been detected as the major proteins in a shotgun proteomics analysis of the CF-2612 culture grown on cellulose (unpublished data) and thus have been considered as core enzymes in *T. cellulolyticus* cellulase system. Interestingly, all core enzymes included a carbohydrate-binding module 1 (CBM1); this is particularly rare in the case of β -glucosidase, and no CBM1 in GH10 and GH11 xylanases are found in the core enzymes from *H. jecorina* (Banerjee et al. 2010; Gao et al. 2010). CBM1 is found in fungal cellulases and hemicellulases and generally targets the enzyme to the cellulose surface. Xyl10A CBM1 has been found to play a critical role in the efficient removal of xylan obstacles on lignocellulosic substrates that interrupt the cellulase activity and to contribute to a reduction of the enzyme loading in the hydrolysis of pretreated lignocelluloses (Inoue et al. 2015a). These reports mean that *T. cellulolyticus* CF-2612 cellulase system is based on the enzyme types suitable for the hydrolysis of crystalline cellulosic substrates and has a potential advantage for the hydrolyses of pretreated lignocellulosic substrates.

The composition of enzymes in native fungal cellulase systems is not necessarily suitable for the hydrolysis of pretreated biomass. The optimization of cellulolytic enzyme composition for the hydrolysis of target substrate, such as the

supplementation of additional enzymes and the use of statistical modeling to create core enzyme mixtures using purified enzymes, promotes further understanding and improvement of fungal cellulase systems for industrial applications. Supplementations of *T. cellulolyticus* CF-2612 cellulase with purified β -xylosidase and β -mannosidase have been reported to enhance total sugar yields from rice straw and softwood, respectively (Inoue et al. 2015b, 2016), since CF-2612 has been known to secrete trace amounts of β -xylosidase and β -mannosidase using cellulose as a carbon source (Fujii et al. 2009). In addition, homologous expression of β -xylosidase in *T. cellulolyticus* has been found to improve the hydrolysis of xylo-oligosaccharides by the recombinant cellulolytic enzyme preparation (Kanna et al. 2011). On the other hand, supplementations of *T. cellulolyticus* cellulase with purified core enzymes have been examined using a hydrothermal sugarcane bagasse; the results showed that the strengthening of Cel7A, Cel6A, and Bgl3A activities in the crude enzyme enhanced the hydrolysis yield, although the strengthening of EGs (Cel5A and Cel7B) and Xyl10A activities had little effect on the hydrolysis yield (unpublished data). A purified enzyme mixture for the hydrolysis of dilute acid-pretreated corn stover (PCS) glucan has been reconstructed statistically for Cel5A, Cel6A, Cel7A, and Xyl10A in the presence of Bgl3A. The optimized core mixture achieved 80% hydrolysis of PCS glucan at a protein loading of 11.2 mg/g glucan, which was at the lower loading than using CF-2612 crude enzyme or a commercial enzyme preparation (Inoue et al. 2014). We have recently constructed a CF-2612-based recombinant strain that additional Cel7A and Bgl3A genes were controlled under Cel7B promoter. The recombinant cellulolytic enzyme preparation has exhibited an increased activities of FPase and β -glucosidase as well as a decreased EG activity, compared with CF-2612 enzyme preparation. The use of recombinant enzyme preparation has allowed for further reduction of enzyme loading to the PCS hydrolysis. This preliminary study demonstrates that the improvement of core enzyme composition by genetic engineering is a practical way to develop cellulolytic enzyme preparation suitable for the hydrolysis of target substrate.

Some accessory enzymes, such as α -L-arabinofuranosidase, α -galactosidase, feruloyl esterase, and acetyl esterase, that remove substituents linked to hemicellulose backbone and one polysaccharide monooxygenase belonging to auxiliary activity family 9 (AA9) that catalyzes oxidative cleavages of cellulose chains in the crystalline regions have been identified in a shotgun proteomics analysis of CF-2612 culture grown on cellulose. Our group is currently conducting the homologous expression of various *T. cellulolyticus* cellulolytic enzymes including accessory enzymes and AA9 and collecting them as a cellulolytic enzyme library. The supplementation of core enzyme mixture with ancillary enzymes from the library would enable a prompt diagnosis of *T. cellulolyticus* cellulase system suitable for the hydrolysis of each target substrate. These results are useful for construction of further improving strategy of this fungal cellulase system fitting with each target biomass.

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Chapter 8

Heterologous Expression of Lignocellulolytic Enzymes in *Aspergillus niger*



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Abstract *Aspergillus niger* is a genetically tractable model organism for scientific discovery and a platform organism used for the production of heterologous enzymes. Academic strategies to increase production include the use of strong promoters, multiple gene copies, gene knock out and knock in strains, secretion pathway engineering, and multi omics-based approaches. However, yields of heterologous proteins are still lower than desired. Industrial approaches are more effective and straightforward: optimizing bioprocess conditions, strains engineering through multiple rounds of mutagenesis and reverse genetics, and new expression constructs design and testing, which are based on high-throughput screening in automated bioreactor/fermenter. High-level production of heterologous enzymes remains elusive in *Aspergillus niger* but more promising than ever.

Keywords Heterologous expression · Lignocellulolytic enzymes · *Aspergillus niger*

1 Introduction

Lignocellulosic biomass is primarily composed of cellulose, hemicellulose, and lignin. These cellulose and hemicellulose are composed of hexose and pentose sugars that can be fermented into substitutes, such as organic acids or biofuel. And lignin can be used for the aromatic compounds and other valuable chemicals (Pérez et al. 2002). Efficient and economical deconstruction of biomass is critical for the success of lignocellulosic biorefineries. One of the challenges of using lignocellulosic

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biomass for bio products production is the recalcitrance of plant biomass, but the promising pretreatment and saccharification technology has accelerated to overcome the barrier from the last decade (Tadesse and Luque 2011). And pretreated biomass saccharification with lignocellulolytic enzymes is efficient at lower enzyme loadings. However, the costs of enzymes for lignocellulosic bioproducts production are still high but decrease by innovative technologies, such as new enzymes discovery, less enzyme loading, and improved enzyme technologies (Schubert 2006). The enzyme production by microbes is the most critical step. And here, we focus on large-scale application, not lab scale.

2 Lignocellulolytic Enzymes

The cell wall of feedstock is made of lignocelluloses, which are the most abundantly regenerative raw materials. Lignocellulytic enzymes play an important role as biocatalyst to efficiently hydrolyze lignocelluloses.

Lignocellulytic enzymes are a crucial component of various industrial processes and attract more and more attentions in agricultural and bio energy application, especially for the production of renewable biofuels, like ethanol (Bhat 2000). Lignocellulytic enzymes in worldwide market are going to become the largest group of industrial enzymes (Wilson 2009).

Basically, lignocellulytic enzymes have three types of enzymes, cellulase and xylanolytic and ligninolytic enzymes. Cellulase is well known and the most valuable player in the category. Cellulase is also made from three types of enzymes, cellobiohydrolases (CBHs), endoglucanases (EGs), and β -glycosidases (BGLs) and deconstructed the cellulose into glucose (Van Dyk and Pletschke 2012). Recently, auxiliary activities family 9, AA9 (formerly GH61), was characterized and approved to be important for the oxidative disruption of the cellulose (Quinlan et al. 2011).

Cellulases used in the saccharification have to be functional during or after pretreatment process, since pretreatment approaches vary and create different environment, which may impair the enzymes. For instance, one of the promising pretreatment is ionic liquids (ILs) pretreatment technology, which can strongly inhibit the downstream enzymatic saccharification. Because of the ionic liquids, the cellulases must be IL-tolerant during the process. And indeed, certain IL-tolerant cellulases were identified and characterized from natural environments (Park et al. 2012; Gladden et al. 2014). But the cellulases need to be produced in heterologous hosts for large-scale application, such as, *E. coli*, fungi.

3 *Aspergillus niger* as Host for Recombinant Enzymes Production

Many filamentous fungi, particularly species of the genera *Aspergillus* and *Trichoderma*, secrete a wide variety of enzymes into their environment, allowing them to utilize many different types of carbon sources. These fungi have therefore

been utilized as hosts for industrial-scale production of native and heterologous enzymes with applications in many industries, such as food, textile, and bioenergy (Gupta 2016). In *Aspergillus*, heterologous protein production has been studied in many species, including *A. nidulans*, *A. awamori*, *A. niger*, and *A. oryzae* (Ward et al. 1990; Broekhuijsen et al. 1993; Gouka et al. 1997a, b; Conesa et al. 2001). Unfortunately, early efforts to produce heterologous non-fungal proteins achieved considerably lower enzyme titers relative to native enzymes. However, more recently several strategies have been employed to improve production of heterologous proteins in *Aspergillus* (Meyer et al. 2011). In *A. niger*, the most successful expression system is glaA fusion expression, in which the desired protein is fused to the signal peptide of the highly secreted indigenous enzyme glucoamylase (Ward et al. 1990; Joosten et al. 2003). In addition, the strain would be engineered by gene knockout, mutagenesis, and multiple gene copy integration (Verdoes et al. 1994). These efforts have enabled some modest improvements in heterologous enzyme production, but more work is needed to leverage the full potential offered by the powerful secretion machinery possessed by these fungi, including gaining a better understanding of the secretory machinery, enhanced process optimization, and the development of expanded genetic engineering toolboxes (Nevalainen and Peterson 2014). Here we won't focus on specific enzymes production in *A. niger* but give a general idea about the challenges and improvement in *A. niger* host for heterologous enzymes production.

4 Genetic Tools and Manipulation

In the past three decades, *Aspergillus niger* as microbial cell factory has been well established in academic and industrial fields. New genetic tools and techniques resulted in big discoveries, including genetic transformation systems, gene targeting tools, and expression toolbox for protein production (Meyer 2008; Lubertozzi and Keasling 2009; Fleissner and Dersch 2010).

For a long time, protoplast-mediated transformation is the canonical approach. And also the only approach for efficient and stable transformation in *A. niger*, even though *Agrobacterium*-mediated transformation appears and is more efficient in other aspergilli (Nevalainen et al. 2005; Meyer 2008).

Without efficient genetic tools, the exploitation of gene functions is time-consuming and labor costly. This situation could be improved, if there are more selective markers and higher gene targeting efficiency.

In *Aspergillus niger*, commonly used selectable markers include antibiotic resistance markers (hph, ble), auxotrophic markers (pyrG, pyrE, argB, niaD), and nutritional markers (amdS, ptrA) (Ruiz-Díez 2002). Especially, the reversible markers amdS and pyrG/E are dominant. The amdS, acetamidase-encoding gene, can be used for selection on acetamide medium and counter selection on fluoroacetamide medium. And the pyrG/E, encoding orotidine-5'-phosphate decarboxylase, can be used on uracil medium and counter selection on 5-fluoroorotic acid (5-FOA) medium. However, the counter selection is more difficult than the forward

selection. Recently, Cre-loxP recombination system was approved to work well for selectable marker rescue in *Aspergillus oryzae*, which needs to prepare DNA and transformation. Therefore, it is still low efficient and time-consuming (Mizutani et al. 2012).

However, still, a limited number of genetic tools are currently available for efficient transformation, high-throughput gene targeting and screening. And there is no breakthrough for hyper production in *A. niger*. However, the gene targeting efficiency did jump from less than 5% to more than 80%, even 100%, when the ku genes in the non homologous end joining (NHEJ) pathway were deficient. This important discovery elevates the throughput of gene targeting and made genome-wide gene editing feasible (Zhang et al. 2011). CRISPR-Cas9 system is unique and accurate in genome editing, and it works in *Aspergillus niger*. But the inefficient transformation hampers the application of CRISPR-Cas9 system in *Aspergillus niger*. If all the parts of CRISPR-Cas9 are in one plasmid, the plasmid is huge and not easy to introduce into *Aspergillus niger* protoplast. If the parts were introduced separately, the co-transformation efficiency was much lower than single plasmid transformation. CRISPR-Cas9 system is more useful in those fungi without established gene targeting approach (Nødvig et al. 2015).

5 Protein Secretion and Degradation

Significant progress has been made regarding genetics of protein secretion in the secretory pathway in filamentous fungi, particularly through the endoplasmic reticulum (ER) compartment; however, information on this pathway is still fragmentary. In *A. niger*, two major cellular mechanisms in the ER are involved: “unfolded protein response” (UPR) and “ER-associated protein degradation” (ERAD), which ensures that only correctly folded proteins are delivered to the subsequent compartment, i.e., Golgi apparatus (Travers et al. 2000; Kleizen and Braakman 2004). Fungi which secrete heterologous proteins or are treated with dithiothreitol (DTT; a reducing agent which causes formation of unfolded proteins) display regulation of chaperones, transcript factor, and foldases such as *bipA*, *hacA*, and *pdiA*, which are over expressed to improve heterologous protein production under ER stress (Hijarrubia et al. 1997; Jeenes et al. 1997; Saloheimo et al. 2003; Mulder et al. 2004). Recent studies indicate that ER stress also leads to transcriptional down regulation of the *gla* gene in *A. niger* (Al-Sheikh et al. 2004). Another feedback mechanism may occur upon ER stress at the *gla* translational level, in addition to repression under ER stress (RESS) (Sims et al. 2005; Mikko et al. 2006; Guillemette et al. 2007).

And constitutive induction of the UPR did increase the heterologous enzyme production in *Aspergillus niger* (Valkonen et al. 2003). UPR and ERAD regulation are still elusive in *Aspergillus niger*. However, proteolytic degradation is a major issue during protein production and well known (van den Hombergh and van de Vondervoort 1997). Following the whole-genome sequencing of *A. niger* (Pel et al.

2007), approximately 200 predicted proteases have been identified. Proteolytic degradation affects mainly heterologous proteins evidenced by improvement of heterologous protein production upon deletion of the protease genes. Four extracellular proteases with acid pH optima (an aspartic protease, PEPA; a glutamic protease, PEPB; and two serine carboxypeptidases, PEPF and PEPG) have previously been characterized (Krishnan and Vijayalakshmi 1985; Thompson 1990; Inoue et al. 1991; Mattern et al. 1992; van den Hombergh et al. 1994). The PEPA and PEPB proteases appeared to constitute 84% and 6% of the extracellular acidic proteolytic activity, respectively, whereas the PEPE protease is responsible for 68% of the intracellular acidic protease activity. Most of the industrial strains for heterologous enzyme production are protease deficient (Hombergh et al. 1997).

6 Genome-Wide Omics Analysis in *A. niger*

Aspergillus niger CBS 513.88 genome was sequenced and published in 2007, which was the first *A. niger* whole-genome sequence (Pel et al. 2007). The well-annotated sequence data plus transcriptomics and proteomics will significantly enrich the enzymes and metabolites to become produced. However, big data set did not make life easier but even confusing, since omics data results in too many predictions and possibilities and most of the possibilities are hard to take insights by experimental biology. But multi omics analysis is still the most powerful tool to predict gene functions and pathways (Gilbert and Dupont 2011).

7 Examples for Improving Heterologous Production in *A. niger*

Four lab-scale approaches were listed to illustrate the combination of omics and molecular genetics (unpublished).

7.1 Promoters and Signal Peptides to Lead Enzyme Expression and Secretion

In nature, *Aspergillus niger* is a starch-based hydrolases producer, e.g., glucoamylase, amylase, and α -glucosidase. Therefore, the promoters and signal peptides of those enzymes are used for heterologous gene expression, which are all inducible promoters. On the other hand, several constitutive promoters like the glyceraldehyde-3-phosphate dehydrogenase *gpdA* are also useful for expression at moderate titer, like metabolic pathway engineering, not for high titer industrial enzyme production

(Blumhoff et al. 2013). For industrial application, the heterologous enzymes are preferred for extracellular secretion. Therefore, both promoters and signal peptides are equally important. Promoters and signal peptides from secreted proteins were identified by proteomics, which can be used for heterologous enzyme expression and secretion. The idea is quite simple.

1. Growth in liquid media with variety of carbon sources
2. Measurement of total proteins in the supernatants by Bradford and SDS-PAGE
3. Identification of promoters and signal peptides by proteomics analysis
4. Strength validation of the promoters and signal peptides by expression of heterologous enzyme

Several promising promoters signal sequences had been characterized based on proteomics analysis of secretome. This approach is fruitful, and proteomics did help to sort the secretome out to a manageable list.

7.2 The Influences of N-Glycosylation Motifs in Heterologous Enzyme Expressed in *Aspergillus niger*

N-Glycosylation has important impacts on enzyme trafficking through organelles (Davies et al. 2001), secretion of the enzymes (Linden and Kaushansky 2000), and enzymatic properties of enzymes (Mistry et al. 1996; Egrie and Browne 2001; Gao et al. 2012). And N-glycosylation modification pathway in the cytoplasm and endoplasmic reticulum was highly conserved across all the filamentous fungi, but the sugar chain varies (Deshpande et al. 2008). In the final stages of N-glycan synthesis ended in fungal cell, but the final trimming of the sugar chain could be completed in the extracellular side, since fungi secreted glycan-trimming enzymes to the environment (Stals et al. 2004; Varki et al. 2009; Beckham et al. 2012).

In filamentous fungi, N-glycosylation modification pathway was more closed to mammalian cell, but yeast (Jin 2012), which made *Aspergillus niger* have the potential for humanized engineering (Gerngross 2004). For industrial application, N-glycosylation of enzyme could be engineered. N-glycosylation site is the motif with asparagine-X-serine/threonine (NXS/T), where X is any amino acid except proline. And N-glycosylation might be beneficial for heterologous protein expression when the glycosylation sites are placed correctly. The corollary is that misglycosylation, either improper placement of the N-glycosylation site or improper extension of the core chitobiose unit of N-glycosylation sites, is detrimental to protein expression. To test this hypothesis, our approach is more straightforward.

1. A prokaryotic enzyme without N-glycosylation motif in the amino acid sequence was engineered, and N-Glycosylation motifs were introduced into regions of the enzyme that were predicted to be surface exposed and least likely to disrupt the secondary structure of the enzyme. Ten single N-glycosylation motifs were incorporated into heterologous prokaryotic enzyme by single amino acid

changes. These in silico-designed putative N-glycosylation sites were then analyzed by the structure feature analysis tool (SFAT) (Lam et al. 2013), which predicted the sites to be glycosylated.

2. These N-glycosylation engineered genes and wild type were expressed in *Aspergillus niger*. An expression cassette driven by glaA promoter and signal peptide was designed to express heterologous enzymes wild type and ten variants with artificial N-glycosylation motifs.
3. All engineered enzymes were purified to verify the asparagine within the motif glycosylated. Nine out of ten variants were not glycosylated, and one variant with N-glycosylation motif was secreted with two forms, N-glycosylation modified and non modified.
4. In addition, transcriptomic analysis of the *A. niger* strains, harboring heterologous artificial N-glycosylation engineered gene, was employed to detect cellular responses to the expression of glycosylated heterologous enzymes.

Comparative transcriptomic analysis of partially N-glycosylation-modified variant versus the other nine variants and wild-type strain helped to find glycosylation-related genes. In general, there were very few significant changes of N-glycosylation-associated genes in all strains, and only three genes were upregulated but just increased 40%.

In summary, the effect of N-glycosylation on heterologous enzyme expression, modification, and trafficking remains an open question. We are trying to analyze a set of data to address these questions. At least, artificial engineered N-glycosylation motif works in specific case. Interestingly, the modification was not 100%, and we have identified three genes involved in glycosylation process by transcriptomics. And the N-glycosylation-modified variant could be used as a platform for characterization of glycosylation-related genes in further study.

7.3 The Impact of Temperature on the Production of Heterologous Enzyme in *Aspergillus niger*

Optimization of growth conditions were one of the approaches for improvement of heterologous enzyme titer in industrial scale in *Aspergillus niger*. The protocols of the optimization including the growth medium, temperature and pH, etc. in submerged and solid fermentation had been described in detail (Enshasy 2007; Rab et al. 2013). And different host strains with heterologous enzyme had specific optimal cultivation conditions. There was no gold standard. In addition, cultivation temperature not only had impact on extracellular proteins secretion (Adav et al. 2013), but also secondary metabolite aflatoxin production (Ogundero 1987). Therefore, with omic analysis, we are trying to illuminate the temperature effects on heterologous enzyme production in *A. niger*.

1. The *A. niger* strain with heterologous enzyme production was cultivated at 30 °C, 33 °C, 35 °C, 37 °C, and 40 °C in liquid media. Our results revealed that the temperature optimum T_{opt} was 37 °C and the cultivation temperature of 37 °C showed more than twofold higher heterologous enzyme titer than 30 °C. In addition, the total amount of secreted proteins in supernatants was also affected by temperature.
2. Transcriptomic analysis of the strains in different temperature.

The mRNA level of heterologous gene was lower, but the amount of protein was higher at 37 °C. Fewer transcripts gave more secreted proteins, which indicated that cell responses for heterologous gene expression and protein secretion are not only at gene transcriptional level but more importantly at posttranscriptional stage. And the transcriptomic data showed that half of the genes changed significantly, which indicated that temperature is a global parameter.

7.4 Random Mutagenesis for Improvement of Heterologous Enzyme Production in *Aspergillus niger*

Aspergillus niger and *T. reesei* have been developed as heterologous enzyme producers for more than 30 years. Random mutagenesis was one of the powerful tools for strain improvement. Some of the high producing strains such as *T. reesei* RUT-C30 and *A. niger* CBS 513.88 were generated by random mutagenesis (Pel et al. 2007; Peterson and Nevalainen 2012). In the past the disadvantage of this approach was difficult to identify the mutation sites, and random mutagenesis always resulted in multiple loci changes. Now re-sequencing technology is available and more convenient than before, which is a breakthrough for identification of mutants. However, the published functional analysis of mutagenesis data was quite little in *Aspergillus niger*, perhaps because of high commercial sensitivity or few successful cases. UV mutagenesis approach was used quite well in *A. niger* (Lotfy et al. 2007), and we followed the old protocol plus re-sequencing analysis.

1. UV mutagenesis and high producers screening. Tens of high-level producing mutants were identified. Some of them were “small colony” strains with morphological difference, and some were no changes in morphology.
2. Re-sequencing the mutants and characterizing the genomic mutations. The re-sequencing data was massive and multiple loci mutation so. For instance, the number of SNPs in mutants was from 28 to 140. After comparison of the data, there were three strains sharing the same gene mutation at CreA locus, which was a well-known carbon catabolite repressor (Ruijter et al. 1997; Ries et al. 2016). And there was no other overlap among the mutants.

The results indicated that random mutagenesis was a useful approach for industrial application, but academic research. And that is the reason why there is very few published information about the gene characterization by random mutagenesis.

8 Discussion and Perspectives for Heterologous Enzyme Production in *A. niger*

Aspergillus niger is an excellent protein production host as evidenced by its wide use in industry to produce a variety of enzymes for hydrolysis of polysaccharides to sugars. However, a gap in our collective scientific knowledge is a fundamental understanding of the factors that provide high titer production of heterologous enzymes in fungal hosts.

After the genome sequence was available, large-scale omics information from transcriptomic proteomic and metabolomic data were generated, which were heavily reliant on computation (Meyer et al. 2015). And computational methods guiding protein redesign have also been reported to improve protein production in *A. niger* (van den Berg et al. 2012, 2014).

With the development of synthetic biology, filamentous fungi lagged far behind other microbes like *E. coli* and *Saccharomyces cerevisiae*. Synthetic biology is the trend and takes matters for constructing biological systems. In *Aspergillus niger*, highly efficient transformation system, high-throughput gene deletion, and screening need to be developed to support the synthetic biology (Nevalainen and Peterson 2014).

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Chapter 9

Thermophilic Cellulolytic Enzymes: From Discovery to Design



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Abstract Compared with current commercial lignocellulolytic enzymes from fungi, their analogues from thermophilic organisms own excellent properties on thermostability and catalytic efficiency. However, isolating and culturing the pure extreme microorganisms are big challenges. It is a huge difficulty to collect sample from inaccessible environments, subsequently culturing these microorganisms with expensive laboratory instruments and special biotechnological skills. Recent advances in microbial biotechnology, especially for pure anaerobe cultivation, single cell sequencing, low-cost genome sequencing, and bioinformatics, allow us to thoroughly understand intrinsic mechanisms within these thermophilic lignocellulose deconstruction systems. In this chapter, we focus on four short topics: (1) key cellulolytic enzymes from filamentous fungi, (2) discovery of potential lignocellulosic cellulolytic enzymes from thermophilic biomass degraders, (3) characterization and application of thermophilic cellulolytic enzymes in filamentous fungi enzyme cocktails, and (4) design for more thermostable cellulases. Variation in cellulase protein structures and acting mechanisms will also be introduced.

Keywords GH family · Thermophilic cellulolytic enzymes · Thermostable cellulases

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1 Introduction

Plant biomass is a renewable, sustainable, and the most abundant raw material for worldwide energies and chemicals in our life (Blumer-Schuetz et al. 2008). It consists of a great abundant of polysaccharides (e.g., cellulose and hemicellulose) and aromatic polymer (lignin) (Kumar and Sharma 2017). The structural complexities of plant polysaccharides highly protect plant, for resistant to attack from environmental microorganism (Garvey et al. 2013). Thus, the research on cellulolytic enzymes is always a hot issue, due to its important effect on the lignocellulosic biomass bioconversion. From plant polysaccharides to carbohydrate intermediates such as clean sugars or carbohydrate derivatives, multiple strategies have been applied, such as chemical/physical pretreatment, thermochemical process, and enzyme hydrolysis (Tang et al. 2017). It remains significantly important for our technology to decrease the cost of biomass bioconversion, especially on enzyme hydrolysis (Li et al. 2017).

In the kingdom of nature, many organisms own the biomass-degrading systems to digest polysaccharides to the utilizable oligo- or mono-sugars (Chen et al. 1995; Garvey et al. 2013; Hess et al. 2011). Of all biomass-degrading organisms, fungi are the most important sources for the application of cellulolytic enzyme due to it widely colonized in many biomass-degrading niches from the terrestrial and marine environments of nature (Mitra et al. 2011; Shida et al. 2016; Ikeda et al. 2007). Another reason is a high production titers of “free” cellulolytic enzymes in many fungi (Ikeda et al. 2007). In the 1940s, the soft-rot filamentous fungus *T. reesei* was first isolated in the South Pacific and characterized by the Natick Research Laboratories (Druzhinina et al. 2010). Subsequently, a series of biomass-degradable filamentous fungi, such as *Aspergillus*, *Neurospora*, *Penicillium*, and *Talaromyces*, were reported (Yazdi et al. 1990; Prasanna et al. 2016; Okuda et al. 2016; Kaur et al. 2014; Ikeda et al. 2007). Till now, the filamentous fungus *Trichoderma reesei*, with a series of industrial-engineering strains, is a classic model for the industrial-scale production of cellulases and hemicellulases. This strain is also suitable for simultaneous saccharification and fermentation (SSF) (Samuels 1996). In the past decade, the research has focused on the structures, catalytic function, thermostability, and substrate specificity of cellulolytic enzymes by significant governmental and commercial investments. In order to reduce enzyme cost and enhance the enzymatic hydrolysis efficiency, researchers focused on the improvement of cellulase productivity and optimization of enzymatic cocktails. Several industrial strains from *T. reesei* and *Aspergillus* sp., such as *T. reesei* Rut-C30, RL-P37, and MCG-80, have been developed and characterized (Peterson and Nevalainen 2012; Escamilla-Alvarado et al. 2013; Tholudur et al. 1999).

Due to its high substrate loading and mass turnover rates, thermostable enzymes with improved capabilities for cellulose degradation provide new route for cost efficiency of biofuel production (Bhalla et al. 2013; Blumer-Schuetz et al. 2010; Mir et al. 2014). Thermostable enzymes showed several advantages, including greater stability, higher catalytic efficiency, higher solid-substrate loading, and lower

contaminate risk resistant to high temperature in the enzymatic hydrolysis of cellulose (Miller and Blum 2010). This approach opens the significant potential to reduce amounts of loading enzymes for hydrolysis and thus reduces the cellulase cost for the second-generation bioethanol production. To date, discovery and application of thermostable enzymes for cellulose hydrolysis are widely studied (Li et al. 2017). Due to the great advances on the industrial-scale DNA-sequencing technology and bioinformatics, the cost of genome and metagenome sequencing is much lower than decade before (Yang et al. 2016). A huge number of uncultured environmental microorganisms were isolated and genomic-sequenced (Voget et al. 2006). Moreover, the technical advances on pure cultivation of microorganisms and single-cell sequencing also allow us to characterize the physiological roles of extremophiles and discover the novel thermostable enzymes from nature (Monkoondet et al. 2016). In our previous studies, we screened many thermophilic microorganisms, such as the members of *Caldicellulosiruptor* and *Clostridium* from extreme environment (the detailed information will be discussed in the following section). In this chapter, applications of thermophilic cellulolytic enzymes are discussed, which includes three sections which are described as followed.

2 Key Cellulolytic Enzymes from Filamentous Fungi

The primary action mode of fungal-derivative enzyme includes endoglucanases (EGs), cellobiohydrolases (CBHs), and β -glucosidases (β Gs). Non-processive EGs firstly act by cleaving the amorphous regions of cellulose chains, and then processive CBHs recognize and depolymerize the ends of cellulose chain into soluble disaccharides. These cellobioses are then utilized by organisms and hydrolyzed into glucose by β Gs in the cytosol (Payne et al. 2015). In this section, the primary components of cellulolytic enzymes from filamentous fungi, including glycoside hydrolase (GH) families of 5, 6, and 7 will be mainly discussed.

2.1 GH-5 Family from Filamentous Fungi

GH-5 family is one of the largest and most diverse families of GHs with more than 10 thousand GenBank accessions (Pickersgill et al. 1998). The hydrolases from GH-5 are key EGs of the “free” enzyme cocktails in filamentous fungi. In 2012, Aspeborg et al. assigned GH5 family into 51 subfamilies (Aspeborg et al. 2012). Till now, there are 53 currently recognized subfamilies that have been defined by a phylogenetic-based CAZy system. In structures, GH5s belong to the GH-A clan with a $(\beta/\alpha)_8$ -fold characteristic of enzymes capable of degrading equatorially oriented glycosidic linkages. In catalytic actions, GH5 hydrolase is endo-initiated action mode, with a few of β -1,4-cellobiosidases (EC 3.2.1.91) (Morag et al. 1991). Due to their variation in protein sequences and structures, the GH-5 hydrolases

Fig. 9.1 Several key cellulolytic enzymes from filamentous fungi. (a) a GH5 family endoglucanase (PDB id 5I77) from *A. niger*, (b) a GH5 family β -mannase (3WH9) from *A. niger*, (c) a GH6 family CBH II type (1HGW) from *T. reesei*, (d) a GH7 family endoglucanase (1EG1) from *T. reesei*, (e) a GH7 family CBH I-type (1CEL) from *T. reesei*

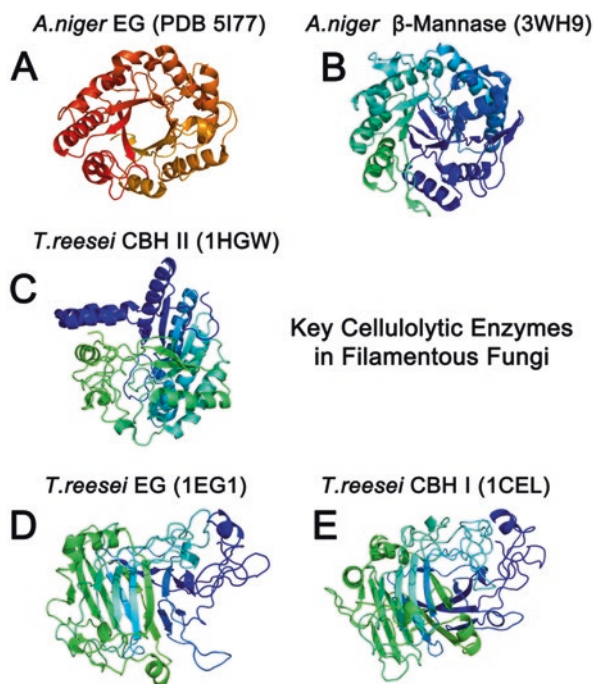


exhibit the specific activities on the substrates of 1,4-glucan, 1,6-galactan, 1,3-mannan, 1,4-xylan, xyloglucan, etc. Based on the scope of this chapter and the widespread body of GH-5 data, we briefly describe the filamentous fungal GH5s with the specific activities of β -1,4-glucanase (EC 3.2.1.4, subfamily-5) and β -1,4-mannanase (EC 3.2.1.78, subfamily-7).

The GH5_5 is the largest subfamily with endo-Glucanase (EG) activities, which are the significant portions of the filamentous fungi's EG action. Especially in *T. reesei*, subfamily-5 EGs represent at nearly 55% of total EG activity (Suominen et al. 1993). Near half of fungal GH5_5 enzymes harbor a family 1 CBM module at the N- or C-terminus, whereas bacterial GH5_5 proteins are not modular ones. Two crystal structures have been solved from *Aspergillus niger* (PDB ID: 5I77) (Fig. 9.1) and *Trichoderma reesei* (3QR3) of filamentous fungi (Wyman et al. 2011). In case of subfamily GH5_7, only β -1,4-mannanase activities were reported in filamentous fungi, and three crystal structures have been determined from *A. niger* (3WH9) (Fig. 9.1), *T. reesei* (1QNO), and *Neurospora sitophila* (4AWE) of filamentous fungi (Goncalves et al. 2012; Sabini et al. 2000).

The filamentous fungal GH5_5 and GH5_7 hydrolases perform their activities by the classical Koshland double-displacement retaining mechanism. The GH5-participant reactions occur with acid/base and nucleophilic assistance with two glutamates within a 5–7 Å distance. Two conserved glutamates were recognized as the catalytic acid/base (in the position of β -strand 4) and nucleophile (β -strand 7). In addition, a water molecule is also required for nucleophilic attack in this mechanism

(Davies et al. 1998). The first step is a covalent intermediate formation between glycosyl and GH5 enzyme (also called a glycosylation step). A proton from the catalytic acid/base glutamate is transferred to the leaving group sugar, a dinitrophenyl group (Davies et al. 1998). The nucleophilic glutamate then attacks the anomeric carbon of the -1 glucopyranose moiety and subsequently binds to the oligomeric substrate. Finally, the nucleophilic water molecule catalyzes the second step of the double-displacement mechanism, deglycosylation. A proton from the nucleophilic water is transferred to the catalytic acid/base, and the remaining hydroxide caps cleaved glycosidic linkage. The nucleophilic glutamate is returned to its original negative charge state, and the enzyme is reset to its original state ready to hydrolyze another glycosidic bond (Liu et al. 2010).

2.2 GH-6 Family

GH6 is a relative smaller identified family with over 1100 accessed protein sequences in GenBank. Due to biochemical data, GH6 hydrolases from filamentous fungi primarily exhibit the cellobiohydrolase activities (EC 3.2.1.91). After hydrolysis by endo glucanases, the reducing and nonreducing ends could be recognized by CBH I and CBH II, respectively. GH6 is currently the only known family which acts from the nonreducing cellulose chain end (CBH II-type action mode). The GH6-CBHII in filamentous fungi is thus the primary component in the biomass degradation cellulose cocktails. The only solved CBH II structure of GH6 from filamentous fungal is the catalytic domain of TrCel6A with a variant of the classical β - α -fold TIM (PDB code: 1HGW) (Fig. 9.1). Slightly different like the classic (β/α)8 barrel, TrCel6A obtains seven parallel β -strands. The first is replaced by an α - α helix insert with twenty residues, and the connection between No. 6 and 7 strands is another unusual α -helix (Rouvinen et al. 1990). Here, the structure and catalytic function of GH6 cellulases from filamentous fungal are summarized based on the data of TrCel6A structure.

According to a series of solved TrCel6A-ligand (or inhibitor) complex structures, the GH6 catalytic mechanism is consented as a Grotthuss mechanism (or called as “water wire”) (Beckham et al. 2014; Koivula et al. 2002). The active site region of TrCel6A is a 20-Å-long tunnel formed by two loops, loops A and B (Fig. 9.1) (Rouvinen et al. 1990), and this tunnel (especially in the part of -1 site) is more spacious when the substrate is absent. Two loose loops in this catalytic tunnel are vital for the first-step binding of an open cellulose chain and surround several water molecules. In subsequent structures, the flexibility of these loops is important as the potential relevance in nucleophilic attack (Zou et al. 1999). The enclosure somewhat restricts the tunnel volume and thus the substrate flexibility. Rouvinen et al. proposed that the tunnel shape is typical for processive attack of cellulose chains and suggested the loops surrounding the active site appear to be ideally positioned to assist in this processive action. In addition to uncovering the fold of GH6, the TrCel6A structure illustrated how the enzyme binds a cello-oligomer

within the tunnel-shaped active site. The new structure showed two carboxylate oxygens belonging to Asp¹⁷⁵ and Asp²²¹ located less than 5 Å from the glycosidic bond between the −1 and +1 subsites (Rouvinen et al. 1990). This location is the cleavage site, and these two residues likely participate in catalysis. Specifically, the authors put forth the hypothesis that Asp²²¹ was protonated, and thus the likely catalytic acid, and that Asp¹⁷⁵ was charged aiding in protonation of Asp²²¹ as necessary. A water molecule in position to attack the anomeric carbon at the cleavage site was also captured.

2.3 GH-7 Family

According to CAZy data, near all the members of GH7 enzymes are discovered in eukaryote. Undoubtedly, GH7 cellulases are the most important enzymes, since they exhibit the primary hydrolytic abilities on the fungal cellulose depolymerization. In *T. reesei*, one processive GH7 CBH I and one GH7 EG are the dominant secreted proteins (Samuels 1996). A large number of works have been applied to figure out the catalytic mode of GH7s, the structural basis of processivity of GH7 CBH I, protein engineering of thermostability and specific activity. Here, we focus on structure and activity studies of GH7 EG and CBH I from filamentous fungi.

The first crystal structure of a GH7 member is the catalytic core of the TrCel7A CBH published in 1994 (PDB code, 1CEL) (Fig. 9.1). This large β-sandwich GH7 enzyme formed by two large antiparallel β-sheets is a complex with inhibitor (o-iodobenzyl-1-thio-β-D-cellobioside). In mechanism, two glutamates have an approximately 5.5 Å apart in the catalytically active conformation (McCarter and Withers 1994). The acid/base offers a proton to the glycosidic oxygen in the initial step and removes a proton from a H₂O molecule that is considered as the next step. In TrCel7A CBH I, Glu²¹² is the nucleophile for the first glycosylation step, and Glu²¹⁷ is the acid/base. In addition, a third acidic residue, Asp²¹⁴ in TrCel7A, was noted to potentially be involved in the chemical steps¹⁷² due to its proximity to the active site and close contact with the putative nucleophile, Glu²¹². TrCel7A is CBH I type with cleavage of the reducing ends of cellulose chain. These cellulases are the key components in fungal enzymatic cocktails (Fig. 9.2).

3 Discovery of Potential Lignocellulosic Cellulolytic Enzymes from Thermophilic Biomass Degraders

Biotransformation of lignocellulosic materials requires the complex and synergistic reactions by a microbial ecosystem (Zhou et al. 2014; Xia et al. 2014). The microbial community is dynamic during the different cellulose degrading periods, and the ecological roles of some specific micro species are also dynamic. Thus,

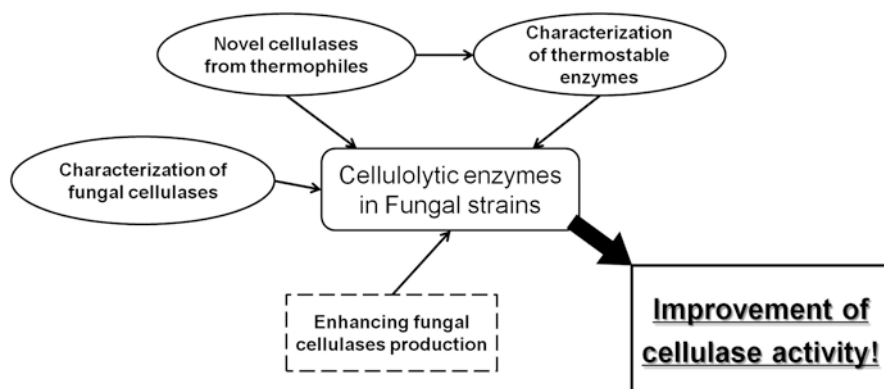


Fig. 9.2 Schematic diagram of application of thermophilic cellulolytic enzymes

understanding the variations of dynamic cellulose-degrading communities by the advantages of next-generation sequencing (NGS) is important. The NGS-based metagenomic analyses of plant biomass-degrading microbial communities have shown unknown and multidimensional genes related to polysaccharide hydrolysis (Warnecke et al. 2007). These cellulolytic enzyme-related genes provide a large enzyme resource for the finding and characterization of novel glycoside hydrolases, especially for thermostable cellulolytic enzymes (Hess et al. 2011). In contrast to metagenomic-based technology, metatranscriptomic sequencings could provide the identification and expression levels of related genes. This information enables a comprehensive understanding of important microbes and DNAs involved in thermophilic bioconversion. Metatranscriptomic-based technology has provided huge numbers of data to analyze the transcription levels of functional genes (Franzosa et al. 2014; Jorth et al. 2014). These brilliant explorations on gene mining enable the evaluation by the biochemical technologies (He et al. 2013). In many cases, the functions of sequenced gene remain ‘putative’. Discovery and characterization on the synergistic effects of thermophilic enzymes cocktails on microbial-community-based biomass degradation is important, since environment provides the auto-optimized multi-species enzyme mixtures under the control of growth environment and substrates. In this section, the recent advances of some (hyper) thermophilic micro-organism and their genome information are discussed (Fig. 9.2).

3.1 Thermophilic Bacterial Biomass Degradors

There are two main thermophilic bacterial biomass degraders, primarily including the species from *Thermotogales* and *Clostridiales*. *Thermotogales* is anaerobic heterotrophic thermophiles, metabolizing the different length saccharides to H_2 , CO_2 , and acetate (White et al. 2017). To now, no less than 31 genomes of the members of

Thermotogales are sequenced and characterized with various research aims (Ben Hania et al. 2015). *Thermotoga maritima* (T_{opt} of 80 °C), one of the most studied specie, has near 7% biomass-degrading related genes in the sequenced genome. *T. maritima* and other *Thermotoga* species could produce various cellulolytic enzymes on the substrates of beta-glucan, hemicelluloses, and pectin (Yu et al. 2016). However, the strains of *Thermotoga* could directly digest crystalline cellulose as carbon source.

Different from the above mentioned organisms, the order *Clostridiales* is much less thermophilic but contains the robust hydrolytic abilities on crystalline cellulose, hemicellulose and pectin. These *Clostridiales* species play vital role in the plant biomass deconstruction. In the members of *Clostridiales*, the species from genus *Caldicellulosiruptor* is the most thermophilic genus (T_{opt} 75~80 °C) (Counts et al. 2017), and *Clostridium thermocellum* (T_{opt} 60 °C) is widely studied (Artzi et al. 2017). Unlike the above species, most strains of *Clostridiales* uptake crystalline cellulose and metabolize the chemicals, such as lactate, ethanol, acetate, H_2 , and CO_2 . In addition, the extracellular cellulolytic enzyme machinery “cellulosome” in *C. thermocellum* shows highest efficiency on cellulose degradation (Bayer et al. 2008). Some anaerobic species produce a large protein machinery with high-molecular mass (also called the cellulosome, firstly found in *C. thermocellum*). Recently, the cellulosome analogues in fungi are also reported (Haitjema et al. 2017). The key protein in this large protein complex is a protein scaffoldin (CipA) with many cohesion modules that interact with catalytic subunits and a carbohydrate-binding modules family 3 (CBM3) that directly interact with cellulose (Ortiz de Ora et al. 2017). The cellulosome binds the cellulose and cell wall at the same time and provides fast and efficient hydrolysis process. Understanding of different catalytic subunits within the cellulosome is not clear (Xu et al. 2016). Different subunits show the significant synergist effects, although the individual proteins have low activity against cellulose. Maybe these subunits lack the CBMs which are important to help catalytic sites to recognize the insoluble substrate. Cellulosomes are firstly found in some anaerobic bacteria and fungi and recently in some aerobic microorganisms, but not from the species of archaea.

Unlike *C. thermocellum*, the strains from *Caldicellulosiruptor* could uptake broader substrates, such as galactose, xylose, glucose, and arabinose, and show good synergist effect with other microorganisms. Till now, 13 genomes of *Caldicellulosiruptor* species are sequenced and characterized (Bing et al. 2015). Primary cellulolytic enzymes from thermophilic *Caldicellulosiruptor* are not part of a cellulosome-like protein complex. The main cellulases from all the members of *Caldicellulosiruptor* are multi-module (Saleh et al. 2017). A well-characterized multi-module in *C. bescii* is CelA (Brunecky et al. 2013; Yi et al. 2013). CelA presents nearly 40% of all secreted proteins in *C. bescii* and contains one family 9 glycoside hydrolase (GH) domain, one family 48 GH, and three family 3 CBMs (Yi et al. 2013). Another multi-modular hydrolase XynF was recently characterized as a novel α -L-arabinofuranosidases. This enzyme plays an important role in hydrolyzing α -1,2-, α -1,3-, or α -1,5-L-arabinofuranosyl glycosidic bonds in arabinose-based polysaccharides (Saleh et al. 2017). Together with a

GH11-family endo-xylanase (XynA), XynF improved the hydrolysis rate of arabinoxylan by a sixfold compared to XynA alone, presumably that XynF could remove the arabinofuranosyl side chains to generate linear xylans. Thus, multi-module enzymes, such as CelA and XynF, in the species of *Caldicellulosiruptor* are thought to be important for its growth on different biomass substrates. Meanwhile, other putative cellulolytic enzymes, such as Csac1080, its GH5 catalytic domain attached to a few of CBMs. In case of Csac0678, its catalytic domain is attached to one CBM and three SHL repeats (VanFossen et al. 2011). In addition, two endo-xylanases, GH11-family XynA and GH10-family XynB from *Caldicellulosiruptor* sp. F32, were systematically studied (Meng et al. 2015). Interestingly, deletion of a CBM in GH11 XynA showed a higher specific activity (215.8 U/nmol of XynA-TM1 versus 94.7 U/nmol of wild-type XynA) and a stronger thermostability (half-life of 48 h versus 5.5 h at 75 °C) compared with those of XynA. However, the truncated mutants of XynB without CBMs exhibited weaker enzyme activities and thermostabilities than its wild type. By protein structure modeling and cross-linking chemical modification, there are interdomain interactions observed in XynB whereas no interdomain interactions in XynA.

In contrast to multi-modular cellulases in *Caldicellulosiruptor* species, several cellulases with single-domain also play the important role in lignocellulosic biomass deconstruction. A novel thermostable lichenase F32EG5 belonging to GH5 was identified from *Caldicellulosiruptor* sp. F32. The enzyme F32EG5 is a cellulose and lichenin-degrading enzyme and exhibited a high activity on β -1,3-1,4-glucan but slight activity on cellulose (Meng et al. 2017). F32EG5 digested the β -1,3- or -1,4 linkage when a 4-*O*-substituted glucose residue connected to a glucose residue by a β -1,3 linkage. The recognition sites of F32EG5 are completely different from GH16 lichenase which strictly hydrolyzed the β -1,4-glycosidic bond adjacent to a 3-*O*-substituted glucose residue in β -glucans. The crystal structure of F32EG5 was determined to 2.8 Å resolution and the crystal structure of the complex of F32EG5 E188Q mutant and cellotetraose was determined to 1.7 Å resolution, which revealed that the exit subsites of substrate binding sites contribute to both thermostability and substrate specificity of F32EG5. The sugar chain showed a sharp bend in the complex structure, suggesting that a substrate cleft fitting to the bent sugar chains in lichenin is a common feature of GH5 lichenases.

3.2 Hyperthermophilic Archaeal Biomass Degrader

In hyperthermophilic species of archaea, only 35 genera could uptake boarder carbon and energy sources. Most of thermophilic biomass-degrading archaeal degraders belong to *Thermococcales* (Marguet et al. 2013). These heterotrophic lives are isolated from hydrothermally heated sea and suffer the temperature above 100 °C. Till now, 26 genomes of the members of *Thermococcales* species are sequenced (Jun et al. 2011) and show a variety of amylases, glucosidases, and glucanases (Cheng et al. 2017). Another archaeal biomass degraders, *Sulfolobales*, are

isolated from acidic thermal pools (Sakai and Kurosawa 2017). Up to now, 19 genomes of *Sulfolobus* species are sequenced (Urbiet al. 2017). Some works reported that a xylanolytic enzyme and a bifunctional xylosidase/arabinosidase hydrolase were isolated from the original hosts of *Sulfolobus solfataricus* (Kambourova et al. 2007; Morana et al. 2007). Most of above mentioned thermophilic archaea have activities on the alpha- or beta-linked polysaccharides. However, the activities are relative low than other cellulolytic enzyme from terricolous microorganisms. Corresponding to the novel ecological niches of *Thermococcales* and *Sulfolobus*, the cellulolytic enzymes produced by both archaea did not show the hydrolytic activities on crystalline cellulose. Till now, a hyperthermophilic archaeon, *Desulfurococcus fermentans*, exhibited a fast growth on filter paper at an thermophilic environment (81 °C) (Perevalova et al. 2005). At acidic pH and high temperatures, strains from *S. solfataricus* could hydrolyze the corn stover with glucose and xylose as hydrolysis products (Maurelli et al. 2008). A beta-1,4-glucanase SSO1354 protein from *S. solfataricus* was thermoacidophilic, with a pH^{opt} of 4.0 and a $T_{\text{m}}^{\text{opt}}$ of 90 °C and half-life of 180 min at 80 °C (Girfoglio et al. 2012). The enzyme showed hydrolytic activities on different substrate such as lichenan, debranched arabinan, carboxymethyl (CM)-cellulose, oat spelt xylan, Avicel, and amorphous-cellulose. SSO1354 exhibited a much lower activity toward intact cellulose, probably because of the lack of a CBM in the sequence, whose activity increased five to ten times after the substrates were acid pretreated. Its thermo/acidic-resistant and high activities on hydrolyzing cellulosic and hemicellulosic substrates confer SSO1354 as an ideal candidate in bio-based industry.

Unlike bacteria and fungus, relatively few *Archaea* are discovered to deconstruct lignocellulose. The first report of *Archaea* that has ability to deconstruct lignocellulose was a three-archaea mixture isolated from a continental geothermal source (Graham et al. 2011). This strictly anaerobic consortium was able to grow on celluloses including filter paper, CMC-Na, and Avicel, at 90 °C. Their 16S rRNA analysis showed similar sequences with the characterized organisms *Ignisphaera aggregans* DSM 17230 (95%), *Pyrobaculum islandicum* DSM 4184 (98%), and *Thermofilum pendens* Hrk (93%). Further metagenomic studies revealed a large number of GHs, four of which are potential GH family 5 endoglucanases by gene annotation. A multidomain cellulase EBI-244 putatively belonging to GH5 family was further characterized (Graham et al. 2011). Thus, the multidomain cellulases was first found in hyperthermophilic archaea. Sequence analysis of EBI-244 unraveled four structural domains, with catalytic module as domain 2 flanked by structural function unknown domains on either side. The recombinant enzyme of EBI-244 showed extremely high thermostability, $T_{\text{m}}^{\text{opt}}$ above water boiling point and a half-life of 5 h at 100 °C. EBI-244 exhibited activity toward a range of soluble and insoluble cellulose containing β -1,4-linked glucans, such as filter paper, CMC-Na, and Avicel.

4 Thermophilic Adaptation of Cellulolytic Enzymes

Application of next-generation sequencing technology on thermophilic environmental samples offers large genomic information and a potential gene source for the application of thermophilic cellulolytic enzyme. Isolation of thermophilic biomass degraders and characterization of several important enzymes are discussed in the above section. Here, we will focus on the characterization and engineering process of cellulolytic enzymes to identify enzymatic parameters and improve the performance during enzymatic hydrolysis (Fig. 9.2). However, it is difficult to compare the different cellulolytic enzymes because of various experimental conditions including temperature, substrates, and experimental instruments.

The cellulase cocktails from *T. fusca* (T_m^{opt} 55 °C) primarily consist of four endo-acting, two exo-acting, and one processive cellulase. The T_m^{opt} s for these enzymes are among 50–77 °C. While many thermophiles have the beta-1,4-glucanase activities on soluble cellulose, such as the species of *Thermotoga* and *Dictyoglomus*. However, the hydrolytic activities on crystalline cellulose are very low. A beta-glucanase (E1) isolated from another thermophile has a similar catalytic mechanism with other GH5 hydrolases. Another case for cost-efficient saccharification by thermostable enzyme has been reported recently (Zhang et al. 2017). An engineered *Clostridium thermocellum* by the supplementation of a beta-glucosidase from *Caldicellulosiruptor* sp. F32 improved the relative saccharification level by over twofolds higher activity than its parent strain by a two-stage saccharification work on the substrate of 100 g/L Avicel.

To hemicellulases, thermophilic GH families 10 and 11 xylanases, GH family 43 xylosidases are widely studied from some species of *Caldicellulosiruptor*, *A. cellulolyticus*, and *T. fusca*. XynA with GH11 catalytic module from *Caldicellulosiruptor* sp. F32 had a T_m^{opt} of 80 °C, and showed the similar specific activity with *T. maritima* GH11 family xylanases (Barabote et al. 2010). Surprisingly, a CBM-deletion mutant of *Caldicellulosiruptor* XynA has an approximately 3.5-fold higher specific activity than its full-length one on the natural substrate of beechwood xylan. This XynA-deletion mutant also has higher expression levels in *E. coli* and *P. pastoris* and thermostabilities than its full-length proteins (Unpublished data). In another case, a novel GH16-participant catalytic domain (Lam16A-GH) within a multi-modular laminarinase/lichenase from *Caldicellulosiruptor* sp. F32 was heterologously expressed in *E. coli*. Lam16A-GH showed a specific hydrolytic activities and strong thermostability on β -1,3-glucan. Lam16A-GH showed an endo-type hydrolysis on the β -1,4-glycosidic bonds with a 3-*O*-substituted glucosyl residue in β -1,3–1,4-glucans. Recently, two GH39 β -xylosidases from *Caldicellulosiruptor* sp. F32 were studied by our group with the distinct modes of action and biochemical characters on the substrates of xylo-oligosaccharides and natural products (e.g. notoginsenoside R1) (unpublished data). Intriguingly, GH39A showed an excellent tolerance to various organic solvents. Mixing the organic solvent, such as methanol,

ethanol, acetone, etc., obviously improved the specific activity, thermostability, and catalytic efficiency. Immobilization of Xyl39A by macroporous cross-linked polystyrene further increase its specific activities on natural products. Thus, characterization of these novel enzymes provides a great source for improving the fungal cellulolytic enzymes performance.

5 Design for More Thermostable Cellulases

5.1 Direct Evolution of Fungal Cellulases

The first aim of protein engineering of cellulases is to increase the thermostability for improving the production of fuels and chemicals. Fungal cellulases attract exclusive interestings on protein engineering because of their high activity and high expression levels in fungal hosts (Heinzelman et al. 2009). The thermostable fungal CBH II chimeras were the first step in building an inventory of stable fungal cellulases (Heinzelman et al. 2009). Heinzelman et al. applied SCHEMA (a structure-guided protein recombination route) on three fungal CBH-IIs and engineered a series of highly thermostable CBH-II variants. The engineered CBH-II variants showed hydrolytic activities on chemical-treated cellulose with higher T_m^{opt} s (7–15 °C), more hydrolysis products, and broader pH/activity profiles than their parent enzymes.

This recombination-based method (SCHEMA) was also used to generate active, thermostable cellobiohydrolase class I (CBH-I) enzymes (Heinzelman et al. 2010). The thermostable CBH-I variants could digest Avicel at 70 °C, which is higher T_m^{opt} than its CBH-I parent (T_m^{opt} <60 °C). A combination of chosen mutations yield a highly thermostable Cel7A with T_m^{opt} of 72.1 °C with a 9.2 °C higher than that of native *T. emersonii* CBH-I. Comparison with native CBH-I, an increased optimal temperature of 65 °C for activity, and a 50% increase in total sugar production from crystalline cellulose were demonstrated,

Based on random mutagenesis-routed direct-evolution and biochemical characterization, a fungal Cel6A from *Hypocrea jecorina* was further thermostablized (Wu and Arnold 2013). Among its mutants, half-life of 3C6P at 75 °C is 280 min and a T_m^{opt} of 80 °C, which was 15 °C higher than the native protein. It is more significant for long-time hydrolysis due to its strong thermostability. After a 3-day Avicel hydrolysis, the mutant 3C6P released ~2.4 times more cellobiose at T_m^{opt} of 75 °C than the wild-type HjCel6A of T_m^{opt} of 60 °C. The total hydrolysis products of 3C6P mutant at 75 °C after 6 h was equivalent to that released by wild-type HjCel6A at 60 °C after 60 h, a tenfold reduction in hydrolysis time. According to structural analysis of HjCel6A and its mutant 3C6P, a typical (β/α)₇ barrel-fold family 6 glycoside hydrolase, and five mutant sites in 3C6P are found on the surface of HjCel6A.

Cel7A (CBH-I) and Cel6A (CBH-II type) respectively target the reducing and non reducing ends of cellulose (Igarashi et al. 2011). The mutants of Cel6A and Cel7A could synergistically digest Avicel and release proximately two times more cellobiose than the wild-type mixture at their T_m^{opt} (Wu and Arnold 2013). For complementing the thermostabilized Cel6A and Cel7A cocktail, a thermostable derivative of *Hypocrea jecorina* Cel5A was generated by combining stabilizing mutations, chimera studies, and structure-based computation (Trudeau et al. 2014).

5.2 Cellulose Deconstruction by Cellulosome-Mediated Design

In case of an artificial cellulosome designer, the stabilities and hydrolytic activities of each subunit were improved by protein engineering. Exo-type glucanase Cel48S and endo-type glucanase are vital components in *Clostridium thermocellum* cellulosome. Using random error-prone PCR an enhanced thermostable Cel8A was generated with a combination of three beneficial mutations (Anbar et al. 2010). On the basis of bioinformatics approach, protein sequence alignment of family 8 glycoside hydrolases was applied to set a mutation data. One benefit was introduced into a triple mutant of Cel8A with increased the half-life (up to 14-fold at 85 °C) (Anbar et al. 2012). From an inventory of Cel48S chimeras obtained by structure-guided site-directed protein recombination, an optimized mutant Cel48S* was chosen, which was as stable as wild type but showed higher activity on Avicel (Morais et al. 2016). To ensure the optimal condition of a hydrolase, error-prone PCR technology was applied in a β -glucosidase to assist the two cellulases in the deconstruction of Avicel. A scaffoldin composed of thermostable modules from three thermophilic species, such as *C. thermocellum*, *C. clariflavum*, and *A. fulgidus*.

The assembled trivalent thermostable designer cellulosome complexes showed the half-lives of 4 h at 65 °C and 3 h at 70 °C, which were only stable at temperature <60 °C. The three thermostable enzymes in complex with mesophilic scaffoldin, however, were even less stable than the conventional cellulosomes, indicating the critical roles of the thermophilic scaffoldin. The thermostable designer cellulosomes showed efficient cellulolysis ability with a significant enhancement of 1.7-fold compared to the conventional designer cellulosomes.

5.3 Mechanisms Responsible for Cellulase Thermostability

Bacterial and thermophilic cellulases from *Bacillus* and *Geobacillus* are important in the biofuel industry. A cellulase GsCelA from *Geobacillus* sp. 70PC53 is more thermostable than BsCel5A from *Bacillus* (Chang et al. 2016). Using SCHEMA recombination method, a series of mutants of GsCelA and BsCel5A was generated. Thermostability and biochemical assays showed one of the blocks was revealed that

might offer the higher thermostability of GsCel1A. On the basis of structural analysis and mutagenesis, a 3¹⁰ helix in the block stabilized the substrate binding loop which could increase the range of cellulase activity to higher temperatures.

6 Conclusion and Perspective

In this chapter, several key cellulolytic enzymes with GH families 5, 6, and 7 from filamentous fungi are briefly discussed. To find a more thermostable alternative one, some thermophiles are listed, and many important and industrial-potential cellulases from these genomes are discussed. These novel microorganisms and enzymes provide a large number of enzymatic sources for changing the formula of fungal enzymatic cocktails in next step.

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Chapter 10

Optimization of Cellulolytic Enzyme Systems for Lignocellulose Hydrolysis



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Abstract The use of cellulase to degrade cellulose during the production of fuel ethanol is of crucial importance for solving energy and environmental problems and has attracted the attention of scientists from all over the world since the 1960s. However, the composition of the cellulase system is complex and key problems remain, such as low degradation efficiency, high production costs, and a still unclear catalytic mechanism, which seriously hinder the use of cellulase. In this chapter, the source and composition of cellulase enzymes and the mechanism of cellulose degradation are discussed. The concept of the minimal or “core” cellulase is introduced, and the engineering of the cellulase system is discussed.

Keywords Cellulolytic enzyme systems · Cellulase diversity · Molecular modification

1 Introduction

Cellulose is the most abundant, renewable, and underutilized resource on Earth. Enzymatic cellulose degradation has recently received substantial attention because cellulose is a seemingly inexhaustible feedstock for the production of biofuels and chemicals. However, cellulolytic enzyme systems from filamentous fungi, such as *Hypocrea jecorina*, *Neurospora crassa*, and *Penicillium oxalicum*, may be optimized to improve enzymatic hydrolysis efficiency by combining specific enzymes from various organisms. In this chapter, recent progress in understanding the enzymatic degradation mechanism, the minimal or “core” enzymes, and optimized cocktails is reviewed, and the future development of cellulolytic enzymes is discussed.

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2 The Enzymatic Degradation Mechanism and Cellulase Diversity

2.1 Enzymatic Degradation Mechanism

Cellulase is a general term for enzymes that degrade cellulose and produce glucose. The degradation mechanisms of cellulases secreted by filamentous fungi include the hydrolytic and the oxidative degradation pathways.

Lignocellulosic biomass is hydrolyzed into fermentable sugar by the synergistic effect of these enzymes, including three major classes of enzymes. First, endo- β -1,4-glucanase (EG), also known as 1,4- β -D-glucan-4-glucan hydrolase (EC 3.2.1.4), randomly hydrolyzes β -glycosidic bonds, resulting in an increase of reducing ends. Second, cellobiohydrolase (CBH), also known as exoglucanase or exogenous glucosidase (exo- β -1, 4-glucanase, EC 3.2.1.91), acts on glycosidic chains from the reducing or non reducing ends, giving rise to cellobiose. Finally, β -glucosidase (BG), also known as cellobiase (cellobiase, CB, EC 3.2.1.21), hydrolyzes β -1, 4-glycosidic bonds in cellobiose, releasing glucose monomers (Eibinger et al. 2014; Harris et al. 2010). In Fig. 10.1, a schematic diagram of the enzymatic hydrolysis of cellulose catalyzed by a fungal cellulase system is shown.

Studies on the three-dimensional structure of cellulase proved that the active site of CBH is in a pocket formed by the coverage of a loop, allowing only the end of the cellulose to gradually enter and be hydrolyzed. Igarashi et al. (2009) found that the average sliding movement velocity (3.5 nm/s) of the catalytic domain of *H. jecorina* Cel7A (without the cellulose-binding domain) on crystalline cellulose was similar to that of the intact TrCel7A enzyme, using high-speed atomic force microscopy. However, *H. jecorina* Cel7A variants, which have no enzyme activity or lack

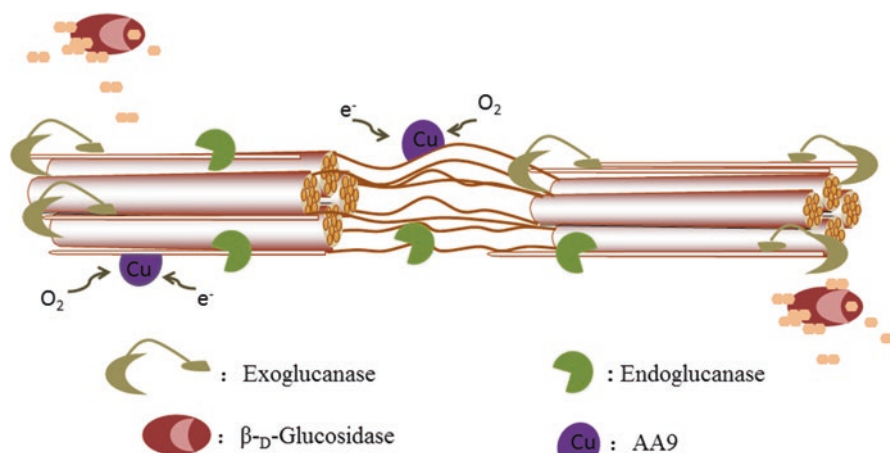


Fig. 10.1 Schematic diagram of the enzymatic hydrolysis of cellulose catalyzed by a fungal cellulase system

a tryptophan at the entrance of the active site tunnel (mutant W40A), could not slide on crystalline cellulose. Therefore, the loading of a cellulose chain into the active site tunnel is essential for enzyme movement, in addition to the hydrolysis of glycosidic bonds. The active site of EG is a large groove, which allows the entire chain of cellulose (more than 1000 basic units) to enter. However, EG cannot break crystallization cellulose that is stiffened by both intrachain and interchain hydrogen bonds. Adjacent sheets overlay one another and are held together (Lee and Paul 2002). Lee and Paul (2002) reported that the hydrolysis mechanism includes both a retention mechanism and an inversion mechanism.

Recently, the enzymes that degrade cellulose through the oxidation mechanism are classified as copper-dependent lytic polysaccharide monooxygenases (LPMOs). LPMOs comprise four families of carbohydrate reactive enzymes: auxiliary activity (AA) 9, AA10, AA11, and AA13. Among them, AA9 and AA10 are related to the degradation of cellulose. The AA9 family includes eukaryotic LPMOs, whereas the AA10 family is bacterial. Unlike hydrolases, AA9 catalyzes the cleavage of glycosidic linkages of cellulose, cello-oligosaccharides, or xyloglucans by oxidation, using O_2 or H_2O_2 as a co-substrate to produce shorter oligosaccharides or monosaccharides and aldehydes or ketones, with which the terminal hydroxyl groups are oxidized (Bissaro et al. 2017; Eibinger et al. 2014; Langston et al. 2011; Moses et al. 2016).

In 2001, Karlsson et al. (2001) first reported that GH61 from *H. jecorina* had a weak endoglucanase activity, and it was subsequently reported that GH61 exists in *Aspergillus kawachii*, *Thielavia terrestris*, and *Thermoascus aurantiacus* (Harris et al. 2010; Koseki et al. 2008). It was found that GH61 has a synergistic effect with cellulase, and Harris et al. (2010) described GH61 as a cellulase boosting enzyme. Synergy between CDH and AA9 was reported in 2011 (Phillips et al. 2011). Furthermore, the copper oxidation mechanism of GH61 from *N. crassa* was suggested (Leggio et al. 2012). The three-dimensional structure of AA9 was analyzed and includes a flat active center that makes interaction with crystalline portions of cellulose possible; metal dependence, especially upon copper, to be catalytically active; a copper ion accommodated in a structure called a histidine brace; conserved histidines found at the C- and N-terminal ends; and dependence on an external electron donor to reduce the copper ion. A common feature of the AA9 domain is the lack of a substrate-binding pocket. The absence of a classical binding site also led to the reclassification of these enzymes as providing auxiliary activity, as opposed to the primary glycoside hydrolases (GHs) (Corrêa et al. 2016; Hemsworth et al. 2013; Karkehabadi et al. 2008; Phillips et al. 2011; Vaaje-Kolstad et al. 2005, 2010).

The AA9 family is categorized into three types based on specificity for cellulose oxidation: Type 1 predominantly oxidizes C-1, Type 2 predominantly oxidizes C-4, and Type 3 oxidizes both C-1 and C-4. Possible products of C1 oxidation by AA9 family members can be aldonic acid ($M + 16$, where M indicates the mass of native oligosaccharides), a sodium adduct of aldonic acid ($M + 16 - H + Na$), and 1,5 δ -lactone ($M - 2$). On the other hand, 4-ketoaldose ($M - 2$) and gemdiol ($M + 16$) are generated by C4 oxidation. In the case of C1 and C4 oxidation, all of the above-listed sugars and doubly oxidized sugars containing both aldonic acid and gemdiol

(M + 32), both 1, 5 δ -lactone and gemdiol (M + 14), or both aldonic acid and 4-ketoaldose (M + 14) can be observed (Kim et al. 2017; Moses et al. 2016).

Notably, about 20% of AA9 sequences were estimated to be associated with C-terminal carbohydrate-binding module CBM1, which has no catalytic activity (Leggio et al. 2012). The CBM in AA9 family members plays an important role in binding to cellulose. Fang and colleagues at Shandong University proposed that the titer of AA9 has a relationship with the degree of polymerization (DP) of the products since products with a lower DP are more easily degraded by β -glucosidase. Thus, AA9 family members producing products with a lower DP have a better synergistic effect with cellulase (unpublished data). In addition, many studies have found that some proteins, which have a non-hydrolytic disruptive activity, can loosen and disrupt the crystalline structures of cellulose, rendering lignocellulosic biomass more accessible to hydrolytic enzymes (Kim et al. 2009; Wang et al. 2014).

In 2002, Saloheimo et al. (2002) reported that a new protein, called “swollenin” in *H. jecorina*, contains sequences similar to plant expansins, which loosen and disrupt polysaccharides (such as cellulose and hemicelluloses) in plant cell walls. This protein also exhibited disruptive activity against cellulosic materials. It was reported that swollenin destroyed the structure of cotton fibers and *Valonia* cell walls but produced no detectable reducing sugars. This swollenin consists of three domains: (1) a cellulose-binding domain at the N-terminus that binds to cellulose, (2) a highly conserved domain of pollen allergens at the C-terminus that swells cellulose similar to expansin, and (3) a region similar to mammalian fibronectin type III repeats. Chen et al. (2010) obtained the *swol* gene encoding swollenin of *Aspergillus fumigatus* and named it *Afswol*. *Afswol* consists of a CBM1 domain and a swollenin domain. *Afswol* exhibits weak endoglucanase activity against carboxymethylcellulose (CMC), binding to both crystalline cellulose and chitin but not to xylan. Subsequently, swollenins were found from *P. oxalicum*, *Kluyveromyces lactis*, *Phanerochaete carnosae*, and others (Jäger et al. 2011; Kang et al. 2013; Santos et al. 2017; Suzuki et al. 2014). There is synergism between cellulase and swollenin during cellulose enzymatic degradation (Verma et al. 2013). Georgelis et al. (2015) suggested that the function of the expansin-like proteins from filamentous fungi greatly differed from those of expansin and cellulase. Therefore, it was proposed that the expansin-like proteins from filamentous fungi should be named “loosenin” instead of “swollenin.”

2.2 The Minimal or “Core” Cellulase

Cellulases are known as a complex enzyme system. Their multiple activities work cooperatively or synergistically to efficiently degrade lignocellulose. The “core” cellulase was proposed by Baker et al. (1998). Three categories of cellulolytic enzymes were required for the degradation of cellulose, one endoglucanase, one exoglucanase (R) specific for the reducing cellulose terminus, and one exoglucanase (NR) specific for the non reducing cellulose terminus, as shown in Table 10.1.

Table 10.1 The minimal or “core” cellulase

Enzymes	Exoglucanase		Endoglucanase				<i>Thermobifida fusca</i> E3	<i>H. jecorina</i> EGI	<i>Acrononium cellulolyticus</i> EI	<i>Thermobifida fusca</i> E5	<i>Microbispora bisporea</i> endo A
	<i>H. jecorina</i> CBH I	<i>H. jecorina</i> CBH II	<i>H. jecorina</i> CBH II	<i>Thermobifida fusca</i> E3	<i>H. jecorina</i> EGI	<i>Acrononium cellulolyticus</i> EI					
Glycosyl hydrolase family	7	6	6	6	7	5	5	6	5	5	6
Cellulose terminal specificity	Reducing	Non reducing	Non reducing	Non reducing	None	None	None	None	None	None	None
Product stereo- chemistry	Retaining	Inverting	Inverting	Inverting	Retaining	Retaining	Retaining	Retaining	Retaining	Retaining	Inverting
Thermal tolerant	No	No	No	Moderately	No	Yes	Yes	Moderately	Moderately	Yes	Yes

Talaromyces cellulolyticus is a promising cellulase-producing fungus for efficient biomass hydrolysis. Inoue et al. (2014) purified six cellulolytic enzymes from *T. cellulolyticus* culture supernatants. The enzymes were critical for the hydrolysis of crystalline cellulose using an enzyme assay based on synergistic hydrolysis of Avicel. Furthermore, these purified enzymes were classified into the following glycosyl hydrolase (GH) families: GH3 (β -glucosidase, Bgl3A), GH5 (endoglucanase, Cel5A), GH6 (cellobiohydrolase II, Cel6A), GH7 (cellobiohydrolase I and endoglucanase, Cel7A and Cel7B, respectively), and GH10 (xylanase, Xyl10A). It was shown that Cel5A, Cel6A, Cel7A, Xyl10A, and Bgl3A play roles as core enzymes in the hydrolysis of acid-pretreated corn stover (PCS) glucan. Therefore, we propose that the core enzyme is not universal and that the core enzymes for hydrolyzing different substrates vary.

2.3 Cellulase Diversity

In nature, many molds and bacteria synthesize cellulase. The enzyme complexes produced by bacteria are named “cellulosomes,” have generally neutral pH optimum, and exist on the cell membrane, making it difficult to collect cell-free enzyme preparations by filtration and centrifugation. The cellulases produced by filamentous fungi generally hydrolyze cellulose substrates under acidic or neutral acidic conditions. Fungal cellulases, which are usually extracellular enzymes, are secreted into the medium, and cell-free enzyme preparations can be obtained easily by filtration and centrifugation (Joglekar et al. 1983; Robert et al. 1984).

The classical enzymatic molecular structure of CBHI (EC 3.2.1.91) from *H. jecorina* consists of three components: a globular catalytic domain (CD), with an active site located deep inside a tunnel; a smaller CBM that serves to bind the enzyme on the surface of the substrate; and a highly glycosylated, flexible linker peptide that tethers the two globular domains together (Tavagnacco et al. 2011).

Many research results have shown that the linker and cellulose-binding domain (CBD) of cellulases produced by fungi significantly differ from those produced by bacteria. The fungal CBD consists of 33–36 amino acids with high homology, whereas, in bacteria, it consists of 100–110 amino acids with low homology. The shape of the CBD in CBHI is a “wedge,” consisting of a hydrophilic side and a hydrophobic side. Only three aromatic amino acids are contained in it, which are located on the flat hydrophilic surface to perform the function of cellulose adsorption. Bacterial exoglucanase has a large CBD and contains many aromatic amino acids. Trp54 and Trp72 are exposed on the surface of the protein to perform the adsorption function. The linker of the bacterial cellulase is rich in Pro and Thr and consists entirely of repetitive sequences, such as Pro-Thr, while the linker of fungal cellulase is rich in Gly, Ser, and Thr. The degree of glycosylation and the specific sugar chains of different cellulases vary but glycosylation is not necessary for cellulase activity. From the structure of cellulase, the angle between the bacterial cellulase CD and CBD is 135°, while the angle between the fungal cellulase CD and

CBD is 180° (Kraulis et al. 1989; Xu et al. 1995). In addition to the amino acid sequence differences, there are also differences in the structure between fungal and bacterial cellulases. The catalytic domain of CBHI from *H. jecorina* contains a tunnel and loops, and the CD alone was shown to be capable of sliding on crystalline cellulose by AFM studies. In contrast, the catalytic domain of *Clostridium thermocellum* CelA contains a (alpha/alpha) six-barrel structure (Alzari et al. 1996; Bodenheimer and Meilleur 2016).

In general, full-value cellulase systems produced by fungi often contain three components: a cellobiohydrolase, an endoglucanase, and a β -glucosidase. Some filamentous fungi are capable of producing a full-value cellulase system, such as *H. jecorina*, *P. oxalicum*, and *T. cellulolyticus* (formerly *Acremonium cellulolyticus*). Specific filamentous fungi are capable of producing only a low-value cellulase system, such as *Poria placenta*, *Poria carbonica*, and others that often produce only endoglucanases. At present, fungi used in the industrial production of cellulase are those producing a full-value cellulase system.

2.4 The Polymorphism of Cellulase

The contents, proportions of cellulolytic enzyme, and activities of endoglucanase and exoglucanase vary among filamentous fungi. In each type of cellulase, different cellulase systems tend to have multiple components that differ in structure, function, and activity, and each component consists of different subcomponents, highlighting the polymorphic nature of cellulases. The same microorganism can also produce a significantly different composition of cellulases due to different regional sources and different culture conditions.

The structural forms of cellulases are polymorphic. On one hand, the extracellular cellulases produced by fungi tend to be monomeric protein molecules, but complexes are also found. In anaerobic fungi and bacteria, cellulases are often found as complexes. On the other hand, extracellular cellulases produced by fungi tend to be glycoproteins. Different cellulase components exhibit a variety of forms due to differences in the amino acids which are glycosylated, the degree of sugar chain polymerization, or the degree of branching (Wood et al. 1988).

Cellulase physicochemical properties are also polymorphic. Different cellulase components have different molecular weights, isoelectric points, and enzymatic activities. These differences are particularly pronounced in endoglucanases.

The molecular structure and function of the enzymes are polymorphic. From the analyses of amino acid sequences and the three-dimensional structures, it has been observed that the active site of CBH (including CBH I and CBH II) is completely different from EG in topology. The active site of an exoglucanase is located in a tunnel formed by a long loop structure, which limits accessibility of the substrate, while the active site of an endonuclease is located in a cleft and the substrate is fully accessible (Davies 1995).

Finally, the catalytic mechanisms are also polymorphic. The hydrolysis mechanism is different for different cellulases, especially with regard to the catalytic mechanism and stereochemistry, which is also a manifestation of polymorphism among components (McCarter and Withers 1997).

3 Molecular Modification of Cellulases

Directed evolution is a general method for modification of cellulases. In this method, random mutagenesis may be accomplished using error-prone PCR or gene shuffling. The improved variants are screened using a high-throughput screening strategy. Examples of modifications of cellulase using directed evolution techniques are shown in Table 10.2.

Lin et al. (2016) reported that approximately 10,000 variants for endoglucanase Cel8M from *E. coli* were obtained using error-prone PCR. Two variants, Cel8ME15 and Cel8ME18, were obtained using a high-throughput screening system, with their activities increased 1.42-fold and 1.61-fold, respectively. Lee et al. (2010) improved

Table 10.2 Directed evolution of cellulase

Cellulase	Source	Directed evolution technology	Content and purpose of research	References
Endoglucanase	Cel8M <i>Escherichia coli</i>	eqPCR	Activity improved	Lin et al. (2016)
Endoglucanase	Cel12A <i>H. jecorina</i>	DNA shuffling	Activity improved	Yenenler and Sezerman (2016)
Endoglucanase	CelA <i>C. thermocellum</i>	Family shuffling	Activity and thermal stability improved	Yi et al. (2010)
Endoglucanase	EngD <i>Clostridium cellulovorans</i>	Family shuffling	Activity and thermal stability improved	Lee et al. (2010)
Exoglucanase	Cellobiohydrolase (HJPlus) Chimeric fungi	epPCR and DNA shuffling	Activity and thermal stability improved	Wu et al. (2013)
β -D-glucosidase	BglA <i>Fervidobacterium</i> sp.	epPCR	Thermal stability improved	Lima et al. (2009)
β -D-glucosidase	1,4- β -D-glucan glucosidase A <i>Thermotoga neapolitana</i>	epPCR	Activity improved	McCarthy et al. (2004)
β -D-glucosidase	CelB <i>Pyrococcus furiosus</i> and LacS <i>Sulfolobus solfataricus</i>	Family shuffling	Activity and thermal stability improved	Kaper et al. (2002)
β -D-glucosidase	BglB <i>Paenibacillus polymyxa</i>	eqPCR, DNA shuffling	Activity and thermal stability improved	Arrizubieta and Polaina (2000)

the thermal stability and catalytic activity of endoglucanase (EngD) from mesophilic *C. cellulovorans* by evolutionary molecular engineering. Thermostable mutants were isolated after a staggered extension process (StEP) to conduct family shuffling, and then the mutant library was screened. The relative activity of the best-evolved clone was about two times higher at 50 °C and showed a higher k_{cat}/K_M value than its parental clone.

In recent years, rational design strategies have been widely used to study the relationship between specific amino acid residues in the cellulase molecule and their function, as well as to direct modification of the cellulase molecule. To date, protein engineering has been carried out on cellulase components belonging to glycoside hydrolase (GH) families GH5, GH6, GH7, GH8, GH9, and GH45, and remarkable progress has been made (Kurniasih et al. 2014; Liu et al. 2014; Puseenam et al. 2015; Wang et al. 2017; Wu et al. 2013).

Ruiz et al. (2016) investigated how the inter-domain region in GH5A from *Bacillus subtilis* affects structure and function. They suggested that flexibility or rigidity in the linker region has a great effect on CMCase activity. When the linker of *BsCel5A* was changed, catalytic efficiency decreased. These results proved that the linker of endoglucanase has a significant role in recognizing and binding substrates. Based on homology modeling and rational design, Zhang et al. (2015) modified a hyperthermostable β -1,4-endoglucanase Cel12B from *Thermotoga maritima* by site-directed mutagenesis. As a result, the activity and thermostability of the two variants were significantly improved, compared with the wild-type *TmCel2B*. The k_i value of the variant Y245G from *A. cellulolyticus* endocellulase Cel5A for the product inhibition of the enzyme increased more than 1480% and, then, the decrease in product inhibition increased released soluble sugars from cellulose, compared with the amounts released by the wild type (Baker et al. 2005). Thus, site-directed mutagenesis is a good method for studying the thermostable mechanisms of thermophilic enzymes and for improving enzyme activity.

Voutilainen et al. (2010) reported that the thermal stability and activity of Cel7A from the thermophilic fungus *Talaromyces emersonii* (*TeCel7A*) was improved by introducing additional disulfide bridges to the catalytic module. The best variant could hydrolyze microcrystalline cellulose at 80 °C. Therefore, the thermal stability of exoglucanase could be improved by increasing the number of disulfide bonds.

Though there are many successful examples of molecular modification of cellulases, there are still no universal rules about the best site-directed mutagenesis strategies to increase cellulase activity on solid cellulose substrates. It is difficult to construct computational models due to the complex heterogeneous nature of a process involving insoluble cellulose cleaved by cellulases.

4 Prospects

Recently, many properties of enzymes, such as activity, thermal stability (Tang et al. 2018; Zhao et al. 2011), stereoselectivity (Reetz 2004), and optimum pH (Ma et al. 2016), have been improved by combining directed evolution with rational design. For molecular biologists, the combination will make it easier to design and modify enzymes (or proteins), making protein engineering even more powerful and attractive. Now, scientists are attempting de novo design of proteins (Chino et al. 2017; Dang et al. 2017; Shepherd et al. 2017), and we believe that we will be able to completely de novo design cellulases with the aid of artificial intelligence. In that case, even more efficient, stable, and useful cellulolytic enzymes will be obtained in the future.

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Chapter 11

Expression of Cellulolytic Enzymes in Yeast



Dongmei Wang and Jiong Hong

Abstract Lignocellulose is the most abundantly sustainable raw material on the earth. To increase the utilization of sustainable lignocellulosic biomass, yeast, especially *Saccharomyces cerevisiae*, is used as a microbial cell factory to produce renewable chemicals like ethanol, butanol, or other fermentative products from lignocellulosic biomass. The cost of cellulolytic enzymes including cellulase and hemicellulase is one of the limitations for industrial applications. Combining the cellulolytic enzyme expression and chemical production is a promising strategy to improve cost efficiency. Although cellulolytic enzyme-encoding genes have been expressed in yeast for enzyme characterization and protein engineering, co-expression of multiple cellulolytic enzyme genes is required to enable the non-cellulolytic yeast to utilize lignocellulose. Types of co-expressed systems include free cellulolytic enzyme, surface-displayed enzyme, or artificial minicellulosome. However, a lignocellulosic biomass utilizing strain with practical applications has been not constructed so far. The low efficiency of cellulolytic enzyme expression is the major roadblock hampering the generation of the desired strain. Although strong promoter, multiple gene copies, and codon optimization improve the gene expression, the protein misfolding, glycosylation, and vesicle transportation during secretion are more critical. Co-expression of multiple cellulolytic enzymes in an optimal ratio is also a challenging in cellulolytic yeast construction. Other than *S. cerevisiae*, nonconventional yeasts such as *Kluyveromyces marxianus* and *Yarrowia lipolytica* with special properties such as thermotolerance, xylose utilization, and lipid production are also good candidates for lignocellulosic biomass utilizing microbial cell factory construction.

Keywords Cellulolytic enzyme · Yeast · Nonconventional · Consolidate bioprocess · Challenge

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1 Introduction

Yeast is known for high expression yields of proteins. The secretory pathway of yeast enables the favored disulfide bonds formation and glycosylation and also enhances the stability and functionality of the enzymes (Lambertz et al. 2014). Yeast, especially *Saccharomyces cerevisiae*, is also an ideal candidate to be used as cell factory for industrial production of commodity products (Kavscek et al. 2015; Davison et al. 2016). These products include biofuels such as ethanol, butanol, and biodiesels and bulk chemicals such as lactic acid, pyruvic acid, succinic acid, xylitol, and ribitol. Several pharmaceutical products such as artemisinin (Paddon et al. 2013), resveratrol (Li et al. 2015), β -carotene (Gao et al. 2017), and lycopene (Chen et al. 2016) are also produced by engineered yeasts. The advantages of using yeast as a cell factory include rapid growth rate, high cell density fermentation, microbial safety, and eukaryotic post translational process (den Haan et al. 2015). Further, tractability and easy cultivation conditions open the possibility of protein engineering for cellulolytic enzyme optimization, such as the generation of more active and stable enzymes (Lambertz et al. 2014; Voutilainen et al. 2014).

In addition to compounds such as glucose and glycerol, sustainable feedstock lignocellulosic biomass is attracting increased attention as substrate and carbon source for microbial cell factories because it is more sustainable, cheaper, and commonly available (Kavscek et al. 2015). Use of non food resources such as poplar, switch grass, and silver grass as well as agricultural by-products like corn cob, corn stalk, rice straw, and bagasse can avoid the competition for resources between food and fuel or other chemicals. Several examples have demonstrated that the fuel and chemical industries are studiously developing novel bioprocesses to change the primary feedstock from oil to lignocellulosic biomass (Hong and Nielsen 2012).

The hydrolysis step is unavoidable in order to utilize lignocellulosic biomass. The cost of added cellulolytic enzymes for lignocellulose saccharification varies widely and does not show obvious reduction with time (Olson et al. 2012). Although Genencor announced that it had achieved an estimated cellulase cost in the range of \$0.10–0.20 per gallon of ethanol in the cost model of NREL, the cost of added enzyme was estimated to be more than 50 cents per gallon of ethanol by other researchers (Lynd et al. 2005). The cost of cellulase is still one of the obstacle in cost-effective utilization of cellulosic biomass (Olson et al. 2012). Therefore, reducing the cost of cellulolytic enzymes is an important criterion to be fulfilled.

Combining the cellulolytic enzyme production and sugar fermentation may enable the yeast to utilize lignocellulosic biomass directly, avoid or reduce cellulolytic enzyme addition, and enhance fermentation. This strategy, termed consolidated bioprocessing (CBP), is a more cost-efficient approach to produce chemicals from biomass because it enables the biological conversion of lignocellulose into desired products in a single step without adding enzymes. Furthermore, the cellulose hydrolysis ability may be improved if the cellulolytic enzyme production is integrated with fermentation in yeast. According to previous reports, enzyme–microbe synergy indicates that the effectiveness of cellulolytic enzyme is enhanced

when it is presented as cellulose–enzyme–microbe (CEM) complexes as compared with cellulose–enzyme (CE) complexes (Lynd et al. 2005). Therefore, a strain with suitable levels of enzyme production without compromising the chemical (such as ethanol) fermentation capacity is the target aimed in this field (den Haan et al. 2015).

In this chapter, the expression of cellulolytic enzymes in yeast is introduced. Specifically, this chapter discusses the expression of cellulolytic enzymes in *S. cerevisiae*, challenges involved in the expression of cellulolytic enzymes in yeast, and cellulolytic enzyme expression in nonconventional yeasts such as *Pichia pastoris*, *Kluyveromyces marxianus*, and *Yarrowia lipolytica*.

2 Expression of Cellulolytic Enzymes in *S. cerevisiae*

Advantages of expressing cellulolytic enzymes in *S. cerevisiae* include the relative ease of growth, similarity of glycosylation to industrially relevant fungi, convenient genetic technology for genes incorporation and deletion, high tolerance to inhibitors, wide usage as host for microbial cell factories, and the potential of producing chemicals from lignocellulosic biomass in single step (Greene et al. 2015). Because most of the cellulosic substrates are insoluble, the cellulolytic enzymes must be secreted to access and hydrolyze them. In the following sections, single cellulolytic enzyme and multiple cellulolytic enzymes secretory expression systems as well as surface display and artificial cellulosome in yeast are introduced.

2.1 Expression of Single Cellulolytic Enzyme

Because efficient hydrolysis of cellulose requires the synergy of multiple cellulolytic enzyme, in most cases the single cellulolytic enzyme expression is not sufficient for the hydrolysis, and this system is generally preferred for recombinant enzyme characterization (Hong et al. 2001; Hong et al. 2003a, b), enzyme engineering (Voutilainen et al. 2010; Larue et al. 2016; Cheng et al. 2015), or expression mechanism analysis (Xu et al. 2014; Zhu et al. 2010). Previous reports have summarized the cellulolytic enzymes expressed in *S. cerevisiae* (Olson et al. 2012; den Haan et al. 2013). In these reports, two critical cellulases Cel6A and Cel7A were efficiently expressed in *S. cerevisiae*. Their expression levels corresponded to 4–5% of total cellular protein content, which meets the estimated levels for growth on cellulose at the rates required for an industrial process and to overcome the barrier of cellobiohydrolase (CBH) sufficiency (Ilmen et al. 2011; Olson et al. 2012). The single-enzyme-system-three-cellulase gene (sestc) isolated from *Ampullaria gigas* was expressed in *S. cerevisiae* to enable the constructed strain to utilize wheat bran (Yang et al. 2016).

2.2 Expression of Multiple Free Cellulolytic Enzymes

An ideal CBP microorganism should be of efficient cellulolytic enzyme producing and fermenting ability. To complete the hydrolysis of cellulose, co-expression of many cellulolytic enzyme genes, including genes of *endo*- β -1,4-glucanase (EG), CBH, and β -glucosidase (BGL) in the yeast cell, is required.

The first analysis of multiple cellulolytic enzyme genes co-expression in the ethanol-producing yeast *S. cerevisiae* was reported by Van Rensburg et al. The genes of cellobiase from *Endomyces fibuliger*, *endo*- β -1,4-glucanase from *Butyrivibrio fibrisolvens*, cellobiohydrolase from *Phanerochaete chrysosporium*, and cellodextrinase from *Ruminococcus flavefaciens* were co-expressed, and free enzymes were secreted into the growth media (Van Rensburg et al. 1998), although the ethanol fermentation ability of the constructed strain was not evaluated. *S. cerevisiae* L26128GC, which was constructed through the expression of β -glucosidase and *exo*-*endo*-bifunctional cellulase from *Bacillus*, produced approximately 4 g/L ethanol from cellulodextrins (Cho et al. 1999). *S. cerevisiae*-eb, constructed by integrating *endo*-1,4- β -glucanase and β -glucosidase from *Trichoderma viride* into the genome, produced 4.63 g/L ethanol with 20 g/L carboxyl methyl cellulose (CMC) (Gong et al. 2014).

Compared to systems involving surface display and cellulosome, the advantage of the approach involving freely secreted cellulolytic enzymes is that the quantity of secreted enzymes is not limited by the incorporation capacity of the yeast cell wall (den Haan et al. 2015). Moreover, free cellulolytic enzymes can penetrate the biomass (Resch et al. 2013), increase the accessibility of cellulose, which is one of the critical factors in enzymatic hydrolysis (Liu et al. 2015). However, even with high cell density, high level of free cellulolytic enzymes in the medium needs to be re expressed upon transfer into fermentation medium due to enzymes diffusion and difficulty in enzyme recycling (den Haan et al. 2015). Therefore, the cellulose utilization ability of the constructed strain was lower than surface display system. Because free cellulolytic enzymes differed from cellulosome in the mechanism adopted for cellulose hydrolysis (Resch et al. 2013), the combination of free enzyme and cellulosome may significantly improve the cellulolytic efficiency. Therefore, integrating free cellulolytic enzymes and surface display or minicellulosome in a single yeast cell or consortium may be a promising strategy.

2.3 Surface Display of Cellulolytic Enzymes on *S. cerevisiae*

Display of heterologous cellulolytic enzymes on *S. cerevisiae* cell surfaces enables it to be promising biocatalysts for direct ethanol production from cellulosic biomass (Inokuma et al. 2016). Displaying cellulolytic enzymes on the yeast cell surface results in easy recovery of enzymes along with the cells that can be reused in the next batch fermentation. Therefore, the cost of yeast propagation is reduced and the

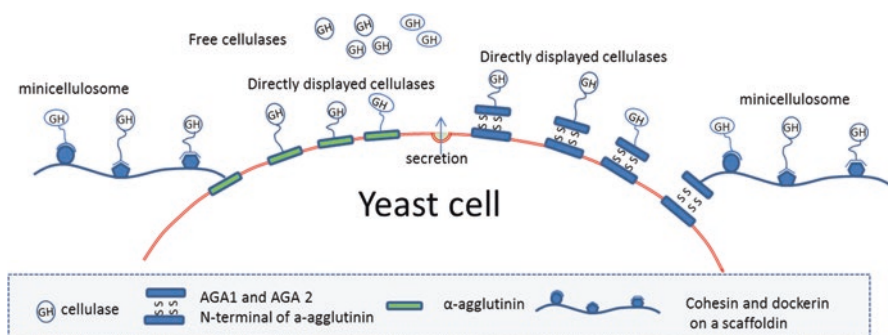


Fig. 11.1 Surface display of cellulase and minicellulosome

regeneration of cellulolytic enzymes is avoided in multiple batch fermentation. In addition, cell surface display method improves the effective concentration of cellulolytic enzymes and brings a greater degree of synergy (Bae et al. 2015). Furthermore, glucose molecules released from lignocellulosic biomass close to the cell surface lead rapid uptake; thereby, product inhibition is minimized (Liu et al. 2015). Finally, cell-surface display system may alleviate the adsorption of free cellulolytic enzymes onto the cellulose and improve the efficiency of cellulose hydrolysis and ethanol production (Matano et al. 2013b). Presently, although the surface display of cellulolytic enzymes could not achieve highly efficient fermentation with lignocellulosic biomass, this strategy successfully reduced the addition of commercial cellulolytic enzymes during the fermentation of lignocellulosic biomass (Matano et al. 2012; Liu et al. 2015).

The most widely used surface display systems in yeast are based on agglutinin. The first system is based on the α-agglutinin receptor which consists of two subunits AGA1 and AGA2. The AGA1 protein is covalently attached to β-glucan in the extracellular matrix of the yeast cell wall after it is secreted. Further, the N-terminal portion of AGA2 binds to AGA1 through two disulfide bonds after secretion and remains attached to the cell surface. Proteins such as cellulolytic enzymes can be fused to the C-terminus of AGA2 and displayed (Fig. 11.1) (Ueda and Tanaka 2000). Another system uses α-agglutinin as the anchor protein wherein the cellulolytic enzyme can be fused to the N-terminus of α-agglutinin and displayed on the yeast cell surface directly (Fig. 11.1). Although both systems are used to display cellulolytic enzymes, Goyal et al. reported that the display ability of α-agglutinin was higher than α-agglutinin system (Goyal et al. 2011). Other proteins such as SED1 (Inokuma et al. 2014), FLO1, and CWP2 were also used as anchor proteins, albeit not commonly (Liu et al. 2015).

Many cellulolytic enzymes, including EG, CBH, and BGL, were co-displayed on the yeast cell surface to complete the hydrolysis of cellulose. The co-display of endoglucanase II from *T. reesei* and β-glucosidase 1 from *Aspergillus aculeatus* enabled the strain to directly utilize β-glucan to produce 16.5 g/L ethanol (Fujita et al. 2002). With the additional display of cellobiohydrolase I from *T. reesei*, the

strain could produce ethanol from amorphous cellulose without adding cellulolytic enzymes (Fujita et al. 2004). Moreover, compared with freely secreted cellulolytic enzymes, the co-display of cellulolytic enzymes on cell surface enhanced the efficiency of direct ethanol fermentation from amorphous cellulose and improved the ethanol titer from 1.6 to 2.1 g/L (Yanase et al. 2010b). The authors of this study concluded that surface display is superior to free enzymes system when constructing cellulolytic *S. cerevisiae*. However, this situation could vary depending on several factors such as the host strain, total amount of expressed cellulolytic enzymes, and fermentation conditions (Yamada et al. 2013). Because hemicellulose can be easily hydrolyzed and the challenge lies in the pentose utilization, there are only few reports about multiple hemicellulolytic enzyme display. Through display of xylanolytic enzymes on the surface of xylose-utilizing *S. cerevisiae* cells, the obtained strain produced 7.1 g/L ethanol from birchwood xylan with a yield of 0.30 g/g (Katahira et al. 2004).

The surface display system has disadvantages such as the limitation of yeast cell wall incorporation capacity. Further, surface-displayed cellulolytic enzymes cannot easily penetrate crystalline cellulose leading to decreased access to cellulose; thus, the enzymatic hydrolysis efficiency is reduced, and crystalline cellulose conversion is weaker compared to free cellulolytic enzyme (Liu et al. 2015). Combining cell-surface display and secretion strategies in same recombinant yeast strain could achieve stronger hydrolysis of cellulose than either surface displaying or secreting cellulolytic enzymes strategies. The combination of the two strategies in a single yeast strain was studied by Liu et al. They found that placing cellulolytic enzymes in the same space (on the cell surface or in the medium) was favorable for ethanol fermentation with amorphous cellulose as substrate (Liu et al. 2015). Moreover, the strains displayed one cellulase and secreted one cellulase simultaneously were less efficient. However, they did not combine the display three enzymes and free three enzymes in a single strain.

2.4 Artificial Minicellulosome

Artificial minicellulosome can be introduced on the *S. cerevisiae* surface to enhance the cellulose conversion. Many anaerobic bacteria display cellulosome on the cell surface with only a limited amount of enzymes through enzyme-substrate synergy to maximize the cellulose hydrolysis efficiency (Tsai et al. 2009). The specific cellulose hydrolysis rates of *Clostridium thermocellum* displayed cellulosomes are more than four folds higher than free enzymes. The synergistic effects are due to (1) targeting the enzymes to cellulose through cellulose binding module (CBM), (2) relative high concentration of the enzymes near the substrate (Wen et al. 2010), and (3) avoiding substrate inhibition through the quick uptake of hydrolysis products (Goyal et al. 2011). Therefore, the artificial minicellulosome is constructed through imitating cellulosome to enhance the cellulolytic activity of yeast cells.

To construct an artificial cellulosome, it is necessary to achieve the secretion of all its components. The major components of the cellulosome complexes include structural scaffoldin and dockerin domain-tagged cellulases (Tsai et al. 2009). In general, scaffoldin containing at least one CBM and repeated cohesins is attached to the yeast surface, and various cellulolytic enzymes are attached on the scaffoldin through the interaction of dockerin and cohesins. To attach the scaffoldin and cohesin on the yeast surface, they are expressed and secreted as a fusion protein as part of α - or α -agglutinin as described in the surface display (Sect. 2.3) in this chapter. The dockerin-tagged cellulolytic enzymes can be expressed in the same or different strains. A consortium including four strains which expressed scaffoldin-cohesin, dockerin-tagged endo- β -1,4-glucanase, cellobiohydrolase, or β -glucosidase, respectively, was used to assemble a minimal cellulosome (Tsai et al. 2010; Goyal et al. 2011; Kim et al. 2013) (Table 11.1). Endoglucanase, cellobiohydrolase, β -glucosidase, and scaffoldin were successfully expressed in *S. cerevisiae* HZ1886 enabling the assembly of the trifunctional minicellulosome (Wen et al. 2010). This constructed strain is the first recombinant yeast strain with capability of producing cell-associated trifunctional minicellulosomes. Subsequently, a more complex trifunctional surface-displayed minicellulosome was constructed (Fan et al. 2012). In this minicellulosome, two levels of scaffoldin significantly improved the scale of the cellulosome. Consequently, the cellulose hydrolytic activity increased significantly, and ethanol fermentation (1.41 g/L) with microcrystalline cellulose was achieved (Fan et al. 2012) (Table 11.1). Minihemicellulosome was also successfully constructed through the expression of endoxylanase, arabinofuranosidase, and β -xylosidase, to enable the recombinant *S. cerevisiae* strains to directly convert birchwood xylan to ethanol with a yield of 0.31 g/g (Sun et al. 2012).

3 The Challenges Associated with Expression of Cellulolytic Enzymes in Yeast

Although the successful expression and secretion of cellulolytic enzymes in *S. cerevisiae* have been reported (Greene et al. 2015) and CBP *S. cerevisiae* strain construction has tremendously progressed, there are still some challenges related to the cellulolytic enzyme expression. As shown in Table 11.1, majority of CBP strains required high cell density ($OD_{600} = 50$) to use insoluble cellulose (phosphoric acid swollen cellulose, PASC) (Liu et al. 2015). *S. cerevisiae* EBY100 (*cdt-1*, *ghl-1*, *celcca*, CA_C0911), in which glucose inhibition/repression on cellulase expression and mixed-sugar uptake was released, could utilize cellulose and fermented with a low start cell density ($OD_{600} = 0.1$) using galactose as co-substrate. However, the ethanol-producing ability with insoluble cellulose was not improved (Fan et al. 2016). Therefore, a practical strain using cellulose as carbon source to grow fast and efficiently ferment is still desired.

Despite multiple attempts to construct cellulosic substrate utilizing strain (den Haan et al. 2015; Hong et al. 2007), high efficient conversion of insoluble cellulosic

Table 11.1 Fermentation with insoluble cellulose by using consolidate bioprocessing *S. cerevisiae* strain or consortium

Strains	Strategy	Initial cell density	substrate	Ethanol titer (g/L)	Yield (g/g)	References
Y294[CEL5]	Free	2×10^5 cells/mL	PASC	1.0	0.37	den Haan et al. (2007b)
EG-D-CBHI-D	Surface display	150 g wet cell/L	PASC	2.9	0.42	Liu et al. (2015)
MT8-1/pBG211/pEG23u3IH6/pFCBH2w3	Surface display	$OD_{600} = 50$	PASC	2.9	0.45	Fujita et al. (2004)
MT8-1/AGEGII-AGCBIII-AGBGL	Surface display	$OD_{600} = 50$	PASC	2.1	~0.5	Yanase et al. (2010b)
MINII/cocδBEC3	Surface display	200 g wet cell/L	Pretreated rice straw	7.5	0.38	Yamada et al. (2011)
HZ1886	Minicellulosome	$OD_{600} = \sim 50$.	PASC	~1.8	0.31	Wen et al. (2010)
Four strains consortium	Minicellulosome	$OD_{600} = 50$	PASC	1.87	0.475	Tsai et al. (2010)
EBY (EA2-2)	Minicellulosome	$OD_{600} = 50$	PASC/Avicel	1.09/1.41	NA	Fan et al. (2012)
Four strains consortium	Minicellulosome	8×10^6 cell/ml	PASC	1.25	0.43	Goyal et al. (2011)
Four strains consortium	Minicellulosome	$OD_{600} = 50$	PASC	1.80	NA	Kim et al. (2013)
EBY100 (cdt-1, gh1-1, celcca, CA_C0911)	Minicellulosome	$OD_{600} = 0.1$	PASC	1.09	0.44	Fan et al. (2016)

NA Not available

substrates by heterologous *S. cerevisiae* strains is not yet achieved. Most results are reported only for highly reactive, non crystalline model substrates such as soluble carboxymethyl cellulose or PASC (Brethauer and Studer 2015). The titer of ethanol produced from micro crystalline cellulose and amorphous cellulose without addition of cellulase was very low (several gram/L in most cases) (Table 11.1). In some cases, the amount of extraneous cellulases for the utilization of lignocellulosic biomass was reduced. However, the cost of additional cellulase was still too high to an economic industrial process (Matano et al. 2013a; Liu et al. 2015).

Efficient expression of cellulolytic enzymes in yeast is challenging. Both weak growth and reduced fermentation with insoluble cellulose are due to the inefficient cellulolytic enzymes expression in the constructed strains. As mentioned above, multiple attempts to express recombinant cellulolytic enzymes in yeast to enhance the lignocellulosic biomass utilization were carried out. However, the expression of most cellulolytic enzymes in *S. cerevisiae* is low, and in particular, the expression of cellobiohydrolases and β -glucosidases in *S. cerevisiae* is difficult (Njokweni et al. 2012; den Haan et al. 2013). It was estimated that 20- to 120-fold improvement in *CBH* expression, as well as simultaneous high-level expression of other cellulolytic enzyme genes, is necessary for the slow growth on crystalline cellulose (den Haan et al. 2007a). In contrast, the most studied cellulolytic fungus *T. reesei* can secrete up to 100 g/L of crude cellulolytic enzymes (Ilmen et al. 2011; Cherry and Fidantsef 2003). Currently, the cellulolytic activity of CBP strain is not high enough to use lignocellulosic biomass, which is mainly hampered by the low expression of cellulolytic enzymes.

The potential bottlenecks include the codon usage of the expressed gene, transcription efficiency, secretion efficiency, ratio of each cellulolytic enzyme, and the types of co-expressed cellulolytic enzymes. Furthermore, producing functional cellulolytic enzymes at right concentration without triggering too much stress on the CBP yeast is also important (van Zyl et al. 2007). With the decreased gene synthesis price, the problem of codon usage of the expressed gene could be easily resolved through codon optimization and gene synthesis with relatively low cost. In the following section, the significance of promoter, gene copy number, and cellulolytic enzyme secretion to the successful cellulolytic enzyme expression is discussed.

3.1 Importance of Promoter Selection in Heterologous Cellulolytic Enzyme Gene Expression

Both inducible and constitutive promoters of the host have been used to achieve heterologous cellulolytic enzyme genes expression in yeast. Inducible promoters such as *GAL1* in *S. cerevisiae* are extremely efficient but require relatively expensive inducers. Moreover, carbon catabolite repression often represses the inducible promoters due to the presence of glucose, and therefore inducible promoters are not

suited for industrial lignocellulosic biomass utilization (Kricka et al. 2014). The constitutive promoters such as *TEF1*, *TDH3*, and *PGK1* have been also used for heterologous cellulose genes expression in yeast (Nakatani et al. 2013; Fitzpatrick et al. 2014). However, a strong promoter does not always lead to high expression. The transcription and translation steps should be compatible with the yeast secretory system. Therefore, selection of a compatible promoter for expression system is an important criterion. The selection is probably enzyme-dependent and is a challenge to the co-expression of multiple cellulolytic enzyme genes.

3.2 Improve the Copy Number of Genes to Enhance the Cellulolytic Enzyme Expression

Episomal plasmids are extensively used to express cellulolytic enzyme genes; however, lack of selection pressure leads to the loss of plasmids and is not suited to construct industrial strains due to the high cost of reagents for selection. Multiple gene copy integration in chromosome through approaches utilizing δ site could improve the cellulose expression (Yamada et al. 2010). Irrespective of the method adopted, similar to the case of promoter selection, owing to the limitation of translation, increased copy number of cellulolytic enzyme genes does not always ensure high expression.

3.3 Challenges Associated with the Optimization of Secretion Procedure in Cellulolytic Enzyme Gene Expression

Efficient processing and secretion of cellulolytic enzymes are often difficult to achieve. Irrespective of the approach adopted including free enzyme, surface-displayed enzymes, or cellulosome, the enzymes must be efficiently secreted. Increasing the gene copy number and using strong promoter do not always result in high cellulolytic enzyme expression. After the cellulolytic enzyme gene is transcribed and further translated by the ribosome, high rates of cellulolytic enzyme secretion from *S. cerevisiae* are hampered due to secretory bottlenecks.

In the protein secretion procedure, the secretion is directed by the signal peptide. The signal peptide is co-translated and translocated with the target protein into the endoplasmic reticulum (ER), and then the signal peptide is cleaved and removed. Next, N-glycosylation is processed when the protein is transported to the Golgi, where glycosylation is further modified. Finally, the protein is released to the cell surface in secretory vesicles (Fig. 11.2) (Lambertz et al. 2014). In this procedure, signal peptide, misfolding, transportation, glycosylation, and process capacity of yeast cell all possibly limit the secretion.

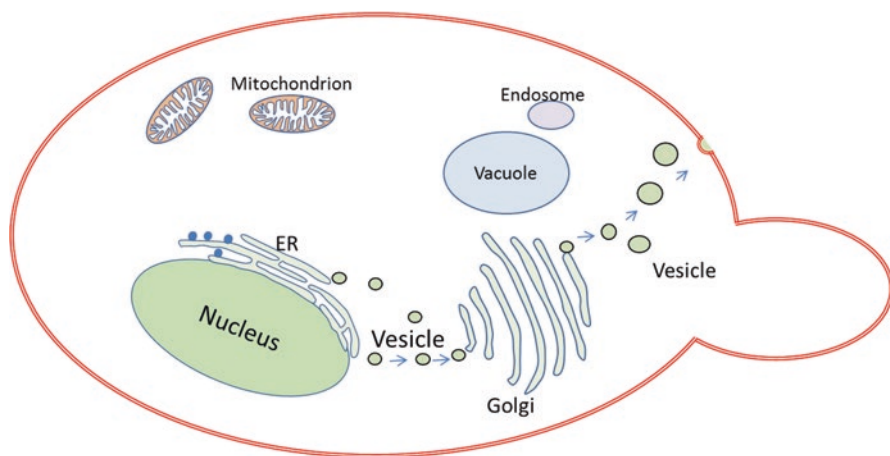


Fig. 11.2 Illustration of the main cellular compartments involved in recombinant protein expression in yeast cell. Arrows indicate the secretory pathway, whereby cellulolytic enzymes are expressed on the ER and transferred via Golgi apparatus to the medium in secretory vesicles. *ER* endoplasmic reticulum

In yeast, the secretion is directed by a signal peptide located at the N-terminal end of the expressed protein. In *S. cerevisiae*, highly efficient secretion was achieved using α -mating factor signal peptide (Lambertz et al. 2014), although the native signal sequences of fungi cellulolytic enzymes were also used (Hong et al. 2001, 2003a, b; Liu et al. 2015). Similarly, another signal peptide obtained from *Rhizopus oryzae* glucoamylase was also used. *S. cerevisiae* SED1 signal peptide was proved to be more efficient than α -mating factor and glucoamylase signal peptide in cellulolytic enzyme gene expression (Inokuma et al. 2016). Once the protein is directed to secretion, translocation of the translated protein to ER is one of the rate-limiting steps. Insufficient amount of proteolytic cleavage enzymes may be limiting for the secreted proteins with precursor domains. Overexpression of signal peptidases cleaving the signal peptide enhanced the entry to the ER (Davy et al. 2017).

The secretion of cellulolytic enzymes can also be hindered by the misfolding of heterologous proteins which leads to ER stress during secretion. The machinery for proper protein folding may be saturated due to heterologous proteins overexpression, leading accumulation of misfolded or aggregated proteins in the ER (ER stress) (Ilmen et al. 2011; Davison et al. 2016). The misfolding of proteins can evoke the unfolded protein response (UPR) and, consequently, the endoplasmic reticulum-associated protein degradation (ERAD) response (van Zyl et al. 2007; Davison et al. 2016; Ilmen et al. 2011). When a prolonged accumulation of misfolded proteins in the ER exceeds its folding capacity, it can induce an additional UPR, which not only further stimulates the proteolytic activities mediated by ERAD but also changes the expression of various foldases and chaperones which enhance the folding capacity of the ER (Davison et al. 2016). These effects may be enhanced

by the interrupted transport of heterologous proteins in the secretion pathway, or accumulation of larger proteins at the cell wall due to low permeability (van Zyl et al. 2007). Therefore, the delivery of correctly folded proteins from ER could be the one of rate-limiting steps for heterologous cellulolytic enzyme secretion (Schroder 2008). The results of Ilmén et al. indicated that the cellulase expression in *S. cerevisiae* also activated UPR (Ilmen et al. 2011), and this response is protein dependent. Although overexpression of chaperones (holdase and foldase) alleviated aggregation of unfolded proteins and enhance the secretion (Schroder 2008), the effect is also protein dependent. Since alleviating ER stress during heterologous gene expression may be a protein-dependent process, devising a common strategy to relieve ER stress is difficult to achieve.

The transport of heterologous proteins during secretion also poses as a limitation for cellulolytic enzyme expression. Secretory proteins are folded in the ER and subsequently transported to the Golgi through vesicle trafficking (Tang et al. 2017). Vesicle trafficking is a complex process involving protein transport through the ER, Golgi, endosome, and to either the cell membrane or vacuole (Fig. 11.2). However, which step is the limiting step for secreted protein is still unclear. Soluble NSF [N-ethylmaleimide-sensitive factor] attachment receptor proteins (SNAREs) drive intracellular membrane traffic (Van Zyl et al. 2014). The over expression of different components of the exocytic SNARE complex in *S. cerevisiae* enhanced *Talaromyces emersonii* Cel7A and the *Saccharomycopsis fibuligera* Cel3A secretion with ~ 52% and ~ 49% improvement, respectively. Moreover, this modification generally did not cause deleterious growth effects (Van Zyl et al. 2014). The over expression of *SED5* led to the maximum increase in extracellular enzyme activity of 22% for Sf-Cel3A and 68% for Te-Cel7A (Van Zyl et al. 2016). Furthermore, co-overexpression of certain ER-to-Golgi SNAREs (BOS1, BET1, SEC22, and SED5) also improved the expression of two cellulases described above. However, the enhancement of each SNARE did not show synergistic effect in co-expression and in some cases was less than single SNARE expression. The effect of overexpression of the vesicle trafficking components in ER to Golgi or Golgi to cell membrane transport which include SNARE components and other vesicle-related proteins was also analyzed with the secretory expression of *C. thermocellum* endo-1,4- β -glucanase (CelA) and *S. fibuligera* β -glucosidase (BGL1). The overexpression of ER to Golgi vesicle trafficking components and Golgi to plasma membrane vesicle trafficking components enhanced the secretion of CelA and BGL1, respectively (Tang et al. 2017). Modifying the vesicle trafficking process is a promising approach to enhance the extracellular secretion of heterologous proteins. However, it is protein dependent. Therefore, improving cellulolytic enzyme gene expression through this strategy needs further investigation.

Additionally, specific glycosylation can limit the cellulolytic enzyme gene expression. The glycosylation of the cellulase linker regions, which connect the catalytic domain and the cellulose-binding module of cellulolytic enzymes, protects the enzymes from protease degradation and may also assist the binding of cellulase to the cellulose surface (Lambertz et al. 2014). However, glycosylation process in yeast may hinder the expression of cellulolytic enzymes. For instance, the lack of

Golgi mannosidase in *S. cerevisia* leads yeast-specific hyper-mannosylation (Lambertz et al. 2014). It may impair the protein folding and activity and secretion of the expressed protein. Suzuki et al. reported that deletion of some glycosylation genes increased the amount of displayed dockerin and promoted efficiency of saccharification in *S. cerevisiae* (Suzuki et al. 2012).

3.4 Challenges Posed by the Co-expression of Multiple Cellulolytic Enzyme Genes in the Construction of Efficient Cellulose-Utilizing Yeast Strain

Efficient cellulose degradation requires a synergistic hydrolysis by the cellulolytic enzymes EG, CBH, BGL, and in some cases, xylanase. In multiple cases, co-expression of several cellulolytic enzyme genes led to the decreased expression of individual enzymes, and thereby the advantages of expressing multiple cellulolytic enzymes are not achieved. Although hydrolysis of crystalline cellulose can be theoretically achieved by the synergistic actions of EGs, CBHs, and BGLs, in filamentous fungi, the cellulolytic enzymes are a complex family. In *T. reesei* cellulolytic enzymes system, apart from cellobiohydrolase I, cellobiohydrolase II, endoglucanase I, and beta-glucosidase I which constitute the main components, other cellulases and swollenins which are present in minor amounts enhanced the cellulose hydrolysis. Currently, most cellulolytic enzymes expressing yeasts only expressed the minimum set of enzymes, i.e., a single copy of endoglucanase, exoglucanase, and beta-glucosidase each. Moreover, in some cases, despite simultaneously expressing five or six cellulolytic enzymes genes, the desired cellulolytic activity could not be attained. Combined with the insufficiency of cellulolytic enzymes, the yeast took prolonged time periods to hydrolyze and ferment insoluble cellulose. Analysis of the minimum requirement of cellulase in CBP did not give a practically useful answer as generally the substrate in the model was not natural cellulosic material. Examination of the enzymatic systems of *T. reesei* and *C. thermocellum* for the lignocellulose breakdown via proteomic analysis could enable the design of a complete enzymatic system, although expressing this system in yeast will remain a challenge.

3.5 Challenges Related to Co-expressing Various Cellulolytic Enzymes in a Desirable Ratio and at the Right Concentration

The high cellulolytic enzyme producing microbes were obtained through studies involving long time evolution and engineering. The true optimal ratio of cellulolytic enzymes used by natural cellulolytic microorganisms is not fully understood, but

the estimated ratio of the total secreted proteins of *T. reesei* under inducing conditions is 60% CBHI, 20% CBHII, 10% EG, and 1% BGL (Kricka et al. 2014). Yamada et al. attempted to integrate *cbh1*, *cbh2*, *egl2*, and *bgl1* into a chromosome through δ integration with various ratios (Yamada et al. 2010), and the results indicated that optimization of the cellulolytic enzymes expression ratio improved PASC degradation activity. However, the cellulose degradation ability was still limited, and the target substrate was not lignocellulosic biomass which is more complex. Co-expressing several cellulolytic enzyme genes in a reasonable ratio is furthermore difficult.

3.6 Engineering the Supply Chain for Protein Production and Secretion in Yeasts

In addition to overexpressing chaperones, engineering of the UPR or vacuolar sorting pathways to improve recombinant protein secretion, engineering the supply chain, is attracting increasing interest. The supply chain including building blocks and energy is not only for protein synthesis and secretion but also for the synthesis and secretion machinery (Klein et al. 2015). Presently, however, studies on the cellulolytic enzyme gene expression through this strategy are still limited.

4 Expression of Cellulolytic Enzyme Genes in Nonconventional Yeasts

In addition to *S. cerevisiae*, several nonconventional yeasts exhibit properties desirable for microbial factories construction. *Pichia pastoris* has proved to be an excellent heterogenous protein expression host. *K. marxianus* and *Hansenula polymorpha* are thermotolerant yeast capable of xylose utilization. *Y. lipolytica* is known for its ability to produce lipids in the cell. *Scheffersomyces (Pichia) stipitis* is probably the most widely studied yeast for xylose utilization. However, not many cellulolytic enzyme genes were expressed in *H. polymorpha* and *S. stipitis*. Only two studies reported the expression of cellulolytic enzymes in *H. polymorpha* for protein heterologous expression evaluation, and these studies indicated that the enzymes were hyperglycosylated (Kim et al. 2007; Muller et al. 1998). The *celD* gene from *C. thermocellum* was expressed in *S. stipitis* to evaluate the promoter for heterologous protein expression. However, an increasing number of studies reported the expression of cellulolytic enzymes in *P. pastoris*, *K. marxianus*, and *Y. lipolytica*, and these are discussed in the following sections.

4.1 Expressing Cellulolytic Enzyme Genes in *P. pastoris*

P. pastoris can express and secrete glycoproteins with high yields in a cost-effective and rapid manner. Because most of cellulolytic enzyme genes were expressed with a methanol-inducing promoter AOX1, and in most cases, a single kind of cellulolytic enzyme gene was expressed. Therefore, the obtained strains were not suited to construct a cellulose utilizing strain. However, due to the highlight feature of heterologous gene expression, *P. pastoris* was used to express cellulolytic enzymes for characterization, enzymatic engineering, and expression mechanism studies (Kim et al. 2015; Mellitzer et al. 2012). A recent review summarizes the recombinant extremozymes (r-extremozyme) produced by *P. pastoris*. The xylanase, laccase, and cellulase expression in *P. pastoris* was compared and analyzed in this report (Ergun and Calik 2016).

4.2 Expressing Cellulolytic Enzyme Genes in *K. marxianus*

K. marxianus has been attracting increasing interest for its thermotolerance, rapid growth rate, and broad substrate spectrum (Zhang et al. 2017). *K. marxianus* grows at 45 °C with a growth rate of 0.58–0.63/h (Banat and Marchant 1995) and was reported to produce 5.1 g/L ethanol from 10 g/L glucose at 45 °C (Banat et al. 1996). This temperature is near the optimal temperature range for cellulases used in lignocellulosic biomass saccharification (45–50 °C). Therefore, fermentation under this temperature can improve the simultaneous saccharification and fermentation (SSF) of biomass (Zhang et al. 2017). Moreover, the use of thermophilic or thermotolerant microbes in fermentation has other advantages including solvent tolerance, reduced cooling costs, easier stripping of products from the broth, and reduced risk of contamination (Zhang et al. 2016).

In general, cellulolytic enzyme genes expressed in *K. marxianus* were used to construct CBP strains. The co-expression of endo- β -1,4-glucanase from *A. niger* and cellobiohydrolase and β -glucosidase from *Thermoascus aurantiacus* in *K. marxianus* enabled the constructed strain to utilize CMC to grow or use cellobiose to produce 43.4 g/L of ethanol from 10% of cellobiose at 45 °C (Hong et al. 2007). In another case, five cellulolytic enzyme genes and a fungal cellodextrin transporter gene were co-expressed in *K. marxianus* KY3. The obtained strain KR5 utilized beta-glycan, cellobiose, or CMC as the sole carbon source for growth and directly converted cellobiose and beta-glycan to ethanol (Chang et al. 2012). Based on the above strain, strain KR7, expressing a cellulase cocktail and cellodextrin transporter genes, used avicel to produce ethanol. However, the efficiency was very low, with a cell density of $OD_{600} = 20$, only 0.6 g/L ethanol produced (Chang et al. 2013). Yanase et al. successfully displayed *EGII* from *T. reesei* and *BGL1* from *A. aculeatus* on the *K. marxianus* cell surface. The resultant strain *K. marxianus*

KΔU/AGEGII-AGBGL converted cellulosic β -glucan into ethanol directly with a yield of 4.24 g/l from 10 g/l substrate within 12 h at 48 °C (Yanase et al. 2010a). Presently, there is no efficient lignocellulose CBP strain available in *K. marxianus*.

4.3 Expressing Cellulolytic Enzyme Genes in *Y. lipolytica*

Y. lipolytica is an oleaginous microorganism that intracellularly accumulates lipids and is widely used as biotechnological “chassis” to produce chemicals such as organic acids, poly alcohols, carotenoids, alkanes, and aromatics. Therefore, the genetic and physiological properties and biotechnological applications of *Y. lipolytica* were extensively studied (Wei et al. 2014). Some bioengineered strains could accumulate up to 90% of dry cell weight (DCW) as fatty acids with up to 85% of theoretical yields by using glucose as carbon source. It could be served for biodiesel production. However, production costs are still not low enough to make the process economically profitable. Using cheaper substrate such as lignocellulose would be helpful to reduce the costs further (Adrio 2017).

However, *Y. lipolytica* cannot directly utilize lignocellulose for growth. Therefore, cellulolytic enzyme genes were expressed to enable *Y. lipolytica* to utilize cellulose. The endoglucase I gene from *T. reesei* was expressed in *Y. lipolytica* under promoter XPR2, and 22.6 U/mL (CMCase) was achieved in fed-batch culture (Park et al. 2000). Through over expression of a cellodextrin transporter *cdt-1* and a β -glucosidase *gh1-1* from *Neurospora crassa*, a *Y. lipolytica* strain with cellobiose assimilation ability was constructed (Lane et al. 2015). The constructed strain produced citric acid from cellobiose with 0.35 g/g yield. *Y. lipolytica* cell consortia system containing cells expressing *T. reesei* EGII, *T. reesei* CBHII, or *T. reesei*–*T. emersonii* chimeric CBHI was constructed. However, only 23% of the avicel was consumed (Wei et al. 2014) implying significant space for further improvement. Recently, three recombinant *Y. lipolytica* strains expressing BG, EG, or CBH, respectively, were constructed. The enzymatic activities of these enzymes were 14.181 U/mL (0.079 IU/mL), 16.307 U/mL (0.091 IU/mL), and 17.391 U/mL (0.097 IU/mL), respectively, with avicel as the substrate (Song et al. 2017). Finally, a surface display system in *Y. lipolytica* was constructed wherein xylanase TxXYN from *Thermobacillus xylanilyticus* was displayed through fusion of cell wall proteins YIPir, YICWP1, and YICBM. The fusion with YIPir yielded best results and the activity reached 71.6 U/g dry cells (Duquesne et al. 2014).

In summary, multiple studies have been carried out to achieve cellulolytic enzyme expression in *S. cerevisiae*. In addition to characterization and expression mechanism research, construction of CBP strain through co-expression of multiple cellulolytic enzyme genes has attracted more attention. However, a highly efficient lignocellulosic biomass utilizing strain has not yet been constructed. Future studies should focus on the ratio of expressed cellulolytic enzymes and the secretion pathway engineering, which is more complex. Furthermore, other than *S. cerevisiae*, the thermo tolerant and oleaginous yeasts are also excellent candidates to construct cellulolytic microbial cell factory through cellulolytic enzymes overexpression.

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Chapter 12

Determination of Cellulase Activities and Model for Lignocellulose Saccharification



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Abstract The hydrolytic ability of cellulase enzymes being used in the current lignocellulosic “biorefinery” industry has stimulated a huge motivation to make an efficient cellulase preparation. Increasing work has shown how important the role that some basic conceptions and cognitions can play in close relation with an effective enzymatic hydrolysis. To shed light on the lignocellulose hydrolysis behavior in this review, hydrolytic performance of cellulase enzyme on various cellulosic substrates existing in form of carboxymethylcellulose (CMC), Avicel, dissolving pulp, filter paper, and natural lignocellulose was reviewed. A development of defining the enzyme activity of cellulase preparation, including specific enzyme and total enzyme mixture, was considered. Finally, the prediction of activity and dynamics of cellulase enzymes was outlooked.

Keywords Cellulase · Enzymatic hydrolysis · Model · Lignocellulose

1 Introduction

Cellulase enzyme has addressed much attention in the past three decades such as its increased application in the textile and booming biofuel industries (Rubin 2008). Presently, the protein amount of cellulase preparation loaded on substrate is ubiquitously used as a standard to define the cellulase loading as an alternative to the FPase activity (FPA) during the enzymatic hydrolysis of substrates. Actually, developing a quick and reliable cellulase activity assay has become extremely crucial for both the laboratory research and the industrial application (Zhang et al. 2006;

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Wilson 2009; Himmel et al. 2017). Researchers require a simple and consistent method to pick out the target cellulase enzymes in a range of application when developing a better cellulase cocktail during bioprospecting, engineering cellulase-producing strain, customizing cellulase cocktails, and so on (Banerjee et al. 2010; Gowen and Fong 2011). And chemical engineers and industrialists need to assess a cellulase preparation on their target substrates quantitatively and economically by a standard assay protocol (Han and Chen 2007; Geddes et al. 2011). In addition, a uniform and commonly accepted analytic procedure for cellulase enzymes is also invaluable for the communication in the cellulase-relevant fields (Eveleigh et al. 1976; Sharrock 1988; King et al. 2009; Resch et al. 2013).

2 The Traditional FPA Failed to Predict a Hydrolytic Potential of Cellulase Preparations on Natural Substrate

Since the 1950s, various cellulase activity-relevant analytic methods have been developed, and among these methods, the filter paper activity (FPA) assay has been most widely acknowledged. Briefly, the FPA assay analyzes a total cellulolytic potential of the cellulase preparation by using Whatman No. 1 filter paper as the substrate with the standardized FPA assay protocol that was initiated by the International Union of Pure and Applied Chemistry at near 30 years ago (Ghose 1987). Relatively, the filter paper assay is simple, reproducible, and quantitative, and it can predict cellulase enzyme action under a simple and practical saccharification condition (Eveleigh et al. 1976; Sharrock 1988). The FPA assay has become an established and commonly accepted standard method of detecting the hydrolytic potential of a cellulase preparation (Ghose 1987; Cowardkelly et al. 2003; Xiao et al. 2004). However, the filter paper assay is not perfect, whose limitation has been increasingly exposed as it is extensively applied in laboratories and pilot plants (Cowardkelly et al. 2003; Xiao et al. 2004). Results obtained from the standardized FPA assay are not reproduced easily in most laboratories, which has been complained with the complexity of the assay protocol and the sensitivity to operators' deviation (Cowardkelly et al. 2003; Jimenez-Flores et al. 2010). Also, the result reliability is prone to be impacted by the β -D-glucosidase present in the cellulase preparation (Joglekar et al. 1983; Breuil and Saddler 1985). Hence, modifying the FPA assay has addressed lots of attention in recent years, but it is worth noting that most results obtained with these modifications are not similar to those obtained with the standard FPA assay (Berlin et al. 2005; Camassola and Dillon 2012; Silveira et al. 2012).

In addition, the filter paper is subjected to skepticism of a suitable model substrate as the FPA has been commonly applied to predict hydrolytic potential of cellulase mixture on the more complex lignocellulosic substrates. Berlin et al. (2005, 2006) suggested that the hydrolytic potential of a cellulase preparation acting

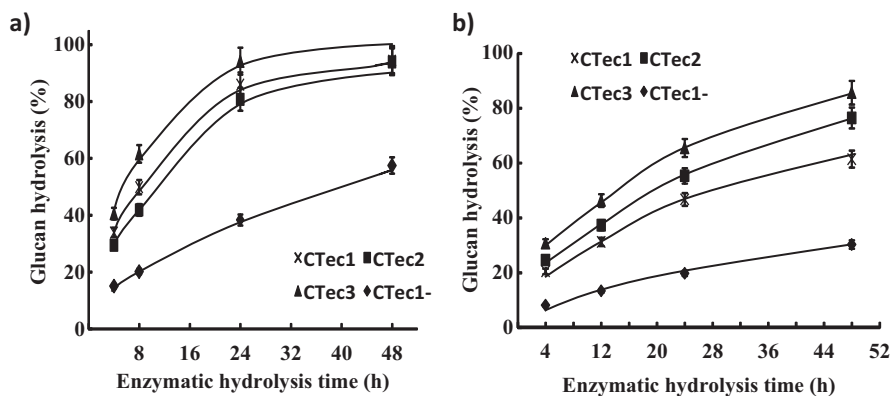


Fig. 12.1 Enzymatic hydrolysis (pH 4.8, 150 rpm, 50 °C) of various cellulase enzymes (Ctec1~3 = 14.2 FPU/ g substrate; Ctec2, 3, 1- = 20 mg protein/g substrate) on 2% (w/w) SPP 200 (a) and SPSSB (b), respectively

on steam explosion or organosolv pretreated lignocellulose was not relevant with its activity on filter paper substrate (Kabel et al. 2006) showed that a relatively low standard activity of cellulase preparations was not always consistent with the degradability of wheat bran xylan and grass cellulose. Moreover Pryor and Nahar (2010) found that the identical loading of three cellulase preparations (Accellerase 1000, Spezyme CP and Celluclast 1.5L) led to different sugar yields from acid/alkaline pretreated switch grass solids. In order to observe such interesting phenomenon, three leading Novozymes cellulase preparations, Cellic® Ctec1/2/3, are assessed on steam-pretreated poplar and sweet sorghum bagasse during the time course of hydrolysis, as shown in Fig. 12.1. At the same enzyme loading, Ctec3 resulted in 94% glucan hydrolysis within 24 h of pretreated poplar, which was higher than those achieved by Ctec1 (86%) and Ctec2 (80%). When acting on the steam-pretreated sweet sorghum bagasse, Ctec3 reached 66% of glucan hydrolysis (24 h), while Ctec1 and Ctec2 achieved only 47% and 55%, respectively. With an addition of Ctec1 at the same protein level as Ctec2 and Ctec3, the glucan hydrolysis of pretreated poplar and sweet sorghum bagasse was only up to 38% and 20%, respectively. The result has showed that the initial cellulase preparation, Ctec1, is relatively less efficient on pretreated lignocellulosic biomass than the new products, Ctec2 and Ctec3. Remarkably, the Ctec3 is more efficient than Ctec2 though the same specific enzyme loading and enzyme activity. It is presumed that the modification was finished not only by concentrating the protein content, also by removing some superfluous proteins irrelevant with lignocellulosic hydrolysis. And the Ctec3 was prepared probably by adding some heterologous proteins independent of the FPA, which was helpful for lignocellulosic hydrolysis.

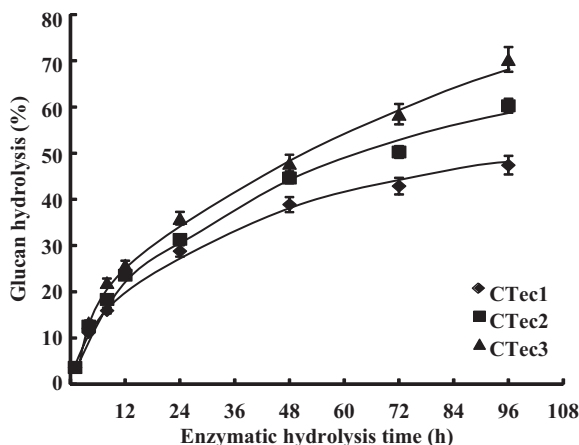
On the other hand, the result showed that even at the same enzyme loading, the three commercial cellulase preparations presented surprisingly different hydrolytic profile on the two different lignocellulosic substrates. Here, The fixed enzyme loading

represents two aspects: firstly, a certain cellulase preparation showed different hydrolysis profile on diversified lignocellulosic substrates; secondly, different cellulase preparations resulted in different hydrolysis on a certain substrate (Pryor and Nahar 2010). The result is beyond all the expectation that cellulase enzyme stocks at the same dosage would give a consistent hydrolysis performance on the same type of substrate. Thus, the FPA cellulase is not reliable and cannot accurately predict the hydrolysis potential of current cellulase preparations. Similar arguments are also made from other researchers (Berlin et al. 2006; Kabel et al. 2006). For example, Kabel et al. (2006) have tested 14 commercial enzymes for their cellulase/xylanase activity and found that these enzyme preparations presented a big difference between standardized activity and the actual hydrolytic performance on grass cellulose and wheat bran xylan. The hydrolytic performance of an enzyme preparation is dependent on the substrate characteristics rather than on the standard enzyme activity measured on model/pure cellulose and/or xylan substrates. Commercial enzyme preparations that comprise various *T. reesei* enzymes such as endoglucanases, exoglucanases, and β -glucosidases are generally deficient in their activity to degrade lignocellulosic substrate due to the more complex cell wall structure (Berlin et al. 2006; Chundawat et al. 2008; King et al. 2009). A functionally based model developed by Zhang and Lynd (2006) has shown that total cellulase activity assay is too complex, inferring that the result of the total cellulase activity assayed on one cellulosic substrate is not supposed to be consistent that on another substrate. Therefore, it seems that the equal enzyme loading of FPA should not suppose give equivalent sugar yields on the heterogeneous lignocellulosics when using different enzyme loading and cellulase preparation. Although the applicability of FPA is in doubt to assess the enzymatic potential of a cellulase preparation, there is not yet any entire and detailed information on this issue to date. Consequently, the protein amount of cellulase preparation loaded on substrate was ubiquitously used as a standard to define the cellulase loading as an alternative to the FPA during the enzymatic hydrolysis of substrates.

3 What Have Resulted in the Incapability of FPA to Represent Hydrolytic Potential of Present Cellulasarations on Native Lignocellulosic Substrate?

To ascertain why the traditional FPA failed to predict a hydrolytic potential of cellulase preparations on natural substrate, based on the FPA assay protocol, the hydrolytic performance of these cellulases was carefully observed at an a prolonged hydrolysis of 72 h (not only the exact 1st hour), at a shaking hydrolysis and at a hydrolysis of substrate alternative to filter paper, respectively.

Fig. 12.2 Enzymatic hydrolysis of 50 mg filter paper with Ctec1, 2 and 3 (0.185 FPU) at the standard FPA assay condition



3.1 Prolonged Hydrolysis of Filter Paper Based on FPA Assay

In order to clarify what is responsible for the above result, the standardized FPA protocol was revisited again. To follow the analytical procedure of FPA, 0.5 mL diluted cellulase enzyme preparations, namely, 0.1850 FPU, was used for hydrolyzing 50 mg filter paper (Fig. 12.2). As expected, three cellulase enzyme stocks, Ctec1/2/3, all produced 2.0 mg of reducing sugars from 50 mg filter paper and gave the same hydrolysis extent (3.6%) at the rigid 1 h hydrolysis. With the hydrolysis time extending, however, the cellulose hydrolysis showed different profile. The Ctec1/2/3 presented a significantly different hydrolysis performance, resulting in 40%, 55%, and 70% conversion of filter paper after 96 h, respectively. The data showed that various cellulases acting on a standardized condition based on FPA assay cannot predict the longtime hydrolysis. To our knowledge, there is no literature that has yet described this phenomenon. These results indicate that the FPA can only formulate hydrolytic activity of the same enzyme preparation with different dilution while not suitable to evaluate and predict the hydrolytic behavior of various cellulase preparations, especially during a longtime hydrolysis.

3.2 Shaking Hydrolysis of Filter Paper Based on FPA Assay

The experiment of “FPA” assay was proposed at shaking condition to assess whether there would be a different cellulase dilution that releases exactly 2.0 mg reducing sugars at 60 min. In terms of the standardized analytical procedure of FPA, properly diluted cellulase preparations of 0.5 mL were added to 50.0 mg filter paper for an accurate 60 min hydrolysis. Then the two data points that were very close to release 2.0 mg reducing sugars would be considered, by which a straight line was drawn then to interpolate the enzyme dilution. As shown in Fig. 12.3, with just the release

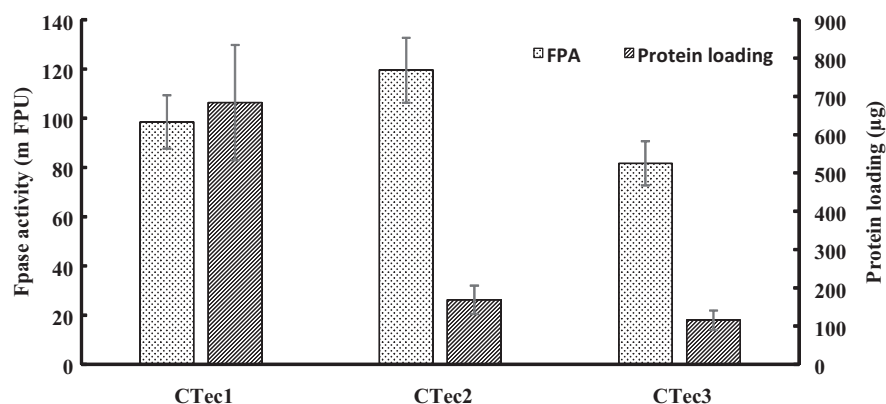


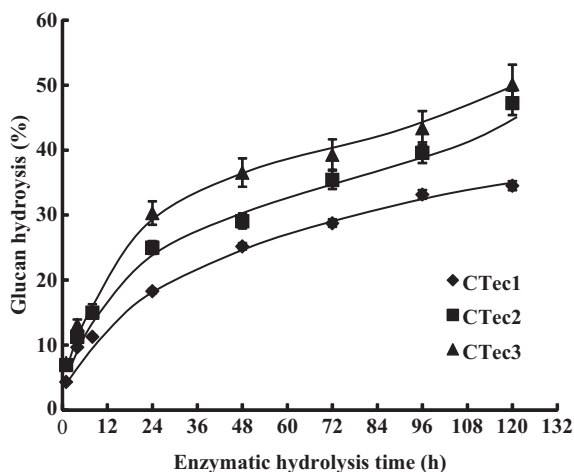
Fig. 12.3 FPA-based assay of three cellulase preparations, Ctec1, Ctec2, and Ctec3, at an oscillation (150 rpm) condition

of 2.0 mg reducing sugars at 60 min, the dosage of three 0.5 mL properly diluted cellulase preparations, Ctec1/2/3, was 0.0985 FPU, 0.1195 FPU, and 0.0817 FPU, respectively, which was far lower than that of 0.1850 FPU determined in the standardized FPA assay. Correspondingly, the protein loading of each enzyme dilution reduced by 48%, 35%, and 56% compared with that (1321 µg, 261 µg, and 261 µg) used in the original FPA assay. These data showed that the shaking activity of three cellulase preparations increased to a different degree. The oscillation enhanced the activity of Ctec3 greatly, thus resulting in a low enzyme dosage to produce equal reducing sugars. Results indicated that the FPA defined at a static hydrolytic condition did not reflect the actual activity of cellulase enzyme in the oscillation condition. Given that the practical enzymatic hydrolysis of “real-life” lignocellulosic substrate is engineered at the shaking condition to facilitate enzyme catalytic reaction, it is within the expectation that various cellulase preparations at an identical dosage would exhibit irrelevant saccharification results. Therefore, the FPA defined in the rigid condition cannot represent the real hydrolytic performance of cellulase preparation under a different condition.

3.3 Hydrolysis of SPCS 180 Similar to FPA Assay

In order to assess the enzyme performance on the natural lignocellulosic biomass, 50 mg SPCS was used as the substrate instead of filter paper strip, with 0.5 mL diluted cellulase enzyme preparation (0.1850 FPU). The hydrolysis profile is shown at Fig. 12.4. Even at an exact 60 min hydrolysis, the three cellulase enzymes, Ctec1/2/3, released 1.6 mg, 2.6 mg, and 2.8 mg, reducing sugar and resulted in 4.3%, 6.9%, and 7.3% hydrolysis of SPCS cellulose, respectively. During the time course

Fig. 12.4 Enzymatic hydrolysis of SPCS 180 with Ctec1/2/3 at 0.185 FPU (8.4 FPU/g glucan)



of hydrolysis, three cellulase preparations performed different hydrolysis profiles on SPCS, reaching 33%, 40%, and 44% conversion of cellulose at 96 h, respectively. Results indicate that the hydrolytic ability of cellulase preparations on filter paper substrate does not represent their performance on the pretreated lignocellulosic substrate, even at the standardized experimental condition.

4 Mechanistic Analysis of This Elusive Phenomenon

Unlike classical enzyme reaction, the cellulase enzymatic hydrolysis is an exceptionally complex course that involves heterogenous substrate and enzyme mixture. The natural lignocellulosic substrate has evolved complex structural and chemical recalcitrance of its structural sugars to the microbial and animal kingdoms. Moreover, various pretreatment (method and condition) before the enzymatic hydrolysis has been demonstrated to intensify the heterogeneity of substrates, though it can deconstruct the recalcitrant composition. The heterogenous substrate necessitates a large pool of glycoside hydrolases efficiently to depolymerize. These enzymes include cellulase hydrolases, mainly consisting of endo- β -1,4-glucanases (EG I, EG II, EG III, and EG IV), cellulose 1,4- β -cellobiohydrolase (CBH I and CBH II) and β -glucosidase (BG I, BG II), and hemicellulases (endo- β -1,4-xylanase (EX), β -xylosidase (BX), α -arabinosidase, α -glucuronidase, and esterase). These various hydrolases are capable of acting in concert to hydrolyze the lignocellulose. With no doubt, there is frequently not a simple relationship between the importance of an enzyme and substrate abundance, and the cellulase hydrolysis of natural lignocellulosic biomass dooms to be complex beyond the expectation.

4.1 Reasonable Analysis

The diversity of substrate is a primary reason that the FPA is not representative of actual cellulase hydrolysis profile (Pryor and Nahar 2010). Such discrepancy can be caused by two aspects, namely, physicochemical characteristics of the lignocellulosic biomass and active role of noncellulolytic enzymes contained in the cellulase preparation. To verify it, the main composition of these substrates was analyzed as shown in Table 12.1. For the filter paper substrate, the composition was pretty simple, which contained almost pure cellulose. However, in the natural lignocellulosic feedstock, there were some other components such as hemicellulose and lignin (Chen et al. 2011; Phitsuwan et al. 2013). Further, the content of various components was unequal. The SPSSB had a high glucan (55%) and lignin content (37%) with pretty low hemicellulose content (5%), while the SPCS180 contained an obviously high hemicellulose (23%) and lignin content (27%). In addition, the content of each composition was altered significantly and depends on the nature of biomass and the pretreatment strategy (Berlin et al. 2010; Galbe et al. 2012). Therefore, the composition of pretreated lignocellulosic feedstock was quite complex, very different from the simple cellulosic substrate as such filter paper (Demartini et al. 2013). The composition complexity of substrates intensified by various pretreatment resulted in their different hydrolytic profile even with the same enzyme, much less with different cellulase preparations. The result confirms the above presumption and at least partly explains that the FPA can't represent the actual activity of cellulase enzymes on native lignocellulosic biomass.

Besides, enzyme composition of three cellulase preparations, Ctec1/2/3, was analyzed. It was found that the cellulase preparation was tailored gradually in the variety and proportion of enzyme proteins to a realistic conversion of lignocellulosic biomass. And in the cellulase preparation, there are some enzymes, whose activity is not detected indeed with the traditional FPA, since the FPA is used at most to predict hydrolytic potential of the canonical cellulase enzymes such as CBH, EG, and β -glucosidase. These enzymes, even without FPA, can synergistically cooperate or not with canonical cellulase enzymes on filter paper substrates, and these synergistic effects would be dominant to a great extent on the pretreated lignocellulosic substrate by hydrolyzing their respective target substrate (Hu et al. 2011, 2013). Accordingly, it is credible that some accessory enzymes and helpful proteins involved deeply in lignocellulosic hydrolysis should be ignored to the FPA assay. This helps to explain well with some findings by Kabel et al. (2006) and Pryor and Nahar (2010) that the FPA of cellulase enzymes did not correlate well with their true hydrolytic ability to different lignocellulosic substrates. This is very probably the reason that these researchers support a rational customization of cellulase cocktails for particular feedstocks and pretreatments in order to optimize hydrolysis (Zhang et al. 2006; Berlin et al. 2010; Hu et al. 2013).

Based on these, some major accessory enzymes and proteins such as endoxylanase, β -xylosidase, and AA 9 (auxiliary activity) were directly added into the Ctec1 cellulase hydrolysis of SPSSB. As shown in Fig. 12.5, only with the cellulase Ctec1

Table 12.1 Chemical composition of pretreated lignocellulosic substrates

Substrate	Composition of pretreated feedstock (%)							
	Glucan	Xyl	Ara	Gal	Man	AIL	ASL	Moisture
FP	99.9	None	None	None	None	None	None	6.5 ± 0.4
SPP200	52.4 ± 1.6	6.3 ± 0.4	0.1 ± 0.0	0.4 ± 0.0	1.2 ± 0.1	31.6 ± 2.2	2.3 ± 0.9	61.1 ± 0.6
SPSSB	55.3 ± 1.7	4.4 ± 0.3	0.3 ± 0.0	0.1 ± 0.0	0.5 ± 0.0	35.5 ± 2.6	1.6 ± 1.1	76.9 ± 0.8
SPSC180	46.3 ± 1.4	19.8 ± 1.2	1.6 ± 0.1	0.7 ± 0.1	1.1 ± 0.1	24.1 ± 1.7	2.9 ± 0.9	71.7 ± 0.6

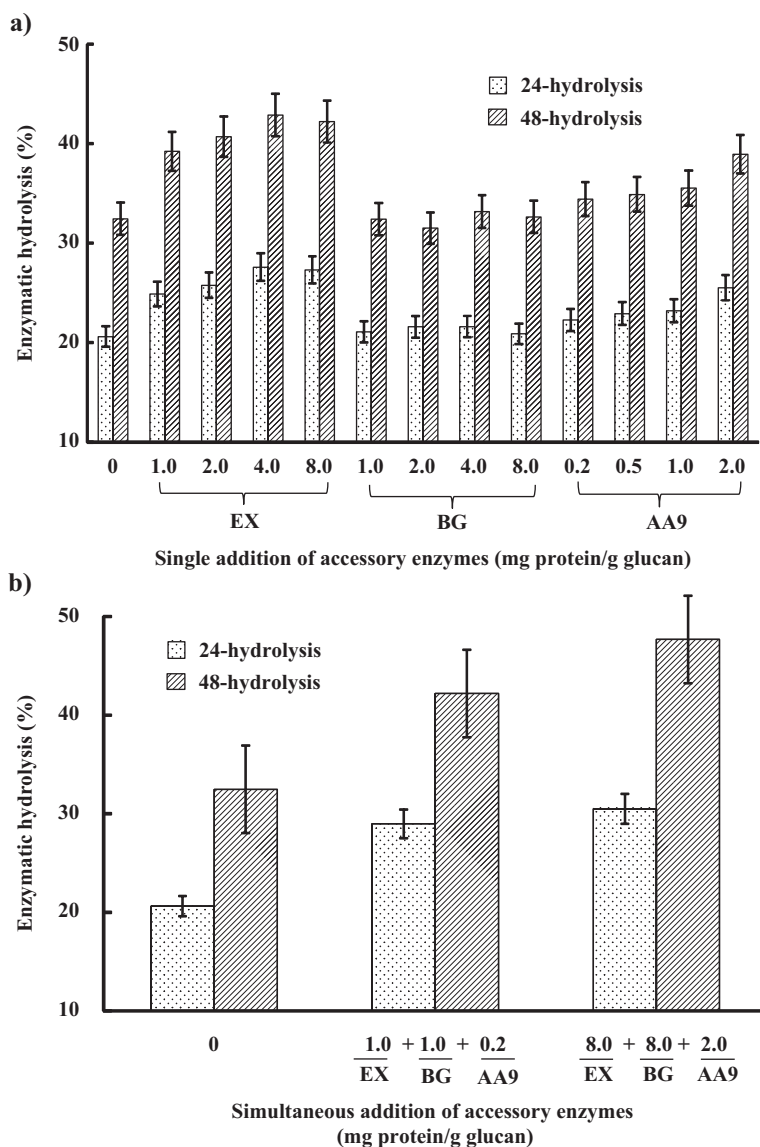


Fig. 12.5 Direct addition of accessory enzymes with Ctec1 to hydrolyze SPSSB

at 20 mg protein/g substrate loading, the glucan hydrolysis of SPSSB was 21% at 24 h and 33% at 48 h. The hydrolysis increased significantly with an addition of some accessory enzymes. With an addition of the endoxylanase preparation Htec2, the 48-h enzymatic hydrolysis reached a maximum of 43% at 4.0 mg g glucan. The data show that the xylanase activity of Ctec1 is deficient and an extra supply is necessary for the efficient hydrolysis of SPSSB, which also testify the above analysis

of specific activity of cellulase preparations. However, an addition of the Novozyme 188 (β -glucosidase preparation) at 1.0–8.0 mg/g glucan nearly had no obvious role on improving the hydrolysis, suggesting that the β -glucosidase activity was seemingly sufficient for Ctec1 to degrade the SPSSB. The result did not accord with the above analysis of specific activity because the analysis showed that the β -glucosidase activity of Ctec1 was weak as compared to the Ctec2 and Ctec3. To the present knowledge, it may be due to the physicochemical complexity of SPSSB, and the detailed information has yet to explore.

The AA9, formerly classified as GH61, is of copper-dependent lytic polysaccharide monooxygenases. As reported extensively, the AA9 is helpful for cellulase hydrolysis of lignocellulose by oxidative cleavage of crystalline cellulose region (Quinlan et al. 2011; Resch et al. 2013; Hu et al. 2014). In the work, the AA9 was also added with the Ctec1 to hydrolyze the SPSSB. With the addition of 0.20 mg AA9, the hydrolysis was 22% at 24 h and 33% at 48 h, which increased slightly as compared to that of no addition. With the more AA9 addition at 2.0 mg, the hydrolytic performance of Ctec1 was enhanced greatly, achieving 26% at 24 h and 39% at 48 h. These indicated that the AA9 addition exerted a positive role on the activity of Ctec1. The AA9 has significantly strengthened the cellulase enzymatic hydrolysis of lignocellulosic substrate. Finally, the combination of endoxylanase, β -glucosidase, and AA9 significantly improved the Ctec1 hydrolysis of SPSSB. With the simultaneous addition of 1.0 mg endoxylanase, 1.0 mg β -glucosidase, and 0.20 mg AA9 protein, the Ctec1 produced a hydrolysis high up to 29% at 24 h and 42% at 48 h, which was higher than that of the sole addition with 2.0 mg AA9 protein and almost equaled to that of 4.0 mg endoxylanase protein. The data confirmed that many accessory enzymes in the current cellulase preparation did play a synergistically cooperative role on cellulase hydrolysis of lignocellulose, though not reflected by the traditional FPA (Berlin et al. 2005; Hu et al. 2014).

For the complex composition of substrate, it is challenging to degrade one component with the sole or several enzyme activities (Chundawat et al. 2008; Berlin et al. 2010). Other components in the natural lignocellulosic biomass, particularly hemicellulose and lignin, exert significant restraints on cellulose hydrolysis (Berlin et al. 2010; Phitsuwan et al. 2013). For example, the lignin tends to bind enzyme components nonproductively, thus to reduce hydrolytic performance of cellulase components. Hemicelluloses and pectin probably restrict the access of cellulolytic enzymes by coating cellulose fibers. Accordingly, complete hydrolysis requires relatively large quantities of cellulase enzymes and also requires the cooperation between cellulases and several accessory enzymes with different catalytic targets (Berlin et al. 2005; Hu et al. 2011). Therefore, the same cellulase preparation with the same FPA might exhibit differences in performance on the different lignocellulosic substrate. And the preparations with different components also resulted in unequal sugar yields from the same substrate if they contain different varieties or amounts of accessory enzyme (Silveira et al. 2012; Hu et al. 2014). This can help to explain why the simple filter paper is not a good candidate for a standard substrate.

Summarily, since the FPA is assayed for the single cellulase to hydrolyze 4% of the uniform filter paper at a low consistency (3.2%) in a static condition for the exact 60 min, it dooms an end up of reflecting the hydrolytic potential and realistic conversion level of current “cellulase cocktail” on a complex matrix of lignocellulosic substrate at a shaking condition for commonly 24–48 h. In addition to the hydrolytic condition, the physicochemical complexity of substrates (cellulose/hemicellulose/lignin content and distribution) and the non-ignorable role of accessory enzymes (EX, BX, and AA9) in cellulase preparation are also responsible for this phenomenon.

4.2 Model Analysis of Enzymatic Lignocellulose Degradation

Modeling enzymatic hydrolysis kinetics is necessary to better characterize and understand the interaction between cellulase and lignocellulosic substrate, to integrate present and future enzyme improvement, and to design optimized process reactors and equipment. Also, developing models for enzymatic lignocellulose degradation help researchers clarify the data analysis. Therefore, a desirable model is of great importance for producing biofuels and biochemicals from lignocellulosic biomass.

Dozens of scientists have attempted to build the kinetics on enzyme degradation of lignocellulose (Kadam et al. 2004; Liao et al. 2008; Khamseh and Miccio 2010, Wang and Feng 2010, Griggs et al. 2012; Kostylev and Wilson 2013; Chien et al. 2014). A well-known hydrolysis model was based on Langmuir-type enzyme adsorption and Michaelis-Menten kinetics (Chien et al. 2014). However, these models are too complex for practical use. In 1951, the Weibull distribution was initially used to calculate the failure probability of metallurgical structure (Weibull 1951). Up to now, this model is still an important tool for application in various fields. With good surprise, Wang et al. (2015) reported that Weibull statistics can accurately predict the lignocellulose degradation process, regardless of the enzyme variety, substrate type, and saccharification condition.

In this model, the glucose production from enzymatic lignocellulose saccharification reactions can be expressed in the following equation (Wang et al. 2015).

$$\frac{Y}{Y_m} = 1 - e^{-\left(\frac{t}{\lambda}\right)^n} \quad (t > 0, \lambda > 0, n > 0) \quad (12.1)$$

In this equation, Y is the cumulative production of glucose, Y_m is the theoretically maximum glucose production, t is the reaction time, and λ and n are independent parameters (Wang et al. 2015). Furthermore, it has been proved that the λ value, defined as the time characteristic, means the overall performance of the hydrolysis system. Accordingly, the λ parameter maybe used to evaluate the overall performance of lignocellulosic hydrolysis. These models are helpful to make clear the

various factors that are at play, but these models that have been reported have not pinpointed the exact role mechanism of enzymes acting on lignocellulosic substrates (Bansal et al. 2009).

However, the lignocellulose degradation process is very complicated because the degradation involves multicomponents (cellulose, hemicellulose, and sometimes pectin) in the heterogenous substrates and tens of enzyme mixtures (CBHs, EGs, BGs, and some accessory enzymes) in a common cellulase preparation. The complication is intensified by the synergistic reactions occurring between different varieties of enzymes. Thus, such the complex hydrolysis environment has posed formidable challenges in developing the modeling (Zhang et al. 2009). For example, there is not yet a clear relationship between the hydrolysis rates obtained on soluble and insoluble substrates, mainly because of large variations in limited solid substrate accessibility to cellulose (Zhang et al. 2009). A functionally based model has been developed to suggest the complexity among endoglucanase, exoglucanase, their ratio, cellulose accessibility, DP, enzyme concentration, and reaction time (Zhang and Lynd 2006).

5 Concluding Remarks

As reported previously, the *T. reesei* genome possesses 200 glycoside hydrolases, in which above 20 of them can be found in the secretome. Except several better-characterized cellulases and hemicellulases, most of these glycoside hydrolases are recognized to extremely limited extent in deconstructing lignocellulosic substrates. Consequently, the way forward for the development of more efficient lignocellulose-degrading enzyme preparations will require deeper and more precise knowledge about the specific enzymes that are involved in the degradation of lignocellulose (Banerjee et al. 2010). However, it is not possible to gain this knowledge working only with partially defined complex mixtures (Banerjee et al. 2010). In the future, huge efforts should be made to elucidate the hydrolytic performance of each specific enzyme/proteins in the cellulase preparation acting on the substrate, especially the lignocellulosic biomass.

At that time, it can be envisioned that huge experimental data would be produced from a common cellulase hydrolysis process. To deconvolute the data, mathematical modeling that is supposed to consider more substrates and enzyme properties is an important tool of the hydrolysis process. A robust model is also necessary to elucidate the hydrolysis process. At all events, modeling the kinetics of the lignocellulosic substrate hydrolysis process is encountered with the below two aspects of main challenge: (1) to obtain a deep insight to the enzyme-related factors contributing to the hydrolysis (enzyme concentration, cellulase composition, adsorbed cellulase concentration, synergism) and substrate-related variables (substrate concentration, accessibility, adsorption capacity, polymerization degree, and crystallinity) and (2) to identify rate-limiting factors (Bansal et al. 2009). In short, a desirable cellulase kinetics on insoluble lignocellulosic substrates is under control

of the above two aspects of the hydrolysis process, which is also supposed to influence by other factors (product inhibition, enzyme deactivation, substrate crystallinity, substrate accessibility changes, substrate reactivity changes, fractal nature of the reaction, changes in enzyme synergism, lignin inhibition). Additional insight will be made possible by models consisting of the major substrate and enzyme properties (substrate concentration, DP, accessible fraction, size distribution of chains, crystallinity, enzyme concentration, synergistic/competitive factors, and adsorbed concentration of individual components) (Bansal et al. 2009).

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Chapter 13

Substrate Factors that Influence Cellulase Accessibility and Catalytic Activity During the Enzymatic Hydrolysis of Lignocellulosic Biomass



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Abstract The development of a renewable, biomass based, “biorefinery” process for fuels and chemicals will be crucial if we are to transit to a more environmentally friendly economy. However, the limited efficacy of the “cellulase mixture” to break down the polysaccharides within lignocellulose into sugar platform is still the bottleneck. Although the catalytic activities of cellulases are comparable with other polysaccharide-degrading enzymes such as amylases, the hydrolytic potential of cellulase enzymes toward pretreated lignocellulosic substrates is much lower. This is primarily due to the limited accessibility of the enzymes to most of the glycosidic bonds and the inhibitory compounds naturally existed and/or derived from biomass deconstruction process.

In this chapter, the major substrate characteristics of pretreated biomass (e.g., gross fiber characters, lignin/hemicellulose content/location, and cellulose allomorph/crystallinity/DP) that influence the accessibility and the hydrolytic performance of cellulase enzymes will be systematically discussed, in combination with various methods that have been used to quantify the changes in the accessibility of lignocellulosic substrate at the macroscopic (fiber), microscopic (fibril), and nanoscopic

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(microfibril) levels. In addition, the influence of potentially inhibitory biomass-derived soluble compounds on the slowdown of enzymatic hydrolysis, as well as their possible inhibitory mechanisms such as reversible/irreversible inhibition and adsorption/precipitation of the major enzyme activities (exo-/endo-glucanase, β -glucosidase, xylanase activities, etc.), will be elucidated. The possible solutions/strategies to improve cellulose accessibility and to overcome various inhibitors will be also introduced. This chapter will show how overall protein/enzyme loading required to achieve effective cellulose hydrolysis can be significantly reduced by tailoring enzyme mixture for different biomass substrates and the various types of pretreatment used.

Keywords Pretreatment · Cellulose hydrolysis · Enzyme synergism · Enzyme inhibition

1 Background

Concerns about increasing levels of carbon emissions, global warming, and energy security have encouraged governments around the world to pursue alternative, sustainable clean energy strategies (Sims et al. 2010). In the same way that oil, coal, and gas are composed of finite hydrocarbons, the carbohydrates in sustainably produced biomass found in forests and agricultural crops can be used as the feedstock for “bioconversion technologies” that can be the basis of sustainably derived products such as bioenergy, biomaterials, and biochemical (Stephen et al. 2012). However, the utilization of biomass for biofuels or biochemicals first requires the breakdown of the carbohydrates within the biomass to their component sugars. In nature, fungi, bacteria, and other microorganisms produce enzymes to break down these sugar polymers (Lynd et al. 2002; Wilson 2009). However, because these polymers serve as structural materials in plants, they have evolved to withstand fast and effective microbial/enzymatic degradation (Himmel et al. 2007). Consequently, in order to efficiently utilize this biomass, the natural degradation process that normally occurs over a long period of time (typically several years) needs to be shortened into a matter of days or even hours (Sims et al. 2010; Stephen et al. 2012). Over the past 50–60 years, many excellent research groups have been studying ways to accelerate this biodegradation process to enable technical, and hopefully economic, biomass conversion to these higher value products. It is widely acknowledged that an efficient biomass pretreatment process is required to not only recover as many of the original biomass components (lignin, hemicellulose, cellulose, extractives, etc.) in a useable form but to also “open up” the biomass matrix and increase enzyme access to the cellulosic component, with a limited production of inhibitory components for the downstream biological conversion processes. In parallel, a robust enzyme cocktail that can quickly and efficiently break down the cellulose component at high substrate and low protein concentrations is also an essential requirement if we are to establish an economically viable biomass-to-fuels/chemicals industry (Himmel et al. 2007; Sims et al. 2010; Gao et al. 2011).

In this chapter, we have briefly discussed the nature of the recalcitrant characteristics of lignocellulosic biomass and the major strategies (e.g., biomass pretreatment, enzyme cocktail optimization, and detoxification) that have been applied to overcome these biomass recalcitrance. In particular, the collective research of our Forest Products Biotechnology/Bioenergy (FPB) Research Group is described, summarizing how we have tried to better understand how the forest and agricultural residues might be efficiently broken down into useful sugars for the production of “green” fuels and chemicals.

2 Biomass Recalcitrance and Pretreatment

Plants are composed of various cell types with a thick and complex cell wall structure to protect the plant from biological, chemical, and physical attacks by the microbial and animal kingdoms, imparting a high level of biomass recalcitrance (Himmel et al. 2007). Biomass recalcitrance is a complex phenomenon that is governed by various physicochemical properties. Among them, several biomass properties, such as the heterogeneity of the cell wall constituents (e.g., the type, amount, and distribution of hemicellulose and lignin) and the highly organized cellulose structure, significantly restrict the accessibility of cellulolytic enzymes to cellulose (Himmel et al. 2007; Gao et al. 2011).

In cellulose, every glucose molecule interacts through four intramolecular hydrogen bonds with the adjacent two glucose molecules in the same glucan chain and also through two intermolecular hydrogen bonds between chains in the same layer (Lynd et al. 2002; Saxena and Brown 2005). The inter- and intra molecular hydrogen bonds keep the chains straight and stacked in a sheetlike structure. Although no hydrogen bonds are found between the sheets, van der Waals forces appear to hold the sheets together (Saxena and Brown 2005). Cellulose forms a highly organized, crystalline structure (interspersed by some disorganized amorphous or paracrystalline regions) under these strong hydrogen bonds and van der Waals forces (Cosgrove 2005). This restricts the penetration of enzymes or even small molecules, like water, into the structure entities (Arantes and Saddler 2010). It has been proposed that the tightly packed crystalline regions are one of the major reasons for the low saccharification efficiency of cellulose, due to the limited accessibility of cellulase enzymes (Arantes and Saddler 2010; Chundawat et al. 2011).

In the plant cell wall, individual cellulose chains join together, shortly after biosynthesis, into elementary microfibrils, which consist of about 36 cellulose chains (3–5 nm in diameter). The elementary microfibrils are further packed into a hemicellulose-lignin matrix to form the rigid cell wall structure (Somerville et al. 2004; Cosgrove 2005). These noncellulosic components further increase the difficulty of the cellulose bio-decomposition process by limiting the accessibility of cellulases to cellulose, causing unproductive adsorption of cellulase enzymes, and inhibiting the catalytic activity of cellulase enzymes (Lynd et al. 2002; Zhang and Lynd 2004).

Table 13.1 Summary of various pretreatment technology used for lignocellulosic biomass

Pretreatment technology	Examples	Major effects	Advantages	Disadvantages
Biological	White-/brown-rot fungi	Degradation of lignin and hemicellulose	Low energy input, environmentally friendly	Very slow
Physical	Ball milling	Reduce particle size, decrease cellulose crystallinity	Suitable for various substrates, no chemical input	Large energy consumption
Chemical	Dilute acid, alkaline, organosolv, ionic liquid	Hemicellulose degradation, lignin transformation/delignification, saponification of intermolecular ester bonds	Improve cellulose accessibility/digestibility	Equipment corrosion, sugar degradation, toxic substrates, difficult to recover chemicals
Physiochemical	Steam explosion, ammonia fiber expansion (AFEX)	Disrupt lignin carbohydrate complex (LCC), hemicellulose degradation, decrystalline cellulose	Short time, cost-effective, improve cellulose accessibility/digestibility	Sugar degradation, produce inhibitors for the following biological process

Due to the recalcitrant biomass characteristics discussed above, a pretreatment step is usually first required to disrupt the highly organized cell wall structure before the bioconversion process. Ideally, the pretreatment should be performed at conditions that allow the various biomass components to be fractioned and recovered in an effective manner while “opening up” the cellulosic fraction to enhance the rate and extent of enzymatic hydrolysis (Chandra et al. 2007; Yang et al. 2011). Pretreatment strategies have generally been categorized into biological, physical, and chemical processes or a combination of these approaches (Table 13.1). Some of the advantages and disadvantages of different strategies are also listed in Table 13.1. Among them, pretreatments that combine both chemical and physical processes are generally referred to as physiochemical processes, and this type of pretreatment has received most attention in recent years. For example, steam explosion with the addition of chemical catalyst such as SO_2 has shown significant advantages for generating easily hydrolysable substrates from most potential lignocellulosic biomass substrates (Chandra et al. 2007; Arantes and Saddler 2011). Unfortunately, many pretreatment conditions that are optimized to result in easily digestible cellulose (usually at quite high severity) typically result in significant loss of the hemicellulose component, which can account for 20–40% of the plant cell wall polysaccharides (Bura et al. 2003; Chandra et al. 2007). The degradation of the hemicelluloses not only reduces the sugar yield of the process but also results in the formation of degradation products that can be inhibitory to the hydrolysis step but particularly to the yeast used in the subsequent fermentation of the sugars to biofuels such as ethanol (Palmqvist et al. 1996; Ohgren et al. 2007). Lower-severity pretreatments allow for the preservation of hemicelluloses in a useable, insoluble form, resulting in an

increase in overall sugar recovery. However, the following enzymatic hydrolysis of the cellulosic component becomes more difficult due to the recalcitrant nature of the remaining, lightly pretreated substrate (Chandra et al. 2007). Therefore, it is crucial for developing pretreatment methods that both maximize recovery of the lignin and hemicellulose components in a useable form while enhancing cellulose accessibility to the cellulase complex.

3 Substrate Characteristics that Influence Cellulose Hydrolysis

After biomass pretreatment, many substrate properties such as lignin/hemicellulose content and location, gross fiber properties, and cellulose crystallinity (CrI)/degree of polymerization (DP)/accessibility have been considered for influencing cellulose hydrolysis (Hu et al. 2014). The measurement of these substrate physicochemical characteristics have been established in the past several decades. For example, the chemical composition and gross fiber properties of the substrates can be assessed by the Klason protocol and fiber quality analysis (FQA), respectively, while the cellulose crystallinity (CrI) and degree of polymerization (DP) can be measured by X-ray diffraction (XRD) and gel permeation chromatography (GPC), respectively. However, it has been difficult to accurately assess certain substrate properties and also been challenging to draw a solid line between these substrate properties and the hydrolyzability of pretreated lignocellulosic biomass. For instance, although the determination of cellulose CrI and its relationship with cellulose hydrolyzability has been investigated at a long time, it is still unclear which CrI determination method is more accurate and whether or not the CrI measured by currently available methods can predict the digestibility of cellulose by cellulase enzymes (Zhang and Lynd 2004; Park et al. 2010). A recent study (Park et al. 2010) compared the most commonly used methods for determining cellulose CrI (e.g., XRD, NMR, infrared (IR), and Raman spectroscopy) and emphasized that even the relatively more accurate methods such as XRD may not be capable of extracting exact information of crystal lattices within the cellulose structure. In addition, considering that the cellulose CrI of pretreated biomass represents the relative amount of crystalline material in the substrates (Nishiyama et al. 2002), CrI may not be able to predict the trend of cellulose crystallinity of different pretreated substrates as they contain different amounts of noncellulosic components. Therefore, the results regarding the changes of cellulose CrI during enzymatic hydrolysis are often contradictory in the literatures, especially for the pretreated lignocellulosic biomass. Thus, other cellulose properties (e.g., accessibility, DP, particle sizes, etc.) should also be considered for predicting cellulose hydrolyzability.

We believe the limited cellulose accessibility to cellulase enzymes is the major rate-limiting factor for cellulose hydrolysis. However, unlike other substrate properties, the assessment of cellulose accessibility has been tricky. Various methods have been developed to assess substrate accessibility such as nitrogen adsorption, mer-

cury porosimetry, solute exclusion, water retention, NMR cryoporometry, and protein adsorption (Gourlay et al. 2012). But most of these techniques involve a drying process during sample preparation that greatly changed the accessibility of cellulose within the biomass due to the fiber collapse and hornification. In the past decades, we have tried to develop more novel methods to better quantify the specific cellulose accessibility of lignocellulosic substrate at the macroscopic (fiber), microscopic (fibril) and nanoscopic (microfibril) level. As examples we have pioneered the use of the modified Simons' staining (SS) technique (Chandra et al. 2007) and the cellulose-binding module (CBM) assays (Gourlay et al. 2015), to help better quantify changes in cellulose accessibility after both pretreatment and enzymatic deconstruction.

Briefly, Simons, staining assay involves the competitive adsorption of a large (>100 kDa) Direct Orange dye with a higher affinity for cellulose vs a small (998 Da) Direct Blue dye which has a lower affinity for cellulose. This assay can be used to quickly assess the degree of cellulose accessibility (i.e., porosity) in pretreated biomass substrates (Chandra et al. 2016). Beside Simons, staining assay, we have also recently developed a highly specific technique using cellulose-binding modules (CBMs) as probes to quantify the relative amounts of accessible amorphous and crystalline regions present in cellulosic substrates (Gourlay et al. 2015). Two different CBMs, one (CBM2a) including a planar-binding face that preferentially adsorbs to crystalline cellulose and another (CBM17) with a cleft-shaped binding site that preferentially adsorbs to amorphous cellulose, have been applied to substrates. The total amount of both CBMs adsorbed can be used to determine cellulosic accessibility. The ratio between the two adsorbed CBMs can also be used to estimate the specific surface area of accessible cellulose. In addition, the two CBMs are fused with different fluorescent probes, and their binding profile can be monitored using confocal microscopy along the length of the cellulosic fibers. These relatively novel techniques will be paralleled with methods such as microscopy, solute exclusion, and water retention to better elucidate the putative barriers to effective biomass deconstruction and to define how the pretreated substrate and enzyme cocktail can be modified to improve cellulose accessibility.

4 Enzyme Synergism

A significant amount of research has been carried out to both improve the efficiency of the pretreatment step and to improve substrate accessibility to enzymes. However, high enzyme loadings are still typically required (Wilson 2009; McMillan et al. 2011), which contribute to the high costs incurred in the production of biomass-derived biofuels. Past work has generally pursued four main strategies to try to reduce the cost of the enzymatic hydrolysis step either by minimizing the costs of enzyme production (Wilson 2009), and increasing the hydrolytic performance of the enzymes (Gao et al. 2011), or by trying to recycle the enzymes for multiple

rounds for hydrolysis (Gregg and Saddler 1996; Tu et al. 2007), or by minimizing enzyme inhibition (Zhai et al. 2016).

Over the last decade, biotechnology companies, such as Novozymes, Genencor International, etc., have reported a significant decrease in the cost of enzyme production, but it is generally recognized that a further three- to fivefold reduction is still needed in order for cellulosic ethanol to be economically feasible (Aden and Foust 2009; Humbird et al. 2010). However, the further cost reductions for enzyme production has been predicted to be challenging (Wilson 2009; Stephen et al. 2012). Alternatively, the strategy to make better “cellulase cocktails” by identifying the essential enzymes and optimizing their ratios has received considerable attention over the last few years. Most of the initial studies that investigated the optimization of cellulose combinations focused on the hydrolysis of “model” cellulosic substrates such as cotton fiber, Avicel, and filter paper (Baker et al. 1998; Kim et al. 1998; Boisset et al. 2001; Gusakov et al. 2007). However, it has been realized that the optimized cellulase mixtures based on cellulosic substrates could not reflect their hydrolysis potential on “real-life” pretreated lignocellulosic biomass (Meyer et al. 2009; Gao et al. 2010). Thus, it appears that the enzyme mixture should be tailored for the particular lignocellulosic biomass and the specific pretreatment strategy (Berlin et al. 2007; Banerjee et al. 2010).

One way to reduce the amount of enzyme usage is to improve the hydrolytic efficacy of “cellulase” mixtures. Although there has been a considerable amount of work done on improving individual cellulase properties such as binding affinity, catalytic activity, and thermostability (Percival Zhang et al. 2006; Wilson 2009), in most cases the extent of improvement achieved on simple, model cellulosic substrates does not directly translate into improved activity on the more complex, heterogeneous, lignocellulosic substrates (Percival Zhang et al. 2006). In parallel, an alternative strategy has involved utilization of the synergistic cooperation among cellulase monocomponents and various accessory enzymes such as xylanases and AA9/LPMO (Wilson 2009; Harris et al. 2010; Hu 2013, 2014; Hu et al. 2015a, b). This approach has been shown to increase enzyme accessibility to the cellulose and therefore the overall “specific activity” of the enzyme mixture.

Several groups have tried to optimize the “cellulase cocktail” on pretreated biomass (Meyer et al. 2009; Banerjee et al. 2010; Gao et al. 2010). Although different optimization strategies and statistical design software were used in different studies, the common process was identical, as summarized in the flowchart (Fig. 13.1). In short, the essential cellulase enzymes, namely, “core cellulases,” were first identified by fractional factorial design, and then the required accessory enzymes were selected based on their ability to enhance the hydrolytic performance of the “core cellulases” on the specific pretreated substrates. After the “core cellulases” and the required accessory enzymes were identified, the optimized enzyme cocktail was formulated by ratio design of these enzyme components. Finally, the hydrolytic potential of the optimized enzyme cocktail was compared with commercially available cellulase mixtures on the pretreated biomass.

In summary, optimizing “enzyme cocktails” by combining “core cellulases” and accessory enzymes provided valuable information to help better understand the

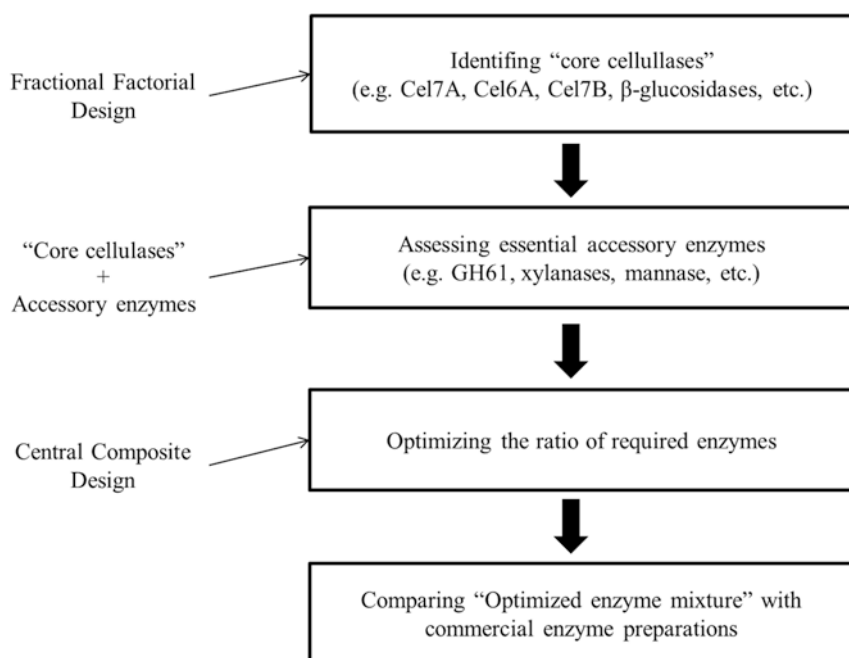


Fig. 13.1 General process for optimizing an “enzyme cocktail” for lignocellulose deconstruction

essential enzymes required for efficient biodegradation of various pretreated biomass and it also significantly improve the efficiency of substrate hydrolysis (Hu et al. 2014). It appears that CBHI, CBHII, and EGI are core enzymes for cellulose hydrolysis, while EGI, xylanase, and BX are the essential ones for xylan hydrolysis. A sufficient amount of BG was an essential requirement in all the optimized mixtures. Accessory enzymes such as LPMO AA9 and xylanases could significantly improve the hydrolytic efficiency of cellulases on a range of pretreated biomass substrates. Small amounts of other accessory enzymes such as arabinofuranosidase (Arb), glucuronidase (Gl), and mannanase, depending on the type of pretreated biomass, also contributed to cellulose hydrolysis. Selecting the “right” enzyme components is crucial to achieve a fast and efficient hydrolysis of pretreated biomass.

5 Enzyme Inhibition

Although pretreatment process are necessary to disrupt the cell wall structure of lignocellulose, it will also generate various degradation products that are strongly inhibitory to the following enzymatic hydrolysis. This, in turn, add extra difficulties for the deconstruction of pretreated biomass owing to the “toxicity” of these inhibitors to the enzyme mixture (Jing et al. 2009; Kim et al. 2011; Cantarella et al. 2014;

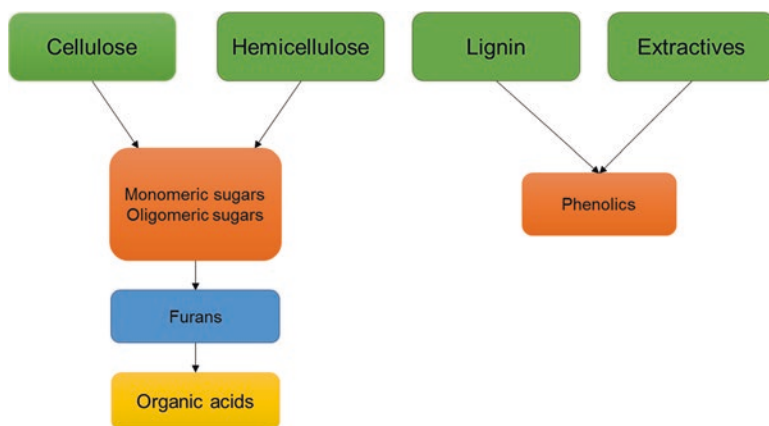


Fig. 13.2 Formation of inhibitors during acid-catalyzed pretreatment of lignocellulosic biomass. (Jönsson et al. 2013)

Humpula et al. 2014). Generally, these inhibitory degradation products include the mono-/oligomeric sugars derived mainly from hemicellulose and the various lignin/sugar degradation products (Jönsson et al. 2013; Jönsson and Martín 2016) (Fig. 13.2). Monomeric sugars (such as xylose, mannose, galactose, and glucose) and solubilized oligomeric sugars (such as glucooligomeric, xylooligomeric, and mannooligomeric sugars) are the major components presented in the liquid phase after thermochemical pretreatment such as steam explosion. In addition, phenolic compounds, generated from both lignin and extractives, are another major group of water-soluble components that generated during steam pretreatment (Jönsson et al. 2013). However, the phenolic content and composition are highly dependent on the nature of the biomass (e.g., the p-hydroxyphenyl/guaiacyl/syringyl (H/G/S) ratio of the original biomass lignin) as well as the pretreatment condition applied (Klinke et al. 2004). Besides sugars and phenolics, organic acids such as formic and levulinic acids and furans such as furfural and hydroxymethylfurfural (HMF) are also generated due to hexose and pentose degradation during pretreatment process (Palmqvist and Hahn-Hägerdal 2000; Panagiotou and Olsson 2007; Jönsson et al. 2013). The HMF and furfural are formed due to hexose and pentose sugar degradation, respectively, while formic acid and levulinic acid are formed due to furfural and HMF degradation, respectively (Palmqvist and Hahn-Hägerdal 2000; Duarte et al. 2012).

Influence of Monomeric Sugars Monomeric sugars derived from biomass include the hexoses (mannose, galactose, and glucose) and the pentoses (arabinose and xylose). Of the five wood-derived monomeric sugars, the inhibitory effect of glucose on cellulases has been the most extensively studied. Competitive inhibition (Asenjo 1983; Mosier et al. 1999), noncompetitive inhibition (Gusakov and Sinitsyn 1992; Philippidis et al. 1993), and uncompetitive inhibition (Fan and Lee 1983) have all been proposed to describe the inhibition mechanism. However, it appears that there is no general agreement on the type of inhibition mechanism so far. It has

been suggested that these inconsistent and sometimes contradictory results could be due to the varied experimental conditions such as different pH, temperature, solid loadings, and initial glucose concentration (Andrić et al. 2010b). Recently, high concentrations of glucose showed to also inhibit enzyme adsorption on cellulose, which might be another factor causing enzyme inhibition (Kristensen 2008). For example, the recent work from our group showed that high concentration of glucose could reduce the amount of adsorbed Cel7A on cellulosic substrate (Pribowo et al. 2013). It has been suggested that high concentration of monomeric sugars could interfere with adsorption of both catalytic domain and binding module of Cel7A to the cellulosic substrate (Pribowo et al. 2013). In another study, it was reported that a decline in enzyme-binding capacity seemed to be associated with increasing concentrations of glucose and cellobiose in high-solid hydrolysis (Wang et al. 2011). Overall, it is likely that glucose not only inhibits cellulases activities but also inhibits enzyme adsorption to the cellulose.

Influence of Oligomeric Sugars Oligomeric sugars can be generated either during pretreatment or enzymatic hydrolysis (Shevchenko et al. 2002). The inhibitory oligomeric sugars that have been reported so far have been primarily xylooligomers and mannooligomers (Qing et al. 2010; Zhang and Viikari 2012; Kumar and Wyman 2014). The inhibitory effect of isolated xylan from woody biomass on enzymatic hydrolysis of cellulosic substrate was shown in 1983 (Mes-Hartree and Saddler 1983). In this study, the presence of xylan decreased enzymatic hydrolysis by ~30% (Mes-Hartree and Saddler 1983). After that, a series of studies looked at the interaction of hemicellulose and cellulose and how hemicellulose affects the catalytic action of cellulases (Hannuksela et al. 2003; Hannuksela and Holmbom 2004). It seems that hemicellulose can interact strongly with cellulose therefore limiting enzyme accessibility to the cellulose. Qingqing et al. reported that xylooligomers (XOS) from the commercial birchwood xylan were also strong inhibitors of cellulase enzymes (Qing et al. 2010). Based on these results, it seems that even at low concentration (2 g/L), xylo-oligosaccharides (XOS) can cause strong inhibition of cellulase activities. However, most of previous studies have only used the “model” xylooligomers, which may not represent the real structure of the oligomers derived from pretreated process. Recently, we have observed that the strong inhibition effect of oligomeric sugars also existed in the pretreatment liquid derived from hydrothermal pretreated corn stover on both traditional cellulase mixture Celluclast and the newest cellulase preparation CTec3 (Fig. 13.3). And it appeared that these oligomers were even more inhibitory than the monomeric sugars since the breakdown of these oligomers to monomers through a mild acid treatment could reduce their degree of inhibition to certain extent (Fig. 13.3).

There are two proposed mechanistic interpretations of the possible inhibitory effect of XOS obtained from commercial birchwood xylan. One is that XOS bind to the cellulose surface, thus restricting the accessibility of cellulose to cellulases (Qing and Wyman 2011; Zhang et al. 2012). The XOSs with a higher degree of polymerization (DP) showed stronger binding affinity toward cellulose, resulting in strong cel-

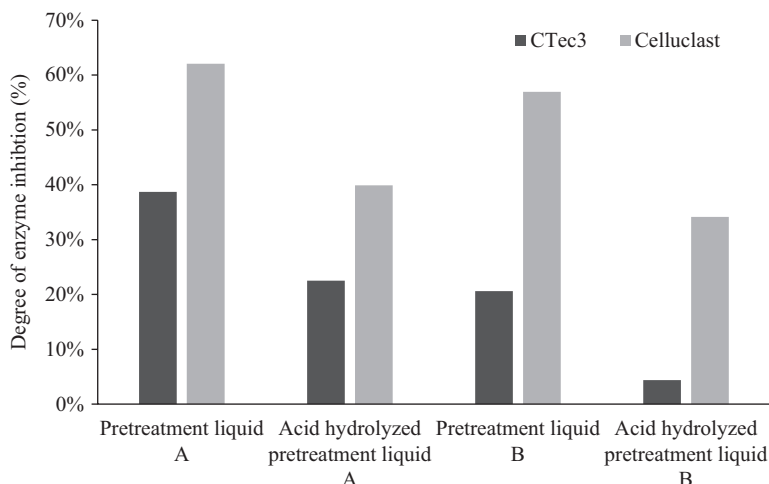


Fig. 13.3 Effect of oligomeric sugars derived from dilute acid pretreated corn stover on cellulose hydrolysis

lulase inhibition (Qing and Wyman 2011). The other proposed mechanism was that the XOS's could competitively bind the active sites of cellulases, leading to a decrease in the formation of productive enzyme-substrate complex (Baumann et al. 2011; Zhang and Viikari 2012). These studies reported that the XOSs could mimic the structure of cellulose and thread into the catalytic tunnel of Cel7A, resulting in nonproductive binding (Baumann et al. 2011). Based on the mechanisms proposed from experimental results using XOS's from birchwood xylan, it is likely that the DP and structure of the XOS's will influence their inhibitory effect on enzymes (Baumann et al. 2011). However, as mentioned above, the XOS's obtained under acid pretreatment conditions may differ in chain structure and degree of polymerization from the XOS's obtained from commercial xylan as the commercial birchwood xylan is usually isolated from birchwood with alkaline extraction, which has already removed the side chains of the original hemicellulose and therefore decrease the xylan solubility in aqueous solution (Christov and Prior 1993). The differences in side chains and degree of polymerization between extracted birchwood xylan and XOSs generated during pretreatment will further affect the binding properties of XOS, not only to cellulose but also to the various enzyme components (Baumann et al. 2011; Köhnke et al. 2011).

Influence of Non-sugar Components The presence of some non-sugar components within the water-soluble fraction after pretreatment has also been reported to be inhibitory to enzymatic hydrolysis. The non-sugar components are primarily composed of furans, organic acid, and phenolics (Mes-Hartree and Saddler 1983; Takagi 1984; Tengborg et al. 2001; Jing et al. 2009; Kothari and Lee 2011). Among these non-sugar inhibitors, the phenolics are known to have the strongest inhibitory effect on enzymatic hydrolysis, even though their concentration is relatively low

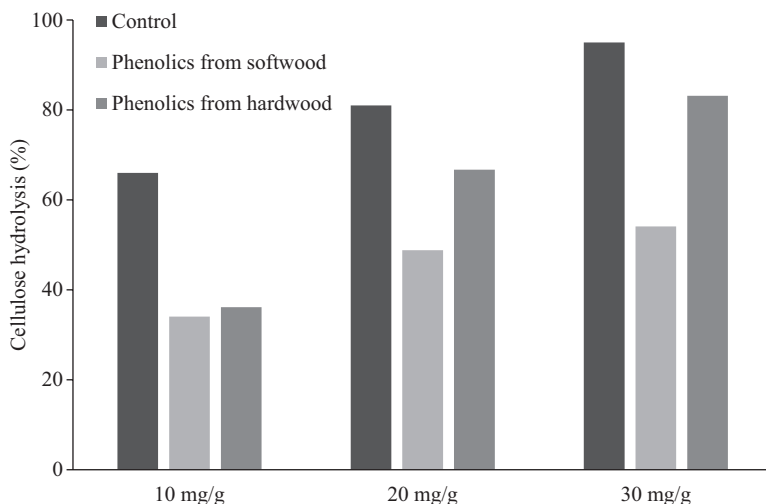


Fig. 13.4 Effect of pretreatment-derived phenolics on cellulose hydrolysis by using the newly developed enzyme CTec3

compared with other inhibitory products such as acetic acid (Tengborg et al. 2001; Hodge et al. 2008; Kothari and Lee 2011; Kim et al. 2011). The inhibitory effect of phenolics (sunflower polyphenols) on cellulases was first reported in 1975 (Sineiro et al. 1997). Although it has been often found that phenolics could directly inhibit cellulose hydrolysis (Kim et al. 2011), other studies have also showed some contradictory results when using “model” phenolics as inhibitors (Cantarella et al. 2004a; García-Aparicio et al. 2006; Hodge et al. 2008). For example, soluble phenolics extracted from hot water pretreated maple wood showed strong inhibition of cellulose hydrolysis, but the “synthetic phenolics” (e.g., vanillin, syringaldehyde, 4-hydroxy-benzaldehyde, catechol, guaiacol, 4-hydroxycinnamic acid, and 4-hydroxybenzoic acid) were used as inhibitors, and a slight or no inhibitory effect on cellulose hydrolysis was observed (Cantarella et al. 2004a; Hodge et al. 2008). By using the pretreatment-derived phenolics from woody biomass, we have also found that the soluble phenolics were highly inhibitory to the cellulose enzymatic hydrolysis (Fig. 13.4). Thus, it appears that the “real-life” pretreatment-derived phenolics, but not the simple “model” phenolic components, are highly inhibitory to the enzyme-mediated cellulose hydrolysis.

There are so far two proposed mechanisms to explain the inhibitory effect of soluble phenolics on cellulose hydrolysis, namely, enzyme inhibition and enzyme deactivation. Enzyme inhibition refers to the inhibition of cellulase, β -glucosidases, and xylanase activities by phenolics. Simple phenolics like vanillic acid, syringic acid, and acetosyringone seemed to inhibit xylanase activity (Ximenes et al. 2010). The other mechanism of inhibition is the enzyme deactivation or precipitation by polymeric phenolics. It was hypothesized (Tejirian and Xu 2011; Ximenes et al.

2010) that polymeric phenolics seem to have a strong affinity to enzymes and may lead to enzyme deactivation/precipitation. It showed that tannic acid (polymeric phenolics) could irreversibly bind to cellulases and cause an exponential decrease of enzyme activities (deactivation/precipitation) with an increased contact time between the inhibitors and enzymes (Ximenes et al. 2010).

Strategies to Minimize Enzyme Inhibition Extensive studies have been carried out on detoxification methods which have mainly involved physical, chemical, and biological detoxification, in situ detoxification, and in situ microbial detoxification (Jönsson et al. 2013). Some of these strategies include enzyme treatment and chemical treatment (Cantarella et al. 2004b; Soudham et al. 2011). Enzyme addition methods can be used to improve the synergism of enzyme cocktails or remove inhibitors. For example, in order to alleviate product inhibition, substantial BG is commonly supplemented into cellulase mixtures to ensure the breakdown of cellobiose, which is a strong inhibitor of CBH (Kumar and Wyman 2009; Andrić et al. 2010a). To remove phenolics generated during pretreatments, laccase was applied to enhance enzymatic hydrolysis of rice straw. It was shown that with laccase treatment, the inhibition by pretreatment liquors on CBH, EG, and BG was greatly alleviated (Niu et al. 2009).

Chemical treatments have also been applied to improve enzymatic hydrolysis efficiency. Addition of specific reducing reagents has been shown to alleviate the inhibition (Soudham et al. 2011). It was shown that reducing agents such as the sulfur oxyanions dithionite and sulfite can improve enzymatic hydrolysis in the presence of pretreatment liquid (Soudham et al. 2011). However, the specific mechanism is still unknown. Surfactants have been shown to prevent detrimental tannin adsorption onto cellulose to form tannin-enzyme complexes, thus alleviating the inhibition caused by tannic acid (Tejirian and Xu 2011). Overliming with calcium hydroxide is also capable of alleviating inhibition with the incorporation of a washing step. When Maria Cantarella et al. compared the effect of calcium hydroxide overliming, water rinsing, water-ethyl acetate two-phase contacting, and in situ detoxification with high level of yeast on enzymatic hydrolysis efficiency, they found that the most effective detoxification was achieved with $\text{Ca}(\text{OH})_2$ (Cantarella et al. 2004b). The mechanism of overliming is that it can reduce the concentration of some inhibitors by precipitation at higher pH. However, the main drawback of detoxification with $\text{Ca}(\text{OH})_2$ remains the large increase in process costs and the fact that it also causes sugar loss in the reaction system (Martinez et al. 2000; Jönsson et al. 2013).

6 Conclusion

In summary, an ideal pretreatment method should be cheap (both capital and operating costs), effective on a wide range of lignocellulosic substrates, require minimum preparation/handling steps prior to pretreatment, ensure recovery of all of the lignocellulosic components in a useable form, and provide a cellulosic stream that can be

efficiently hydrolyzed with low concentrations of enzymes. So far it is unlikely that one pretreatment process will be declared a “winner” as each method has its inherent advantages/disadvantages. It is apparent that by optimizing the “cellulase” enzyme cocktail by the addition of specific accessory enzymes to the cellulases mixture/complex and minimizing enzyme inhibition, we can further decrease the required enzyme loading needed to achieve fast and efficient cellulose hydrolysis. However, it is also clear that the “ideal” enzyme cocktail will be influenced by the nature and source of the biomass substrate, by its gross and detailed characteristics, and by the pretreatment method that is used to both fractionate and open up the cellulosic component. Therefore, it is important to define the ideal, “synergistic conditions” between the compromised pretreatment parameters (i.e., between maximizing overall sugar recovery while enhancing cellulose accessibility) and the “optimized” enzyme cocktail. This combination will be essential to further improving the economics of a biochemically based biomass-to-fuels and chemicals process.

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Chapter 14

Rheology Characterization of Lignocellulose Feedstock During High Solids Content Pretreatment and Hydrolysis



Weiliang Hou and Jie Bao

Abstract The accurate rheological properties of lignocellulose feedstock are crucial basics on designing industrial scale bioreactors by CFD model. Tendency of lignocellulose refining is to acquire more product yield at high solids loading and reduces the cost of subsequent process. This chapter reviewed the physical and rheological properties of raw material and the incompletely and completely pretreated material and hydrolysis slurry based on the dry acid pretreatment and high solids containing enzymatic hydrolysis. The apparent viscosity of pretreated lignocellulose feedstock was significantly related to the efficiency of pretreatment and consequent hydrolysis. The chapter firstly analyzed the rheology evolution during the dry acid pretreatment under extremely high solids ratio and established the basis for scaling up of pretreatment reactor. Due to the disturbance of abundant fiber particles, conventional rheometers or viscometers were impracticable to high lignocellulose solids system. A new on-site method using the torque meter equipped on hydrolysis reactor is suggested for accurate rheological property measurement. The measured torque data were transformed into the apparent viscosity and then the rheological parameters of the power law model after correlation. Based on these measured rheological properties of lignocellulose, this chapter used CFD model to simulate the power consumption and mixing performance of pretreatment reactor and hydrolysis reactor and designed optimal structure of large-scale bioreactors of high solids content pretreatment and hydrolysis system.

Keywords Lignocellulose feedstock · High solids content · Pretreatment · Enzymatic hydrolysis · Rheology · Bioreactor design

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1 Background

High product concentration is crucially important for reducing the downstream recovery cost of fermentation processes (Galbe et al. 2007; Guo et al. 2013; Larsen et al. 2008). In lignocellulose biorefining, the high lignocellulose feedstock loading in pretreatment, enzymatic hydrolysis, and fermentation steps is the prerequisite condition for achieving the high product concentration. The consequent problem for the high feedstock solids loading is the requirement of intense mixing of low enzyme solution with lignocellulose slurry when solids concentration is up to 30–70% (w/w). The poor mixing could cause the nonuniform distribution of enzyme and temperature inside the reactors and result in the poor bioconversion performance. For such a high solids content, high viscous, and high mixing system, the accurate measurement of rheological property is the key to the design of industrial scale biorefining reactors (Carvajal et al. 2011; Hoon et al. 2015; Wu 2010, 2012a, b; Senturk-Ozer et al. 2011). Rheology of lignocellulose slurry is conventionally measured by the rheometers in vane style (Dasari et al. 2009; Du et al. 2014; Dunaway et al. 2010; Roche et al. 2009; Stickel et al. 2009), the plate style, and the helical ribbon agitator (Pimenova and Hanley 2003, 2004). However, these rheometers are only available to the low lignocellulose solids content system and unsuitable for the heavy solid-contained slurry due to the disturbance of solid fibers (Ehrhardt et al. 2010) and particle sedimentation.

This chapter reviewed the rheological characterization of lignocellulose biomass at different biorefining stages toward the original raw lignocellulose feedstock, the pretreated feedstock, and the hydrolyzed feedstock slurry of lignocellulose biomass at high solids loading by the on-site torque method. Based on the accurately measured rheological properties, the industrial scale pretreatment bioreactor and enzymatic hydrolysis bioreactor were designed using CFD simulation.

2 Rheological Characterization of Raw Lignocellulose System Before Pretreatment

The raw lignocellulose feedstock should be premixed with aqueous solution before pretreatment in all pretreatment operations. The aqueous solution includes dilute acid solution in dilute acid pretreatment (He et al. 2014a, b; Gu et al. 2015; Yang et al. 2016; Zhang et al. 2010a, b), dilute alkaline solution in alkaline pretreatment (Cheng et al. 2010; Ibrahim et al. 2011; Liang et al. 2010; McIntosh and Vancov 2010; Park et al. 2010), condensed water in steam explosion pretreatment (Hooper and Li 1996; Kurabi et al. 2005; Mladenovska et al. 2006; Ruiz et al. 2006; Varga et al. 2004), or liquid ammonia in ammonia fiber explosion pretreatment (Gollapalli et al. 2002; Holtzapple et al. 1992; Teymouri et al. 2004). At the high solid loading of lignocellulose-aqueous solution system, the non-Newtonian property is strongly demonstrated, and the characterization of the rheological properties should be

performed using specially designed methods instead of generally applied of the rheometers (Wu et al. 2012a, b). A helical ribbon impeller driven on-site measurement was found to be the proper way to solve the question by Hou et al. (2016a, b) and Zhang et al. (2014). The rheological model was developed using torque measurement method by measuring the torque parameters of lignocellulose-water system and introducing the power law model. The first step of rheological research is to develop a measurement method of apparent viscosity in the lignocellulose-water system. The dimensionless power number N_p was expressed as the function Reynolds number Re_m under the laminar flow (Carreau et al. 1993):

$$N_p = C \cdot Re_m^x \quad (14.1)$$

where C is a geometry parameter of the reactor used and independent of the fluid properties and x is a dimensionless factor. The Re_m value for a non-Newtonian fluid system under laminar flow condition and the N_p value under different rotation rates were calculated by Eqs. (14.2 and 14.3) according to Chen (1981) and Paul et al. (2004).

$$N_p = \frac{P}{\rho N^3 d^5} = \frac{2\pi NM}{\rho N^3 d^5} = \frac{2\pi M}{\rho N^2 d^5} \quad (14.2)$$

$$Re_m = \frac{\rho N d^2}{\eta_a} \quad (14.3)$$

where P is the power consumption of stirred impeller (W), ρ is the density of the fluid (kg/m^3), N is the impeller rotation rate (rev/s), d is the impeller diameter (m), and η_a is the apparent viscosity (Pa·s).

Combining Eqs. (14.1, 14.2, and 14.3) gave the calculation of the apparent viscosity η_a :

$$\eta_a = \frac{2\pi M}{C N d^3} = \frac{\pi M}{73.56 N d^3} \quad (14.4)$$

The pseudo properties of pretreated materials could be well described by the power law model $\eta_a = K_p \cdot \gamma^{n-1}$, where K_p was the consistency coefficient ($\text{Pa} \cdot \text{s}^n$), n was the dimensionless power-law index, and γ was the apparent shear rate (s^{-1}) (Dasari et al. 2009). The linearized power law model was shown in Eq. (14.5):

$$\begin{aligned} \log_{10} \eta_a &= \log_{10} K_p + (n-1) \cdot \log_{10} (K_s \cdot N) \\ &= [\log_{10} K_p + (n-1) \cdot \log_{10} K_s] + (n-1) \cdot \log_{10} N \end{aligned} \quad (14.5)$$

where the apparent shear rate $\gamma = K_s \cdot N$ (Metzner and Otto 1957), N was the impeller rotation rate (rev/s), and K_s was the Metzner constant and expressed according to Delaplace et al. (2006) in Eqs. (14.6) and (14.7).

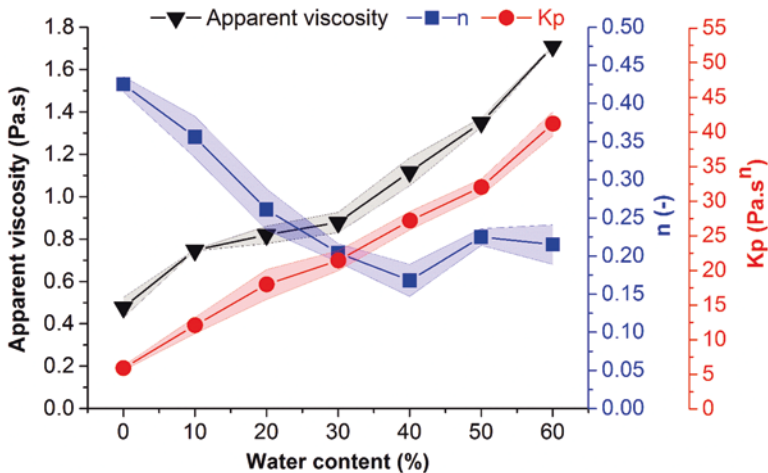


Fig. 14.1 Rheological properties of raw lignocellulose and water mixing system

$$K_s = \frac{2}{N_r} \frac{S^{2/n}}{S^2} \frac{S_e^2 - 1}{S_e^{2/n} - 1} \frac{C}{\pi^2 (l/d)} \left[\frac{n}{2-n} \frac{S^{(2/n)-1}}{S-1} \right]^{1/(n-1)} \quad (14.6)$$

$$S_e = \frac{D}{d_e} = \frac{S}{S - \frac{2w/d}{\ln\left(\frac{S - (1-2w/d)}{S-1}\right)}} \quad (14.7)$$

where S is the diameter ratio of D/d of the helical ribbon impeller, w is ribbon width (m), d is the impeller diameter (m), l is the immersed height of helical ribbon (m), D is the reactor diameter (m), and N_r is the number of helical ribbon.

For a specific reactor and fluid, the power-law index n and K_s value was constant. The n value was calculated by the slope value of linearized power law Eq. (14.5). The K_s value was calculated by Eqs. (14.6 and 14.7) using the n value, and the consistency coefficient K_p was calculated by the intercept of Eq. (14.5).

The rheological properties of the lignocellulose-water mixture were shown in Fig. 14.1. With the increasing corn stover solids content, the n values generally decreased, indicating the weaker shear-thinning behavior of lignocellulose-water system. The K_p values were found to constantly increase with the increasing water content, indicating the stronger viscosity of lignocellulose-water system.

The conventional viewpoint indicates that the viscosity decreases with the increasing water content in solids containing system due to the lubricating effect of water added (Cheng and Li 2015; Wang et al. 2017). On the other hand, for long fiber lignocellulose solid particles, the rheology behavior was significantly differ-

ent. Opposite to the general solids containing system, the viscosity of the lignocellulose-water suspensions increased, instead of decreased, with increasing water content. The reason could be due to the distinct properties of lignocellulose feedstock. After its addition in water (up to 60%, w/w), lignocellulose materials absorb water immediately and completely, hence no free water acting as lubricant to decrease the viscosity of the lignocellulose-water, suspensions. Instead, the long lignocellulose fibers are swelled by the absorbed water, and the intertwined entanglement of the fibers is further enhanced, resulting in the increased friction among lignocellulose fibers. Therefore, an increase viscosity tendency is observed in the lignocellulose suspensions at higher solids content. It is expected that the entanglement of the swelled lignocellulose fibers will be loosed gradually to singular particles with the further increase of water addition to diluted suspensions, and then the rheological performance will be similar to that of the solids containing system.

The corresponding CFD model was established based on the measured rheology property. The mock-up experiments were conducted to validate of the established CFD model. The calculated power consumption and mixing efficiency well agreed with the experimentally measured values. This study provided a practical method for the rheology characterization and CFD model of lignocellulose system at high solids loading. The CFD model could be applied to optimal design of pretreatment reactor, enzymatic hydrolysis reactor, and fermentation reactor in lignocellulose biorefining processes.

3 Rheological Evolution of Lignocellulose Feedstock During Pretreatment

The well-mixed lignocellulose-water system is sent for pretreatment in the specially designed reactors according to the operation types and parameters (Chen et al. 2016; He et al. 2014b; Kim and Lee 2005; Palmaqvist et al. 1996). The rheological property and its evolution during the pretreatment process are an important basis for optimal design of pretreatment reactors with the optimal mixing efficiency and the minimum energy input. Hou et al. (2016b) proposed a rheological evolution profile of corn stover at the very high solids content pretreatment which was recorded by a specially designed method. The pretreatment was dry sulfuric acid pretreatment of corn stover (He et al. 2014a, b; Shao et al. 2017), and it was regularly stopped by interrupting the steam supply and emptying reactor pressure. After that, the incompletely pretreated lignocellulose feedstock was taken out directly from the bottom of the reactor and used for analyzing rheology behavior, as well as the efficiency of physical property and enzymatic hydrolysis. The bulk density of lignocellulose feedstock generally increased with the progress of pretreatment due to the water condensation by steam supply, but still no free water was released. The apparent viscosity slightly increased at the low temperature, but rapidly increased

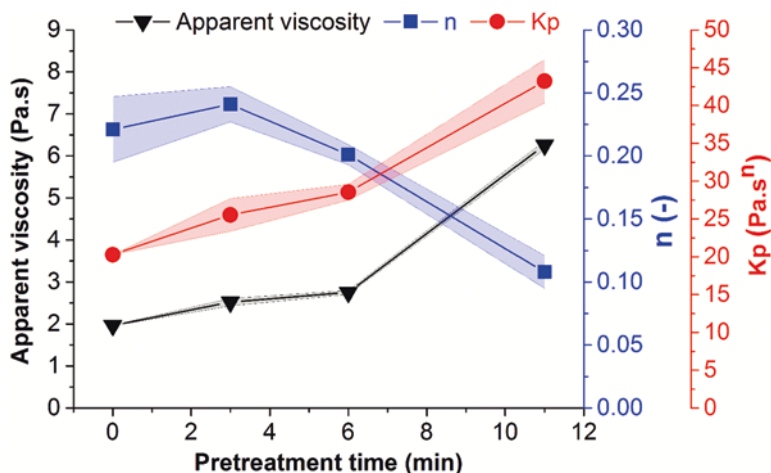


Fig. 14.2 Rheological evolution of lignocellulose biomass during dry dilute acid pretreatment

at the required high pretreatment temperature (Fig. 14.2). The evolution of K_p value was similar with apparent viscosity during pretreatment. The general decrease of the n value indicated the weakened shear-thinning behavior of lignocellulose feedstock during pretreatment.

The rheological properties of pretreated lignocellulose feedstock were used for designing the optimal pretreatment reactor in industrial scale based on the established CFD model. The successful scale-up of pretreatment reactor paved solid foundation for industrial application of biorefining process.

4 Rheological Characterization of Lignocellulose Feedstock During Enzymatic Hydrolysis

In biorefining process, high product concentration is crucially important for reducing the downstream recovery cost (Gerbens-Leenes et al. 2009; Hodge et al. 2008; Humbird et al. 2010; Kristensen et al. 2009; Liu et al. 2015; Liu and Chen, 2016; Roche et al. 2009); therefore, high solids content hydrolysis is inevitably required. Rheology is the requisite condition to achieve scale design of hydrolysate bioreactor, but regular rheometers or viscometers are inapplicable to vast solid particles containing hydrolysate. An on-site method was used for rheology research by measuring the torque value of hydrolysate slurry on bioreactor at a certain agitation rate. The measured torque values were applied for calculating apparent viscosity and the relevant rheological parameters of power law model (Hou et al. 2016a; Zhang et al. 2014). No sampling was withdrawn, and the required data was directly obtained from bioreactor in the overall hydrolysis process. The apparent viscosity measured

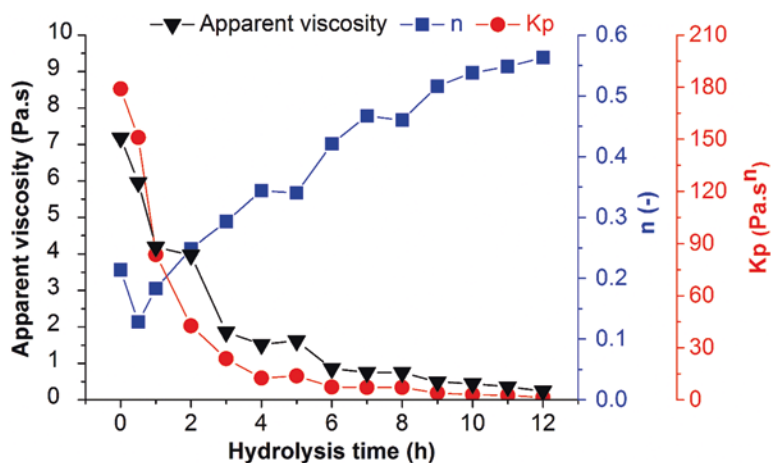


Fig. 14.3 Rheological evolution of pretreated lignocellulose biomass during enzymatic hydrolysis

by this method and rheometer was well agreement after large fiber particles hydrolyzed. The measured rheological properties indicated that the apparent viscosity of hydrolysate slurry decreased and shear-thinning behavior enhanced with enzymatic hydrolysis processes (Fig. 14.3), which was similar with the rheological characterization at low solids loading (Sorensen et al. 2006; Wiman et al. 2011). The phenomenon was mainly caused by the reduced molar mass from polysaccharide chains to monosaccharide by enzymatic hydrolysis disrupting the interaction of fiber polymers and hence creating smaller particles.

The measured rheological properties were applied to establish CFD model and then design industrial scale hydrolysis bioreactors with well mixing performance at economic power cost. The simulated power consumption was consistent at per kg of lignocellulose feedstock in 5 L and 2.5 m³ hydrolysis reactors, but several folds increased mixing time in 2.5 m³ reactor.

5 Conclusion

The review summarized the rheological characterization of raw, pretreated, and hydrolyzed lignocellulose biomass at high solids loading measured by an on-site torque method. The measurement method provided a practical tool for measurement of rheological parameters of high solids content pretreatment, hydrolysis, and fermentation system. Based on the accurately measured rheological properties, the industrial scale pretreatment bioreactor and enzymatic hydrolysis bioreactor were detailedly designed using CFD simulation.

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Chapter 15

Industrial Applications of Cellulases and Hemicellulases



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Abstract Cellulase and hemicellulase products have been developed and widely used in many industrial settings over the last several decades. These applications include textile, animal feed, bakery, brewing, pulp and paper, and biofuel sectors. This chapter illustrates the mechanisms and examples of these applications. Characteristics of enzymes desirable for each of these uses and technologies to gain such properties will be discussed.

Keywords Cellulases · Hemicellulases · Industrial applications

1 Introduction

Despite the complexity and recalcitrant nature of plant cell wall and the diversity of microbial enzyme systems for its biodegradation, tremendous progress has been made over the last half century in terms of developing efficient enzyme technologies to convert plant materials to cost-effective fermentable sugars and to improve industrial processes and products.

Lignocellulose is the structural polysaccharides of plants that consists of cellulose (~50%), hemicellulose (~30%), and lignin (~20%) and is the most abundant and renewable carbon and energy source on earth (Rubin 2008). Due to the complexity of lignocellulose composition and structure, a series of enzymes are required for its degradation. These include the major enzymes (1) cellulases (endoglucanase, EC 3.2.1.4; exoglucanase, EC 3.2.1.91; β -glucosidase, EC 3.2.1.21 (Medie et al. 2012); lytic polysaccharide monooxygenases (Vaaje-Kolstad et al. 2010; Quinlan et al. 2011), swollenins (Saloheimo et al. 2002; Chen et al. 2010), (2) hemicellu-

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lases (endoxylanase, EC 3.2.1.8; β -xylosidase, EC 3.2.1.37; β -mannanase, EC 3.2.1.78; α -L-arabinofuranoside, EC 3.2.1.55; feruloyl esterase, EC 3.1.1.73; α -glucuronidase, EC 3.2.1.139; glucuronoyl esterase, acetylxytan esterase, EC 3.1.1.72 (Druzhinina et al. 2016; Shallom and Shoham 2003)), and (3) ligninases (laccase, EC 1.10.3.2; lignin peroxidase, EC 1.11.1.14; manganese peroxidase, EC 1.11.1.13) (Janusz et al. 2013)). These enzymes have been discovered from diverse sources of eukaryotic, prokaryotic, and archaeal organisms and belong to different families of glycoside hydrolases and carbohydrate esterases (<http://www.cazy.org>; Lombard et al. 2014). For many of these enzymes, beside essential catalytic modules, there are non-catalytic modules facilitating substrate binding as well as protein-protein interactions.

Industries that benefit from cellulase and hemicellulase research and development include textile, animal feed, bakery, brewing, detergent, pulp and paper, and biofuel. Demand for stable, highly active, and specific enzymes used in many industrial applications is growing rapidly. Global market for industrial enzymes was estimated to be about \$4.2 billion in 2014 and expected to develop at a compound annual growth rate of approximately 7% over the period from 2015 to 2020 to reach nearly \$6.2 billion (Singh et al. 2016). Approximately 75% of the industrial enzymes are hydrolases, with cellulases and hemicellulases being the largest groups (Li et al. 2012).

Biotechnology of cellulases and hemicellulases began in early 1980s, first in animal feed followed by food applications. Subsequently, these enzymes were used in the textile, laundry as well as in the pulp and paper industries (Bhat 2000).

2 Application of Cellulases and Hemicellulases in Various Industries

2.1 Textile Industry

The textile industry has been one of the largest contributors to environmental pollution (Ahuja et al. 2004). Therefore lowering or eliminating the use of harsh chemicals without affecting the desired finished effects by using enzymatic process during the fabric and garment manufacturing would decrease pollution and provide safer working conditions. Cellulases were impactful in the textile industry worldwide because of their ability to modify cellulosic fibers in a controlled and desired manner in the process of improving the quality of the fabric (Cortez et al. 2001; Olson 1990). Biostoning and biopolishing are two key textile processes heavily dependent on cellulases.

The textile production process starts with fiber, the raw material that is used to make the fabric. Fibers are spanned into yarn and weaved or knitted into fabric. Natural fibers, like raw cotton, contain noncellulosic materials. These materials are pectin, wax, tannin, and hemicelluloses, mostly in the form of xylan and mannan. Because of the physical blockage and hydrophobicity of the impurities, the process

to remove them, known as scouring, involves cooking the fabric in high concentration of sodium hydroxide, which is an energy- and pollution-intensive process. Promising enzyme-based scouring process has been developed using pectin-degrading enzymes (Solbak et al. 2005; Su et al. 2011). The most important enzyme delivering the scouring function has been identified as pectin lyases. However, other enzymes including cellulase, cutinase, and xylanase may also enhance the performance (Zhang et al. 2010; Battan et al. 2012).

Fabric containing cellulosic materials such as cotton, linen, and rayon tends to leave protruding fibers on the surface, otherwise known as pill formation. Removal of the protruding fibers or depilling by treating the fabric with cellulase under mechanic force achieves a shining appearance and a smoother, softer, and supple feel referred to as biopolishing. Biopolishing is particularly common for knitted fabric and garment. This process is usually carried out during the wet processing stages which also include desizing, scouring, bleaching, dyeing, and finishing. Earlier work demonstrated that acidic cellulases could improve softness and water absorbance property of fibers, strongly reduce the tendency for pill formation, and provide a cleaner surface structure with less fuzz (Sreenath et al. 1996). More recent research has found that cellulase preparations rich in endoglucanases are even better suited for biopolishing in terms of enhancing fabric look, feel, and color with less strength and weight loss. The action of cellulases is capable of achieving multiple benefits such as removing protruding fibers and surface fuzziness, creating smooth and glossy appearance, and improving color brightness, hydrophilicity, and moisture absorbance ability (Araujo et al. 2008). Cellulases fall into many glycoside hydrolase (GH) families (<http://www.cazy.org>), and their abilities to hydrolyze soluble cellulose such as carboxymethyl, acid-swollen cellulose, and crystalline cellulose drastically differ not only between enzymes of different families but also between enzymes of the same family. Research over the years has provided evidence that GH family 5, 12, and 45 enzymes deliver better performance to remove fuzz fibers when acting alone and in combination. A balance between polishing performance and minimizing strength loss has been in favor of using endoglucanase and with no or lower amounts of cellobiohydrolase. To reduce consumption of energy and water and to enhance productivity, the textile industry has showed tremendous interest in developing processes that integrating dyeing and polishing into a single step. The integrated process commonly called dye-polish bath requires the cellulases to perform under high temperature and high salt concentrations. Future development may allow us to integrate desizing and other processes with the dye-polish bath process.

The faded and beaten finish effect of denim apparels, known as stone washed, was achieved in the past by laundering in the presence of pumice stones. The process was labor, and energy-intensive as well as environmentally unfriendly due to the consumption of large amount of the stone and release of stone debris. In the 1980s, the process for achieving similar appearance for jeans using cellulases instead of pumice stones emerged, and now almost all jeans products are manufactured with cellulases to achieve the abrasion effect, and this process is widely referred to as biostoning. During the biostoning process, cellulases break down cellulosic fibers on the yarn surface, thereby loosening the indigo, which is easily

removed by mechanical abrasion in laundry machines. The advantages in the replacement of pumice stones by a cellulase-based treatment include shorter treatment time, increased productivity of the machines, less work-intensive and safer working conditions, flexibility to create and consistently reproduce new finished products, and the possibility to automate the process with computer-controlled dosing devices.

Results have shown that evaluation of abrasion and back-staining of denim garments by using neutral cellulases was better than that by using acidic cellulases (Bhat 2000). However, the reason for this phenomenon was still unknown. Firstly, researcher suspected that this might be caused by the acidic pH during treatment, but other results have shown that some acid cellulases facilitated low level of back-staining while some neutral cellulases showed high redeposition of indigo (Bhat 2000). So the pH profile should not be the sole reason for its potential performance during biostoning. More recent research has provided evidence that back-staining might be related to redeposit of indigo and/or adsorption of indigo dyes to the fabric surface. Low back-staining could be achieved with less strong binding of the cellulase, but the low binding ability tends to decrease abrasion potency. Strategies to maintain the abrasion ability, while minimizing the dye rebinding, included the removal of cellulose-binding module and adding surfactant during the process.

Another industrial process related to textile is dissolving pulp manufacturing. Dissolving pulp is made from wood and bamboo pulp by extracting and purifying cellulose fibers from crude pulp using chemical and biological processes. The material is widely used for making cellulose acetate, cellophane, and rayon that serve as raw materials for textile fabric. The key to producing high-quality dissolving pulp is to remove noncellulosic constituents like hemicellulose, lignin, and resin. Enzymes like xylanase, mannanase, cellulase, and laccase acting alone and in combination have showed potential to remove the impurities and refine cellulosic fibers.

2.2 *Animal Feed Industry*

As the world population has reached over 7.0 billion, the demand for meat, milk, egg, wool, and leather products without burdening the planet will be a huge challenge. More efficient use of raw nutritional ingredients and broaden the scope of plant resources as animal feeds play critical roles to meet the continuous growth of the animal husbandry industry. Animal feed industry has become an important sector of agro-business with an annual production of >600 million tons of feed, worth >1 trillion US dollars. Of the total feed produced, the major share is taken by poultry, pigs, and ruminants (up to 90%) (Singh et al. 2016).

Modern breeding and nutritional management have resulted in dramatic improvements in growth efficiency, particularly muscle growth, for domestic livestock. Conventional diets have become more and more a challenge to meet the nutritional requirements of the high-productivity animals, and the challenge is amplified if low-quality feed materials are used to lower feed cost. Under normal physiological

conditions, animals secrete sufficient levels of enzymes to digest starch, protein, lipid, and polynucleic acid but lack the secretion of enzymes to break down non-starch polysaccharides (NSP). NSPs if not hydrolyzed have been demonstrated to cause high viscosity of digesta and therefore impair the digestion and absorption of many nutrients. For example, recalcitrant polysaccharides like cellulose in plant cell structures impair the animal digestive function due to their physical masking of nutrients accessible to digestive enzymes. Supplementation of NSP-degrading enzymes (NSPases) was able to reduce digesta viscosity and improve overall digestion by increasing the release of oligosaccharides and monosugars from the feed, which serve as prebiotic and energy source. Hence, the technologies using feed NSPases have become a widely accepted practice to enhance animal productivity and health, allowing the use of more varieties of feed ingredients and reduce environmental pollution from animal husbandry industry (Celi et al. 2017).

Two major approaches are commonly utilized to incorporate NSPases for improving animal nutrition. The first one is the addition of enzymes for prehydrolysis of the substrates during silage and whole or ingredient feed fermentation stages. The other is to apply single or complex enzyme preparations into mesh and pellet feed before mixing or post-pelleting spraying (Dhiman et al. 2002). The purpose of both approaches is to improve feed nutritional value through the elimination of the NSPs and converting more of the feed into energy- and health-promoting factors and also by stimulating the secretion of endogenous enzymes like protease, amylase, and lipase (Cortez et al. 2001).

Animal feed manufacturing processes generally include heat treatments that inactivate potential viral and bacterial contaminants followed by extruding feed into pellets. Application of thermostable NSPases in feed production had been shown to enhance digestibility and nutrition of the feed, thereby allowing heat treatment and addition of NSPases to be combined into a single step (Bhat 2000). Cellulases and hemicellulases are responsible for partial hydrolysis of lignocellulosic materials, dehulling of cereal grains, hydrolysis of β -glucans, and better emulsification and flexibility of feed materials, which resulted in the improvement in the nutritional quality of animal feed (Dowman and Collins 1982; Graminha et al. 2008).

Among NSPases, xylanase, β -glucanase, β -mannanase, cellulase, and pectinase are now widely used in monogastric animal diets. α -Galactosidase hydrolyzes the galactosidic bonds in the oligosaccharides (raffinose, stachyose, and verbascose) commonly found in soybean and other legume feed ingredients. Though a broad spectrum of NSPases had been found useful in improving animal nutrition, β -glucanase and xylanase were particularly useful for monogastric diets to hydrolyze NSPs such as β -glucan and arabinoxylan found rich in barley and wheat, respectively (Hesselman et al. 1982; Walsh et al. 1993). The presence of high levels of NSPs in cereal-based diet, if not hydrolyzed, resulted in poor feed conversion rate, slow weight gain, and sticky droppings by young animals, especially chicks (Bedford and Classen 1992; O'Neill et al. 2014). Addition of β -glucanase and xylanase during feed production was found to degrade NSP and markedly improve the digestion and absorption of feed components as well as weight gain of broiler chickens and egg laying hens (Walsh et al. 1993; Rexen 1981; Shrivastava et al. 2010; Saleh et al. 2005).

Most low-quality feedstuffs contain higher concentrations of cellulose and lower amounts of protein and fat. For example, grass only contains less water-soluble carbohydrates, but after treating with cellulase, it can improve silage production for cattle feeding. The forage diet of ruminants, which contains cellulose, hemicellulose, pectin, and lignin, is more complex than the cereal-based diet of poultry and pigs. Enzyme preparations containing high levels of cellulase, hemicellulase, and pectinase have been used to improve the nutritive quality of forages (Zou et al. 2013; Lewis et al. 1996). Beauchemin et al. (1995) reported that the addition of commercial enzyme preparations containing cellulase and xylanase to hay diet increased the live weight gain of cattle by as much as 35%. Similarly, a 5–25% increase in milk yield has been reported in the case of dairy cows fed with forage treated with commercial fibrolytic enzymes (Lewis et al. 1996).

2.3 Bakery Industry

Baking enzymes are used for providing flour enhancement and dough stability; improving texture, volume, and color; prolonging crumb softness and uniform crumb structure; and prolonging freshness of bread. There is rising demand for quality enzymes in current baking market because they are considered as natural and healthy options. The baking enzyme market is expected to reach \$700 million by 2019 with an annual growth rate of 8.2%.

Amylases and proteases have been used in bakery industry for many years (Poutanen 1997; Therdthai and Zhou 2003; James et al. 1996). Nowadays, hemicellulases, especially endo-xylanases, have also been used to enhance the volume and improve the quality of dough, bread, biscuits, cakes, and other baked products (Butt et al. 2008), which hydrolyze arabinoxylan and facilitate the redistribution of water in dough and bread (Moers et al. 2005). In addition, certain types of xylanases have been thought to transitionally convert insoluble xylan to soluble polysaccharides and long oligosaccharides due to the fact that after the addition of xylanases the dough stickiness increased. Furthermore, the addition of endo-xylanases during dough processing is expected to increase the concentration of arabinoxyloligosaccharides in bread, which should serve as prebiotics and hence have added beneficiary effects on human health. Recently, several other hemicellulase, arabinases and α -L-arabinofuranosidases, have been reported to play important roles in improving the quality of bakery products (Vries 2003). However, the ratio of these enzymes in dough progressing and baking should be explored and optimized (Beg et al. 2001).

2.4 Brewing Industry

Many types of enzymes, cellulase, β -glucanase, xylanase, α -amylase, glucoamylase, and protease are now widely used in fermentation processes to produce alcoholic beverages such as beer and wine. With respect to cellulase and hemicellulase,

enzyme cocktails containing both types of activities are utilized during the malting and mashing steps in brewing to improve process efficiency, increase ethanol yield, and enhance product stability. The addition during malting accelerates germinating and boosting overall enzymatic activities in malt products to sufficient levels. During mashing and fermentation stages, microbial β -glucanases and related polysaccharides also play important roles to improve both quality and yields of the fermented products. β -Glucanase is added either during mashing or primary fermentation to hydrolyze glucan to reduce the viscosity of wort and improve the filterability (Bourne and Pierce 1970; Linko et al. 1998).

Beer brewing is based on the action of enzymes activated during malting and fermentation. α - and β -amylases, carboxypeptidase, and β -glucanase act collaboratively to produce high-quality malt. However, due to seasonal variation or poor harvest, unmalted or poor-quality barley was also used frequently. The problem of this type of barley is that they contain 6–10% NSPs, which will form gels, hamper filtration and extraction, and affect the final quality of beer. β -Glucanases can be added to solve this problem. The commonly used β -Glucanases are from *Penicillium emersonii*, *Aspergillus niger*, *Bacillus subtilis*, and *Trichoderma reesei* (Bischof et al. 2016; Peterson and Nevalainen 2012). Besides, β -1,3–1,4-glucanases that specifically hydrolyze the β -1,4 bond following each β -1,3 bond were discovered in both bacterial and fungal sources (Chen et al. 1997). Both types of β -glucanases can effectively reduce the wort viscosity and provide more sugars for alcohol production. However, in an earlier study, Oksanen et al. (1985) observed that endoglucanase II and exoglucanase II of the *Trichoderma* cellulase system also were highly effective in reduction in the degree of polymerization and wort viscosity (Oksanen et al. 1985).

Wine production is a biotechnological process in which both yeast cells and enzymes play a key role. Pectinases, β -glucanases, and other hemicellulases were widely used to improve color extraction, skin maceration, clarification and filtration, quality, and stability. Besides, β -glucosidase can modify glycosylated precursors and improve the aroma of wines (Caldini et al. 1994; Gunata et al. 1990).

2.5 Pulp and Paper Industry

Effort to utilize enzymes in pulp and paper industry dated back to the 1980s with the introduction of xylanase for pulp bleaching enhancement. Tremendous momentum has driven the development and applications of a few enzymatic processes such as (1) pulp bleaching enhancement with xylanase, mannanase, and laccase; (2) pulp refining to reduce energy consumption and machine wearing; (3) deinking and drainage improvement during the process of recycled pulp; (4) enrichment of α -cellulose during dissolving pulp making; and (5) degradation of pitch. However, the pulp and paper industry over many decades has established well-defined large-scale processes, and any modification to these steps will potentially add significant and perhaps prohibitive costs. This has hampered to a certain extent the implementation of enzymatic processes to the industry. The rapid advance in discovering

suitable enzymes from nature and modified through powerful directed evolution approaches (see Sect. 3.2) and with the added tighter pollution control, the pulp and paper industry has been rapidly adapting enzymatic applications for the last 20 years. To implement a sustainable enzymatic technology, one has to keep in mind that significant cost and benefit parameters are realized, no or minimum change to current process setup, and secure supplies of large amount of enzyme products are readily available.

Pulp bleaching requires the consumption of large amount of chlorinated chemicals and ozone. Pollution from pulp bleach traditionally represents a major percentage of the pollution from industrial sources, and hence the reduction of chlorinated chemical use drove the momentum of developing biological bleaching technology. Bleaching involves the removal and oxidation of lignin in both chemical and mechanical pulp. Xylan, the main constitute of hemicelluloses, has been shown to form a matrix physically (possible covalent bond) with lignin (Li et al. 2007). Degradation and removal of xylan should help the removal and exposure of lignin to bleaching chemicals (Paice et al. 1988; Shah et al. 2000). Depending on the bleaching conditions, robust xylanases may have to function under extreme pH/temperature conditions as well as tolerate high concentrations of harsh chemicals.

Refining and grinding of the woody raw material by mechanical pulping lead to pulps with high content of fines, bulk, and stiffness, while the advantage of biomechanical pulping using cellulases from white-rot fungi was substantial energy savings (20–40%) during refining and improvements in handsheet strength (Akhtar 1994).

Unrefined wood chips are generally less accessible to enzymatic modification. Therefore, the addition of an enzyme in mechanical pulping can be effective only after the initial refining. Twenty percent energy saving was achieved by marginal modification of the pulp with cellobiohydrolase I while 30–40% by using cellobiohydrolase I to treat low-intensity refiner (Pere et al. 1995).

Mansfield et al. (1996) studied the action of a commercial cellulase preparation on different fractions of Douglas fir kraft pulp and observed that the fiber coarseness reduced after cellulase treatment. While endoglucanases decrease the pulp viscosity with a lower degree of hydrolysis, cellulases have also been reported to enhance the bleachability of softwood kraft pulp and produce a final brightness score comparable to that of xylanase treatment (Oksanen et al. 1997).

Cellulase and hemicellulase also play an important role in deinking. The proposed mechanism of cellulase and hemicellulase action in the release of ink from the fiber surface is partial hydrolysis of carbohydrate molecules (Kuhad et al. 2010a). Compared with traditional method, enzymatic deinking reduced or eliminated harsh alkali chemicals, improved fiber brightness, enhanced strength properties, and reduced fine particles in the pulp (Kuhad et al. 2010b). Although enzymatic deinking can lower the need for deinking chemicals and reduce the adverse environmental impacts of the paper industry, the excessive use of enzymes need to be avoided, because significant hydrolysis of the fines could reduce the bondability of the fibers (Beg et al. 2001).

2.6 Biofuel Industry

Due to the severe environmental pollution by utilizing fossil fuels and its decreasing resource, the world has focused on developing biofuels, especially bioethanol from renewable resources, which is expected to replace 20% of the fossil fuel consumption by 2020. Enzymatic saccharification of lignocellulosic biomass by cellulases and hemicellulases for biofuel production had gained significant attention (Sun and Cheng 2002; Gamage et al. 2010). Multisteps have been involved in biofuel production: pretreatment (mechanical, chemical, or biological), enzymatic hydrolysis to produce hexose and pentose, fermentation, and the separation and purification of the desired products (Ghosh and Singh 1993; Wyman et al. 2005).

Although technologies are currently available for the bioconversion of lignocellulosics to ethanol and other chemical products (Kuhad and Singh 1993; Mosier 2005), efficiency and price need to be improved, so they can be widely used in industry and compete against conventional fossil fuels. Researchers found that the recalcitrance of the lignocellulosic substrate, thermal deactivation of enzymes, feedback inhibition, nonspecific binding to lignin (Yang and Wyman 2004), and irreversible adsorption of the enzymes to the heterogeneous substrate (Taniguchi et al. 2005) were problems we need to solve.

Cellulase cost is considered as a significant barrier to the efficient conversion of lignocellulosic biomass to fuels and chemicals (Himmel et al. 2007). This problem can be solved by two main strategies: one is the economical improvement in cellulase production, such as cheaper medium, alternative inducer system, and stronger producing strain; and second is the improvement in the specific cellulase activity to reduce the dosage of enzyme for achieving equivalent hydrolysis by cocktails and component improvement (Zhang et al. 2006; Gusakov et al. 2007). Besides, strategies for recycling and reuse of enzymes may also be a direction to reduce enzyme costs (Lee et al. 1995; Singh et al. 1991).

Genome sequencing, protein engineering, and directed evolution are powerful tools that can facilitate the development of more robust and cost-effective cellulases (Baker et al. 2005). Actually, many companies have devoted themselves to developing new cellulase preparations by using these methods and have streamlined production of those enzymes. After two generations of Cellic® releases in 2009 and 2010, Novozymes launched Cellic® CTec3 in Feb 2012 for production of bioethanol from agricultural wastes and residues. This new product is 1.5 times better than Cellic® CTec2 and five times less enzyme dose needed as compared to enzymes products from competing companies for the generation of the same amount of ethanol. After calculation, Cellic® CTec3 could lower the cost of cellulosic biofuels to around \$2.0/gal of ethanol, which was attractive and competitive with traditional gasoline.

The production of biofuel could compete with the world food supply as crops like corn are used as the starting material. Many countries and researchers are looking to 1.5th - or 2nd-generation fuel ethanol from sweet sorghum or corn stalks as the raw materials for the production of biofuel (Ren et al. 2012). The challenge in

using those materials is the release of and access to fermentable sugars. Enzymes, like cellulases and hemicellulases that can overcome the recalcitrant nature of the crop or stalk to release sugar, are crucial. Addition of cellulase during the fermentation of sweet sorghum or strain optimization with increased enzyme activity increased ethanol production (Yu et al. 2014a, b; Du et al. 2015).

2.7 Other Industries

Besides the abovementioned industries, applications of cellulases and hemicellulases in other sectors have emerged quickly. It is impossible to cover all of these emerging areas using these two types of enzymes. Some of the more exciting and promising sectors are listed below.

Plant or herb extraction requires degradation of cell walls. Mechanical and thermochemical means to extract biologically active ingredients from plants may not achieve high yield due to covalent bonds or damage to the structure. Enzymatic degradation followed by extraction under milder conditions could result in higher yield of product recovery and more specific release of the active ingredients. For example, during the extraction of resveratrol from giant knotweed, crude cellulase preparation with addition of β -glucosidase not only increased the total yield of the resveratrol precursor but also converted trans-piceid to resveratrol by β -glucosidase (Beňová et al. 2008).

For corn wet-mill processing, a cellulase and hemicellulase cocktail is added during the grinding of corn kernels to improve starch extraction yield and reduce water content in spent corn fiber. The overall benefit includes increasing starch yield and reducing energy cost during corn fiber drying (Johnston and Singh 2004).

Enzymes for detergent use represent the largest sectors among all industrial enzyme uses. Protease, α -amylase, cellulase, lipase, mannanase, and xylanase have been successfully utilized to serve as functional ingredients in both liquid and solid household and industrial detergent products. Among these enzymes, protease is by far the largest player because protein stains have always been problematic for traditional detergents (Olsen and Falholt 1998). However, cellulase has become a critical component in recent years to depilling damaged fiber and fuzz on the surface of fabric which resulted in newer and polished appearance for the washed clothes.

3 Advancements in Enzyme Engineering

Although cellulases and hemicellulases are already widely used in many industries, the momentum to find and engineer novel enzymes has not decreased. This is primarily because many of these enzyme properties are far from ideal and application in new fields continues to be discovered. The exploitation of new types of enzymes and improvements of enzyme properties and of the production process are moving goals

for the enzyme research and manufacturing. Accordingly, systematic methods in the field of enzyme engineering have allowed better chances to reach the goals, such as (1) screening for novel enzymes from natural environments with improved characteristics, (2) engineering existing enzymes using genetic engineering approaches, (3) fine-tuning enzymatic reactions with close to perfect catalytic properties, and (4) improving downstream processing, formulation, and immobilization.

3.1 Genomics and Metagenomics for Novel Enzyme Discovery

In order to obtain microorganisms with special characteristics, traditional enrichment culture technique and screening of a wide variety of microorganisms for the desired enzymes have been widely employed for decades. However, numerous microbes of the biosphere, more than 99%, are uncharacterized or unculturable due to difficulties in enriching and isolating microorganisms as pure cultures (Gilbert and Dupont 2011). Recent success of genome sequencing programs has resulted in an explosion of information available for providing candidate enzyme genes by creating opportunities to explore the possibility of finding novel enzymes and enzymes with novel properties through data mining. Additionally, tools for metagenome sequencing have advanced rapidly to allow avoiding the labor- and time-intensive processes of isolating and cultivating microorganisms by directly extracting DNA from environmental samples and systematically looking into open reading frames potentially encoding putative novel enzymes. Metagenomic library screening is mostly based on the function-driven approaches: i) direct phenotypical detection (Waschkowitz et al. 2009), ii) heterologous complementation (Simon et al. 2009), and iii) induced gene expression (Handelsman 2005). By accessing different locations such as volcanic vents, deep ocean beds, arctic tundra, etc., the sequence data obtained could potentially provide millions previously unknown genes for novel enzyme discoveries. Some potential cellulase and hemicellulase genes have been isolated from the bovine rumen and termite gut. For example, Wang et al. (2012) have obtained a novel thermostable β -glucosidase, Bgl-gs1 from a metagenomic library of termite gut. The residual activity of Bgl-gs1 was maintained above 70% after the recombinant enzyme was incubated at 75 °C and pH 6.0 for 2 h, and its half-life at 90 °C was approximately 1 h in the presence of 4 mM pNPG. In addition, a novel xylanase, XYL7 with high specific activity (6340 U/mg) and broad pH active range of 5.5–10.0, was isolated (Qian et al. 2015). The rapid development of cloning-independent DNA sequencing technology and the combination of metagenomics, metatranscriptomics, and metaproteomics will accelerate the new biocatalyst discovery for better and more specific enzymes.

With the more recent discovery of the gene modification system using CRISPR/Cas9, it has been much easier to precisely target gene of interest for deletion, addition, or modification. This technology has improved host strains and the expression of desired enzymes (Liu et al. 2015).

3.2 Directed Evolution

The use of enzymes in industrial processes often requires them to function under extreme temperatures and pH as well as in the presence of high concentrations of organic solvents and salts. Sometimes higher specific activity and different substrate and product specificities are desired. Enzymes isolated from natural sources may not be able to meet one or more such specific requirements. To obtain those features, one may choose to modify a candidate enzyme through directed evolutionary approaches. Directed evolution involves repeated rounds of random gene library generation, expression of genes in a suitable host, and screening of libraries of variant enzymes for the property of interest. Both in vitro screening-based methods and in vivo selection-based methods have been applied to the evolution of enzymatic activities and properties. Examples of many successful projects have been reported. Kim et al. (2000) reported a five-fold specific activity increase for a *Bacillus subtilis* endoglucanase by DNA shuffling and display expression. A xylanase mutant generated using the error-prone PCR technique followed by expression and selection in *E. coli* was found to have 250-fold increase in half-life at 55 °C, with a 10 °C increase in optimal temperature compared to that of the wild type (Qian et al. 2015).

Improvement in the low-temperature catalysis (threefold) for the hyperthermostable *Pyrococcus furiosus* β -D-glucosidase CelB (Lebbink et al. 2000) and in thermostability and catalytic efficiency for the *Paenibacillus polymyxa* BgblA and BglA were obtained (Gonzalez-Blasco et al. 2000). In another recent example, after DNA shuffling, a β -glycosidase mutant was found to display lactose hydrolysis rates 3.5-fold and 8.6-fold higher than the parental *P. furiosus* CelB and *Sulfolobus solfataricus*, LacS, respectively (Kaper et al. 2002).

4 Summary and Perspective

Over many decades, cellulase and hemicellulase have not only received much attention from the aspect of basic research but also been successfully utilized in many industries. It is especially notable in large scale processes in delivering performance that may not be able to accomplish with chemicals to reduce energy input, machine wearing, water usage and effluent waste. Cellulase and hemicellulase, in combination with other types of enzymes, are helping many industrial processes to embrace greener footprints and provide healthier products for human consumption. Due to rapid progress in developing native enzymes from diverse ecosystems and engineered enzymes tailored to specific requirements, enzymatic applications have increased in both the number of industries and the total revenue. This trend should continue or even be accelerated as many powerful molecular techniques such as genomics, metagenomics, CRISPR/Cas9, directed evolution, synthetic microbiology, and genome editing are becoming routine and commonly utilized.

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