Engineering Fungi for Boosting Cellulase Production and its Potential Industrial Application

A thesis presented to

The Faculty of Graduate Studies

of

Lakehead Univeristy

by

MEHDI DASHTBAN

In partial fullfillment of the requirements
for the degree of
Doctor of Philosophy in Biotechnology

July 16th, 2012

Abstract

Bioconversion of lignocellulosic residues is initiated primarily by microorganisms such as fungi and bacteria which are capable of degrading lignocellulolytic materials. Fungi produce large amounts of extracellular cellulolytic enzymes including endoglucanases, cellobiohydrolases (exoglucanases) and β-glucosidases that work efficiently on cellulolytic residues in a synergistic manner. The ascomycete *Hypocrea jecorina* (anamorph *Trichoderma reesei*), an industrial (hemi)cellulase producer, can efficiently degrade plant polysaccharides. However, the biology underlying cellulase hyperproduction of *T. reesei*, and the conditions for enzyme induction in this organism are not completely understood. In this study, the optimum conditions for cellulase production by *T. reesei* strains were investigated. Three different strains of *T. reesei*, including QM6a (wild-type), and mutants QM9414 and RUT-C30, were grown on 7 soluble and 7 insoluble carbon sources, with the latter group including 4 pure polysaccharides and 3 lignocelluloses. Maximum cellulase activity of QM6a and QM9414 strains, for the majority of tested carbon sources, occurred after 120 h of incubation, while RUT-C30 had the greatest cellulase activity after around 72 h. Maximum cellulase production was 0.035, 0.42 and 0.33 μmol glucose equivalents using microcrystalline celluloses for QM6a, QM9414, and RUTC-30, respectively. Increased cellulase production with the ability to grow on microcrystalline cellulose was positively correlated in QM9414 and negatively correlated in RUT-C30.

Although *T. reesei* is widely used as an industrial strain for cellulase production, its low yield of β-glucosidase has limited its industrial value. In the hydrolysis process of cellulolytic residues by *T. reesei*, a disaccharide known as cellobiose is produced and accumulates, inhibiting further cellulase production. In order to improve β-glucosidase production and ultimately overall cellulase activity of *T. reesei*, a thermostable β-glucosidase gene from the fungus *Periconia sp.* was engineered into the genome of the *T. reesei* QM9414 strain. The engineered *T. reesei* strain showed about 10.5-fold (23.9 IU/mg) higher β-glucosidase activity compared to the parent strain (2.2 IU/mg) after 24 h of incubation. The transformants also showed very high cellulase activity (about 39.0 FPU/mg) at 24 h of incubation,

whereas the parent strain showed almost no cellulase activity at 24 h of incubation. The recombinant β-glucosidase was thermotolerant and remained fully active after two-hour incubation at temperatures as high as 60 °C. Additionally, it maintained about 88% of its maximal activity after a four-hour incubation at 25 °C across a wide range of pH values from 3.0 to 9.0. Furthermore, an enzymatic hydrolysis assay using untreated, NaOH- or Organosolv-pretreated barley straw or microcrystalline cellulose showed that the transformed *T. reesei* strains released more reducing sugars compared to the parental strain. These features suggest that the transformants can be used for β-glucosidase production as well as improving biomass conversion using cellulases.

Xylitol, a naturally occurring five-carbon sugar alcohol derived from *D*-xylose, is currently in high demand by industries for its sweetening and anti-microbial properties. Biotechnological methods can be used for large-scale xylitol production since its current industrial production relies on chemical methods which are costly and energy intensive. While *T. reesei* is capable of selectively using *D*-xylose for xylitol production as an intermediate metabolite, its production can be enhanced by genetic engineering of the metabolic pathway. In this study, two *T. reesei* mutant strains were used, including a single mutant in which the endogenous xylitol dehydrogenase gene was deleted (Δ*xdh1*), and a double mutant in which L-arabinitol-4-dehydrogenase was additionally deleted (Δ*lad1*Δ*xdh1*). The widely-available agricultural residue barley straw was used for xylitol production by the strains after its pretreatment using NaOH- and Organosolv-pretreatment methods. High xylitol production by both strains was achieved when barley straw (untreated or pretreated) was supplemented with 2% *D*-xylose, whereas the *D*-glucose supplementation did not increase production. The highest production of xylitol was 6.1 and 13.22 g/L obtained after 96 and 168 h of incubation, respectively, using medium supplemented with 2% Organosolv-pretreated barley straw and 2% *D*-xylose by single and double *T. reesei* mutant strains, respectively. Maximum saccharification of barley straw was observed after 120 h of incubation for NaOH-pretreated by single (692 ± 1.01 mg/g reducing sugars) and double *T. reesei* mutant (685 ± 11.9 mg/g reducing sugars) strains, respectively. Moreover, the significant increase of xylitol production by the

T. reesei strains using medium containing Organosolv-pretreated barley straw supplemented with *D*-xylose suggests that the pretreatment in combination with the added sugar favored xylitol production. These results suggest that agricultural residues, such as barley straw, could be a suitable resource for bioconversion to produce value-added products such as xylitol.

Acknowledgments

I am deeply greatful to my supervisor Dr. Wensheng Qin for his guidance, encouragement, continuous support, and allowing me to conduct such an exciting project in my Ph.D. study. I would also like to extend my sincere thanks to the members of my supervisory committee Dr. Heidi Schraft, Dr. David Law and external examiner Dr. Yun Ji, for sharing their valuable time by lending their knowledge and support to my thesis. I am also very grateful to Dr. Heidi Schraft and Dr. Kam Leung for letting me use their lab space during my first year of Ph.D. study. Additionally, I would like to thank Dr. David Law for letting me use his lab equipment during my Ph.D. study.

Additionally, I wish to express sincere gratitude to the hard working honors thesis or research internship students who worked with me throughout my thesis: Crystal Esau, Ryan Jackson and Robert Buchkowski. I would also like to especially thank for the support of all the members of Dr. Qin's laboratory over the period of my Ph.D. My special acknowledgements to Miranda Maki, MaryJane Moses, Bruce Rosa and William Dew for their help and a lot of proof reading. In addition, I further extend thanks to Greg Kepka, technician of the Lakehead Univerisity Instrumentation Lab and Biorefining Institute for his great help and support. This work would not be complete without their help.

This work was further made possible by the donation of *T. reesei* strains by Dr. Bernhard Seiboth, Dr. Monika Schmoll (Vienna University of Technology, Austria), Dr. Xiaobin Yu (Jiangnan University, China) and Dr. Tianhong Wang, (Shandong University, China). My special thanks to Dr. Bernhard Seiboth (Vienna University of Technology, Austria) for kindly providing the pPtef1-hph vector as well as his great suggestions during my thesis. I also would like to thank Dr. Shuna Cheng and Dr. Chunbao (Charles) Xu (Lakehead University, Canada) for their great help with the Organosolv-pretreatment. Also, special thanks for financial support from OGS throughout my doctoral study.

Finally, it is with much gratitude and sincerity I thank my wife Saedeh and my two lovely kids, Niki and Daniel for their patience, and encouragement during my Ph.D.

Dedication

To the loving memory of my mother.

To my wife, who has supported in all my endeavors.

To my lovely daughter and son.

Table of Contents

List of Tables

Chapter 4: Xylitol production by genetically engineered *Trichoderma reesei* strains using barley straw as feedstock

List of Figures

Chapter 4: Xylitol production by genetically engineered *Trichoderma reesei* strains using barley straw as feedstock

Chapter 1: Literature Review

1.1 Introduction

Rising energy consumption, depletion of fossil fuels and increased environmental concerns have shifted the focus of energy generation towards biofuel use. Bioconversion of biomass has significant advantages over other alternative energy strategies because biomass is the most abundant and also the most renewable biomaterial on our planet. Lignocellulose is a renewable organic material and is the major structural component of all plants. Lignocellulosic wastes are produced in large amounts by many industries including those of forestry, pulp and paper, agriculture, and food. Lignocellulose mainly consists of three major components: cellulose, hemicellulose and lignin. Significant efforts, many of which have been successful, have been made to convert lignocellulosic residues to valuable products such as biofuels, chemicals and animal feed. Using lignocellulosic wastes as the main source for ethanol production will eliminate the concern originally caused by bioethanol industries where they have used sugars or corn as the feedstock.

Bioconversion of lignocellulosic residues is initiated primarily by microorganisms such as fungi and bacteria which are capable of degrading lignocellulolytic materials. Fungi such as *Trichoderma reesei* produce large amounts of extracellular cellulolytic enzymes. In filamentous fungi, cellulolytic enzymes including endoglucanases, cellobiohydrolases (exoglucanases) and β-glucosidases work efficiently on cellulolytic residues in a synergistic manner. The breakdown of lignocellulosic biomass involves the formation of long-chain polysaccharides, mainly cellulose and hemicellulose, and the subsequent hydrolysis of these polysaccharides into their component 5- and 6-carbon chain sugars. In biofuel production, these sugars can be converted to bioethanol through fermentation processes.

The primary challenge in biomass conversion to bioethanol is achieving yields that make it cost-competitive with current fossil fuel sources. Cellulose in the plant cell wall is not readily available to enzymatic hydrolysis. Pretreatment of the lignocellulosic residues is necessary because hydrolysis of non-pretreated materials is slow, and results in low product yield. Many pretreatment methods rely on

applying high temperature, and addition of acids or bases at industrial scales. Thus, additional steps are included prior to hydrolysis step and this makes the overall process expensive, slow and inefficient. Additionally, pretreatment releases inhibitors which lower overall yield of the fermentation process.

The main goal of this thesis is to approach the above mentioned challenges in biofuel production mainly using fungi. Obviously, fungi contribute significantly to the decay of lignocellulosic residues in nature by producing many different lignocellulolytic enzymes. Most fungal strains produce various enzymes in large amounts which are released in the environment and act in a synergistic manner. Despite the fact that some fungal strains have the advantages of being thermostable and producing cellulases, most of these fungal strains do not produce sufficient amounts of one or more lignocellulolytic enzymes. These enzymes are required for efficient bioconversion of lignocellulosic residues to fermentable sugars. *T. reesei*, for example, produces small amounts of β-glucosidase which inhibit further cellulose hydrolysis due to accumulation of the end product inhibitor (cellobiose). This became the key bottleneck in the process of biofuel production and thus new biotechnological solutions are needed to improve the efficiency. Many recent studies focused on improving fungal hydrolytic activity as well as finding stable enzymes capable of tolerating extreme conditions.

The main objectives of this thesis include:

1- Understanding the biology underlying cellulase hyperproduction in *T. reesei*, and the conditions for enzyme induction.

2- Improving cellulase activity of the *T. reesei* QM9414 strain, through increasing its β-glucosidase activity using genetic engineering.

3- Applying established fungal culture techniques to produce bio-products such as xylitol using metabolically engineered *T. reesei* strains.

1.2 Fungal bioconversion of lignocellulosic residues; opportunities & perspectives

Adapted from: Mehdi Dashtban[1,2], Heidi Schraft [2] and Wensheng Qin*[1,2]

[1] Biorefining Research Institute
Lakehead University
955 Oliver Road
Thunder Bay, ON P7B 5E1 Canada

[2] Department of Biology
Lakehead University
955 Oliver Road
Thunder Bay, ON P7B 5E1 Canada

*Corresponding author: Wensheng Qin

Abstract

The development of alternative energy technology is critically important because of the rising prices of crude oil, security issues regarding the oil supply, and environmental issues such as global warming and air pollution. Bioconversion of biomass has significant advantages over other alternative energy strategies because biomass is the most abundant and also the most renewable biomaterial on our planet. Bioconversion of lignocellulosic residues is initiated primarily by microorganisms such as fungi and bacteria which are capable of degrading lignocellulolytic materials. Fungi such as *Trichoderma reesei* and *Aspergillus niger* produce large amounts of extracellular cellulolytic enzymes, whereas bacterial and a few anaerobic fungal strains mostly produce cellulolytic enzymes in a complex called the cellulosome, which is associated with the cell wall. In filamentous fungi, cellulolytic enzymes including endoglucanases, cellobiohydrolases (exoglucanases) and β-glucosidases work efficiently on cellulolytic residues in a synergistic manner. In addition to cellulolytic/hemicellulolytic activities, higher fungi such as basidiomycetes (e.g. *Phanerochaete chrysosporium*) have unique oxidative systems which together with ligninolytic enzymes are responsible for lignocellulose degradation. This review gives an overview of different fungal lignocellulolytic enzymatic systems including extracellular and cellulosome-associated in aerobic and anaerobic fungi, respectively. In addition, oxidative lignocellulose-degradation mechanisms of higher fungi are discussed. Moreover, this paper reviews the current status of the technology for bioconversion of biomass by fungi, with focus on mutagenesis, co-culturing and heterologous gene expression attempts to improve fungal lignocellulolytic activities to create robust fungal strains.

Keywords: Biomass, Lignocellulose, Bioconversion, Fungi, Cellulases, Cellulosome

Introduction

Millions of years ago, atmospheric carbon was captured by plants in a process called photosynthesis, and over time was manifested into crude oil and coal. However, since the industrial revolution, we have used much of these energy sources, causing the excessive release of carbon back into the atmosphere. Thus, over the past 150 years atmospheric CO_2 levels have increased from ~280 to ~380 ppm [1,2]. In return, this is potentially causing warmer temperatures worldwide and leading to global climate changes [1,3,4].

Rising energy consumption, depletion of fossil fuels and increased environmental concerns have shifted the focus of energy generation towards biofuel use. Global crude oil production is predicted to decline to one-fifth of its current level by 2050. Based on World Energy Council (WEC) calculations, the world-wide primary energy consumption is approximately 12 billion tonnes coal equivalent per year. United Nations calculations have shown that the world's population will increase to about 10 billion people by 2050 which will in turn increase energy demands to at least 24 billion tonnes coal equivalent per year (twice of what we consume today) depending on economic, social and political developments [5,6].

Lignocellulose is a renewable organic material and is the major structural component of all plants. Lignocellulose consists of three major components: cellulose, hemicellulose and lignin. In addition, small amounts of other materials such as ash, proteins and pectin can be found in lignocellulosic residues, in different degrees based on the source [7]. Cellulose, the major constituent of all plant material and the most abundant organic molecule on the Earth, is a linear biopolymer of anhydroglucopyranose-molecules, connected by β-1,4-glycosidic bonds. Coupling of adjacent cellulose chains by hydrogen bonds, hydrophobic interactions and Van der Waal's forces leads to a parallel alignment of crystalline structures known as microfibril [8]. Hemicelluloses, the second most abundant component of lignocellulosic biomass, are heterogeneous polymers of pentoses (including xylose and arabinose), hexoses (mainly mannose, less glucose and galactose) and sugar acids. Composition of hemicelluloses is

9

very variable in nature and depends on the plant source [9,10]. Lignin, the third main heterogeneous

polymer in lignocellulosic residues, generally contains three aromatic alcohols including coniferyl

alcohol, sinapyl and *p*-coumaryl. Lignin acts as a barrier for any solutions or enzymes by linking to both

hemicelluloses and cellulose and prevents penetration of lignocellulolytic enzymes to the interior

lignocellulosic structure. Not surprisingly, lignin is the most recalcitrant component of lignocellulosic

material to degrade [7,11].

Lignocellulosic wastes are produced in large amounts by different industries including forestry,

pulp and paper, agriculture, and food, in addition to different wastes from municipal solid waste (MSW),

and animal wastes (Table 1) [12-17]. These potentially valuable materials were treated as waste in many

countries in the past, and still are today in some developing counties, which raises many environmental

concerns [18,19]. Significant efforts, many of which have been successful, have been made to convert

these lignocellulosic residues to valuable products such as biofuels, chemicals and animal feed [20].

Interestingly, in 2008 approximately 90% of the global ethanol fuel production (15,472.2 out of 17,335.2

Million of Gallons) was concentrated in two countries, Brazil (6,472.2), and The United States of

America (9,000) [21]. In Brazil, ethanol is usually produced from cane juice, whereas in the USA, starch-

crops such as corn are usually used for ethanol production [7]. Using sugars or corn as the main source for

ethanol production caused a great deal of controversy due to its effect on food production and costs,

which has made it difficult for ethanol to become cost competitive with fossil fuels. These concerns

became a driving force in the generation of new biofuel research using lignocellulosic wastes produced by

many different industries. The Iogen Corporation in Canada (http://www.iogen.ca/) is the world's leading

operating plant for bioethanol production from lignocellulosic residues, and uses up to 30 tonnes per day

of wheat, oat and barley straw to produce up to 0.52 million gallons of ethanol per year [22].

In nature, degradation of cellulosic biomass is performed by mixtures of hydrolytic enzymes

collectively known as cellulases. The cellulases include endo-acting (endoglucanases) and exo-acting

(cellobiohydrolases) enzymes, which act in a synergistic manner in biomass-degrading microbes. Many

microorganisms including fungi and bacteria had been found to degrade cellulose and other plant cell wall fibres. By 1976, over 14,000 fungal species capable of degrading cellulose had been isolated, but only a few of them were subjected to in-depth studies [23]. Obviously, fungi contribute significantly to the decay of lignocellulosic residues in nature by producing many different lignocellulolytic enzymes. Most fungal strains produce various enzymes in large amounts which are released in the environment and act in a synergistic manner. The breakdown of lignocellulosic biomass involves the formation of long-chain polysaccharides, mainly cellulose and hemicellulose, and the subsequent hydrolysis of these polysaccharides into their component 5- and 6-carbon chain sugars. In biofuel production, these sugars can be converted to bioethanol through fermentation processes [24].

Table 1. Some of the lignocellulosic residues produced by different industries and potential for ethanol production

Lignocellulosic Wastes	Annual production	Potential contribution to ethanol production (billion litre/year)	References
World Agricultural Wastes[1]	Trillion grams/year (Tg/y)		
Corn stover	203.62	58.6	[12]
Barley straw	58.45	18.1	[12]
Oat straw	10.62	2.78	[12]
Rice straw	731.34	204.6	[12]
Wheat straw	354.35	103.8	[12]
Sorghum straw	10.32	2.79	[12]
Bagasse	180.73	51.3	[12]
Subtotal	1549.42	442.0	
Municipal Solid Waste (MSW)	Million metric tons (million MT)		
USA (2001)	208	13.7 [2]	[13]
China (1998)	127	8.3 [3]	[14]
Canada (2002)	30.5	2 [4]	[15]
Animal Wastes[5]			
In Canada (2001)	177.5		[16]
In USA (1995)	160		[17]

[1] Average values from 1997 to 2001 have been used to calculate world agricultural waste production [12].

[2-4]Potential contribution of MSW in USA, China and Canada in 2001, 1998 and 2002 respectively, assuming a conservative yield of 66 L of ethanol/MT of MSW [13-15]. [5]The fiber content (including cellulose and hemicellulose) of cattle manure, for example, is 52.6% (dry biomass basis). These sugars can be hydrolyzed and fermented to produce ethanol but the utilization of animal manures is more complicated due to its high protein content [16,17].

The primary challenge in biomass conversion to bioethanol is achieving yields that make it cost-competitive with the current fossil-based fuels. Cellulose in the plant cell wall is not readily available to enzymatic hydrolysis (cellulases) due primarily to (1) low accessibility of (micro-) crystalline cellulose fibers, which prevents cellulases from working efficiently, and (2) the presence of lignin (mainly) and hemicellulose on the surface of cellulose, which prevents cellulases from accessing the substrate efficiently [25]. Thus, pretreatment of lignocellulosic residues before hydrolysis is a prerequisite and this can be performed by different methods (discussed in section 3.1.). High temperature and acid have been used initially for chemical cellulose degradation and they are still involved in pretreatment of lignocellulosic residues at industrial scales. However, this approach is expensive, slow and inefficient [26]. In addition, the overall yield of the fermentation process will be decreased because this pretreatment releases inhibitors such as weak acids, furan and phenolic compounds [27]. Some of these problems could be overcome by applying microorganisms such as fungi. For example, thermophilic fungal species such as *Sporotrichum thermophile* [28], *Thermoascus aurantiacus* [29] and *Thielavia terrestris* [30] have been proposed as good candidates for bioconversion of lignocellulosic residues to sugars and offer the great potential to be used at industrial scales. Applying thermophilic fungal species at industrial scales also allows energy savings because the costly cooling after steam pre-treatment is avoided and saccharification rates are improved. These fungi have been shown to produce cellulases and to degrade native cellulose; however, the enzyme activity in thermophilic organisms (e.g. *S. thermophile*) is usually low compared to mesophilic fungi such as *T. reesei* [28].

The initial conversion of biomass into sugars is a key bottleneck in the process of biofuel production and new biotechnological solutions are needed to improve their efficiency, which would lower the overall cost of bioethanol production. Despite the fact that some fungal strains have the advantages of being thermostable and producing cellulases, most of these fungal strains do not produce sufficient amounts of one or more lignocellulolytic enzymes required for efficient bioconversion of lignocellulosic residues to fermentable sugars. Wild-type *T. reesei* and its best extracellular cellulase producer mutants

12

(e.g. RUT-C30) for example, produce small amounts of β-glucosidase which inhibit further cellulose

hydrolysis due to accumulation of the end product inhibitor (cellobiose). In addition, plant cell walls are

naturally resistant to microbial and enzymatic (fungal and bacterial) deconstruction, collectively known as

"biomass recalcitrance" [11]. These rate-limiting steps in the bioconversion of lignocellulosic residues to

ethanol remain one of the most significant hurdles to producing economically feasible cellulosic ethanol.

Improving fungal hydrolytic activity and finding stable enzymes capable of tolerating extreme conditions

has become a priority in many recent studies.

This review focuses on lignocellulosic bioconversion by the application of different

lignocellulolytic enzyme-producing fungi. In addition, this review addresses recent efforts to create robust

fungal strains using mutagenesis, co-culturing and heterologous gene expression techniques and how

these robust organisms can help overcome some of the critical issues in biofuel production.

Lignocellulolytic enzyme-producing fungi

Lignocellulolytic enzymes-producing fungi are widespread, and include species from the

ascomycetes (e.g. *T. reesei*), basidiomycetes including white-rot fungi (e.g. *P. chrysosporium),* brown-rot

fungi (e.g. *Fomitopsis palustris*) and finally a few anaerobic species (e.g. *Orpinomyces sp.*) which

degrade cellulose in gastrointestinal tracts of ruminant animals [31,32]. Biomass degradation by these

fungi is performed by complex mixtures of cellulases [33], hemicellulases [31] and ligninases [7,34],

reflecting the complexity of the materials. Cellulases and most hemicellulases belong to a group of

enzymes known as glycoside hydrolases (GH). Currently more than 2500 GH have been identified and

classified into 115 families (for more information please visit the CAZy web page; www.cazy.org) [35].

Interestingly, the same enzyme family may contain members from bacteria, fungi and plants with several

different activities and substrate specifications. However, fungal cellulases (hydrolysis of β-1,4-glycosidic

bonds) have been mostly found within a few GH families including 5, 6, 7, 8, 9, 12, 44, 45, 48, 61 and 74 [35,36]. Table 2 summarizes a few different fungi producing different lignocellulolytic enzymes.

Fungal extracellular cellulases

Hydrolysis of the β-1,4-glycosidic bonds in cellulose can be achieved by many different enzymes known as cellulases which use two different catalytic mechanisms, the retaining and the inverting mechanisms. All GH 12 cellulases, for example, hydrolyze glycosidic bonds by the retaining mechanism whereas family 6 cellulases use the inverting mechanism [33,36]. In both mechanisms, two catalytic carboxylate residues are involved and catalyze the reaction by acid-base catalysis. Many different fungal species have the ability to degrade cellulose by producing extracellular fungal cellulose-degrading enzymes including endo-cleaving (endoglucanases) and exo-cleaving (cellobiohydrolases).

Endoglucanases can hydrolyze glycosidic bonds internally in cellulose chains whereas cellobiohydrolases act preferentially on chain ends. The products of the enzymatic reaction are mostly a disaccharide known as cellobiose and, to a lesser extent, cello-oligosaccharides, which will be further hydrolyzed by the third group of enzymes called β-glucosidases [56]. Cellulases mostly have a small independently folded carbohydrate binding module (CBM) which is connected to the catalytic domain by a flexible linker. The CBMs are responsible for binding the enzyme to the crystalline cellulose and thus enhance the enzyme activity [33]. Currently many CBMs have been identified and classified into 54 families, however only 20 families (1, 13, 14, 18, 19, 20, 21, 24, 29, 32, 35, 38, 39, 40, 42, 43, 47, 48, 50 and 52) have been found in fungi. Different fungal cellulolytic enzymes and their main features are summarized in Table 3.

Table 2. Examples of different fungi producing different lignocellulolytic enzymes and their substrates.

	Group	Fungal strain	Enzymes	Substrate	References
Aerobic fungi **(Extracellular lignocellulolytic enzymes)**	**Ascomycetes**	*T. reesei*	Cellulases (CMCase, CBH, BGL), Hemicellulase (xylanase)	Wheat straw	[37,38]
		T. harzianum	Cellulases (CMCase, CBH), β-1,3-glucanases	Wheat bran, wheat straw	[39,40]
		A. niger	Cellulases, Xylanases	Sugar cane bagasse	[41]
		Pestalotiopsis sp.	Cellulases (CMCase, CBH), Laccase	Forest litter of *Quercus variabilis*	[42,43]
	Basidiomycetes	*P. chrysosporium*	Cellulases (CMCase, CBH, BGL), CDH, LiP, MnP, Hemicellulase (xylanases)	Red oak, grape seeds, barley bran, woodchips	[7,44,45]
		F. palustris	Cellulases (CMCase, CBH, BGL)	Microcrystalline cellulose	[32,46]
Anaerobic rumen fungi (Chytridiomycetes) **(Cell-wall associated lignocellulolytic enzymes, "cellulosome")**	***Anaeromyces***	*Anaeromyces mucronatus 543*	Cellulase (CMCase), Hemicellulase (xylanase)	Orchard grass hay	[47,48]
	Caecomyces	*Caecomyces communis*	Cellulases, Hemicellulases (xylanase, β-D-xylosidase)	Maize stem	[48-50]
	Cyllamyces	*Cyllamyces aberensis*	Cellulases, Xylanases	Grass silage	[48,51]
	Neocallimastix	*Neocallimastix frontalis*	Cellulases, Hemicellulase (xylanase, β-galactosidase)	Cotton fiber, wheat straw	[48,52,53]
	Orpinomyces	*Orpinomyces sp.*	Cellulase (CMCase, CBH, β-glucosidase), Hemicellulases (xylanase, mannanases)	Wheat straw	[31,48,53,54]
	Piromyces	*Piromyces sp.*	Cellulases (CMCase, CBH, β-glucosidase) Hemicellulases (xylanase, mannanases)	Maize stem	[31,48,49,55]

CMCase: Carboxymethylcellulases (endoglucanase), CBH: Cellobiohydrolases, BGL: β-glucosidases,

CDH: Cellobiose dehydrogenase, MnP: Manganese peroxidises, LiP: Lignin peroxidises.

Endo-1,4- β-glucanases (EC 3.2.1.4, endocellulase)

Endoglucanases (EG) are also referred to as carboxymethylcellulases (CMCase), named after the

artificial substrate used to measure the enzyme activity. EG initiate cellulose breakdown by attacking the

amorphous regions of the cellulose, making it more accessible for cellobiohydrolases by providing new free chain ends. This has been shown by the effect of the enzyme on carboxymethylcellulose and amorphous cellulose [8]. Fungal EGs are generally monomers with no or low glycosylation and have an open binding cleft. They mostly have pH optima between 4.0 and 5.0 and temperature optima from 50 to 70 °C (Table. 3). Studies have shown that many fungi produce multiple EGs. For example, *T. reesei* produces at least 5 EGs (EGI/Cel7B, EGII/Cel5A, EGIII/Cel12A, EGIV/Cel61A and EGV/Cel45A) whereas three EGs were isolated from white-rot fungus *P. chrysosporium* (EG28, EG34 and EG44) [44,57]. In addition, some EGs lack a CBM while some other EGs with CBM have been described. For example, four of five EGs in *T. reesei* including EGI, EGII, EGIV and EGV have CBM whereas EGIII does not have a CBM [36].

Cellobiohydrolases (EC 3.3.1.91, exocellulase)

Cellobiohydrolases (CBH) preferentially hydrolyze β-1,4-glycosidic bonds from chain ends, producing cellobiose as the main product. CBHs have been shown to create a substrate-binding tunnel with their extended loops which surround the cellulose [58,59]. Similar to EGs, CBHs are monomers with no or low glycosylation with pH optima mostly between 4.0 and 5.0, but the temperature optima are wider, from 37 to 60 °C (Table. 3). Studies have shown that some CBHs can act from the non-reducing ends and others from the reducing ends of the cellulosic chains, which increases the synergy between opposite-acting enzymes. For example, *T. reesei* has been shown to have two CBHs acting from non-reducing (CBHII/Cel6A) and reducing (CBHI/Cel7A) ends, which results in a more efficient cellulolytic degrader. Moreover, both CBHI and CBHII of *T. reesei* have CBM at the carboxy-terminus or at the amino-terminus of the catalytic module respectively. Cellobiose, the end product of CBHs, acts as a competitive inhibitor, which can limit the ability of the enzymes to degrade all of cellulose molecules in a system [36,44,60].

Table 3. Overview of the three groups of fungal cellulolytic enzymes and their main features.

	Optimum Substrate	Molecular mass (kDa)	GH family: corresponding structural fold	Optimum temperature (°C)	pH optimum	Glyco-sylation	References
Endo-1,4- β-glucanases (EG)	Cellulose (amorphous regions)	Monomeric (22-45)	**5**: (β/α)8 **6**: Distorted (β/α) **7, 12**: β-jelly roll **9, 48**: (α/α)6 **45**: β barrel **61**: - **74**: 7-fold β-propeller	50-70	Mostly 4-5	None or very low	[33,35, 44]
Cellobiohydrolases (CBH)	Cellulose (crystalline regions)	Monomeric (50-65)	**6**: Distorted (β/α) **7**: β-jelly roll **9, 48**: (α/α)6	37-60	Mostly 4-5	None or very low	[33,35, 44]
β-glucosidases (BGL)	Cellobiose, cellodextrins	Monomeric, dimeric, trimeric (35-450)	**1**: (β/α)8 **3**: -	45-75	Vary[1]	Usually very high	[33,35, 44,61-63]

[1]pH optima of BGLs vary based on the enzyme localization.

β-glucosidases (EC 3.2.1.21)

β-glucosidases (BGL) have been isolated from many different fungal species including ascomycetes such as *T. reesei*, and basidiomycetes such as white-rot and brown-rot fungi. β-glucosidases hydrolyze soluble cellobiose and cellodextrins to glucose, and are thus competitively inhibited by glucose. BGLs have been placed in families 1 and 3 of glycoside hydrolases based on their amino acid sequences [64]. Family 3 includes β-glucosidases from fungi, bacteria, and plants whereas family 1 includes β-glucosidases of bacterial, plant and mammalian origins which have galactosidase activity in addition to β-glucosidase activity. BGLs from both families hydrolyze β-1,4-glycosidic bonds using the retaining mechanism [65]. BGLs show the most variability among the cellulolytic enzymes due to their structure and localization. While some BGLs have a simple monomeric structure with around 35 kDa molecular mass (e.g. from *Pleurotus ostreatus*) [61] some others have dimeric (e.g. from *Sporobolomyces*

singularis with 146 kDa) [62] or even trimeric structures with an M_r over 450 kDa (e.g. from *Pisolithus*

tinctorius) [63]. In addition, most of BGLs are glycosylated and in some cases, such as the 300 kDa

monomeric BGL from *Trametes versicolor*, the glycosylation degree is up to 90% [66]. Regarding

localization, BGLs can be grouped into three different types including intracellular, cell wall-associated

and extracellular [67]. Not surprisingly, pH optima for the enzymes vary based on enzyme localization.

However, the temperature optima range from 45 to 75 °C (Table 3). In *T. reesei*, for example, two β-

glucosidases (BGLI/Cel3A & BGLII/Cel1A) have been isolated from culture supernatant, but the

enzymes were found to be primarily bound to the cell wall [68]. Moreover, BGL production in *T. reesei* is

very low compared to other cellulolytic fungi such as *A. niger*. Attempts with some success have been

made to improve BGL activity in *T. reesei* by transformation of the *bgl* gene from the thermophilic

fungus *Talaromyces emersonii* (*cel3a*) [69]. More recently, the production of *T. reesei* β-glucosidase I

was enhanced by homologous recombination using xylanase (*xyn3*) and cellulase (*egl3*) promoters which

improved β-glucosidase activity to 4.0 and 7.5 fold compared to the parent, respectively [70].

Fungal hemicellulases

Several different enzymes are needed to hydrolyze hemicelluloses, due to their heterogeneity

[10]. Xylan is the most abundant component of hemicellulose contributing over 70% of its structure.

Xylanases are able to hydrolyze β-1,4 linkages in xylan and produce oligomers which can be further

hydrolyzed into xylose by β-xylosidase. Not surprisingly, additional enzymes such as β-mannanases,

arabinofuranosidases or α-L-arabinanases are needed depending on the hemicellulose composition which

can be mannan-based or arabinofuranosyl-containing [71]. Similar to cellulases, hemicellulases are

usually modular proteins and have other functional modules, such as CBM, in addition to their catalytic

domains. Also similarly to cellulases, most of the hemicellulases are glycoside hydrolases (GHs),

although some hemicellulases belong to carbohydrate esterases (CEs) which hydrolyze ester linkages of

acetate or ferulic acid side groups [71,72]. Hemicellulases belong to 20 different GH families (1, 2, 3, 4,

5, 8, 10, 11, 26, 27, 36, 39, 43, 51, 52, 53, 54, 57, 62 and 67) and all of them except for 4 (families 4, 8, 52 and 57) have been found in fungi. All but 1 (family 7) of the 7 different CE families (1, 2, 3, 4, 5, 6 and 7) reported for hemicellulases have been found in fungi [35]. Similarly to cellulases, aerobic fungi such as *Trichoderma* and *Aspergillus* secrete a wide variety of hemicellulases in high concentrations (8 and 12 hemicellulases, respectively) and these work in a synergistic manner [71].

Fungal ligninases

Lignin, the most abundant renewable aromatic polymer on the Earth, is composed of non-phenolic (80-90%) and phenolic structures [73]. It has been shown that fungi degrade lignin by secreting enzymes collectively termed "ligninases". These include two ligninolytic families; i) phenol oxidase (laccase) and ii) peroxidases [lignin peroxidase (LiP) and manganese peroxidase (MnP)] [74]. White-rot basidiomycetes such as *Coriolus versicolor* [73], *P. chrysosporium* and *T. versicolor* [75] have been found to be the most efficient lignin-degrading microorganisms studied. Interestingly, LiP is able to oxidize the non-phenolic part of lignin, but it was not detected in many lignin degrading fungi. In addition, it has been widely accepted that the oxidative ligninolytic enzymes are not able to penetrate the cell walls due to their size. Thus, it has been suggested that prior to the enzymatic attack, low-molecular weight diffusible reactive oxidative compounds have to initiate changes to the lignin structure (as discussed below) [76,77].

Oxidative (Non-lignocellulolytic) lignocellulose-degradation mechanisms in higher fungi

A few decades ago, non-enzymatic degradation mechanisms for plant cell-wall polysaccharide degradation were also considered and over the time more evidence for these was found. The non-enzymatic degradation mechanism is mostly assisted by oxidation through production of free hydroxyl radicals (•OH). In fact, many white and brown-rot fungi have been shown to produce hydrogen peroxide

19

(H_2O_2) which enters the Fenton reaction and results in release of •OH [78,79]. These free radicals attack polysaccharides as well as lignin in plant cell walls in a nonspecific manner providing some cleavages which make it easier for the lignocellulolytic enzymes to penetrate [80,81]. Three different pathways have been found for the generation of free radicals (discussed below) including cellobiose dehydrogenase (CDH) catalyzed reactions, low molecular weight peptides/quinone redox cycling and glycopeptide-catalyzed Fenton reactions (Table 4) [44].

CDH, an extracellular monomeric protein with some glycosylation, has been identified in a number of wood- and cellulose-degrading fungi including basidiomycetes (mostly white-rot fungi) and ascomycetes growing on cellulose. The enzyme is able to oxidize cellobiose, higher cellodextrins and other disaccharides or oligosaccharides with β-1,4 linkages. In addition, CDH with (in ascomycetes) or without CBM (in basidiomycetes) have been identified however even in the absence of CBM they are able to bind to cellulose through hydrophobic interactions [82]. It has been shown in some fungi that under cellulolytic conditions CDH production increases which helps cellulases and hemicellulases [83,84]. It is now widely accepted that CDH are able to degrade and modify all three major components of the lignocellulosic residues (cellulose, hemicelluloses and lignin) by producing free hydroxyl radicals in a Fenton-type reaction (for detailed information please refers to the review by Baldrin and Valaskova, 2008 [44]).

It has been shown that white and brown-rot fungi produce low molecular weight chelators which are able to penetrate into the cell wall. For example *Gloeophyllum trabeum* produces a low molecular weight peptide (known as short fiber generating factor, SFGF) which can degrade cellulose into short fibers by an oxidative reaction [81,85]. It has also been reported that some of these low molecular weight compounds are quinones which have to be converted to hydroquinones by some fungal enzymes (Table 4) and then through Fenton reaction, free hydroxyl radicals will be produced [73].

Different glycopeptides with different molecular weight (ranging from 1.5 to 12 kDa) have been found in many brown-rot fungi such as *G. trabeum* [86] and white-rot fungi such as *P. chrysosporium*

20

[77,87]. Similar to the other mechanisms, glycopeptides are able to catalyze redox reactions and thus produce free hydroxyl radicals.

Table 4. Different mechanisms involved in production of ˙OH in different fungi

Fungi	Mechanisms	Other enzymes involved/their function	References
White-rot fungi (e.g. *Dichomitus squalens*)	CDH catalyzed reaction	Oxalate decarboxylase/regulation of oxalate concentration	[88,89]
Brown and white-rot fungi (e.g. *Coniophora puteana, P. chrysosporium*)	Quinone redox cycling	Benzoquinone reductases, CDH, sugar dehydrogenases/convert quinones to hydroquinones	[83,90]
Brown and white-rot fungi (e.g. *F. palustris, P. chrysosporium*)	Glycopeptides-catalyzed Fenton reaction	Cell wall-associated reductase/reduction of glycopeptides	[91]

"Cellulosome": non-free cellulases in anaerobic fungi

Anaerobic fungi represent a special group of microorganisms inhabiting the gastro-intestinal tract of ruminants and most non-ruminant herbivores. These fungi, along with some anaerobic bacteria (mainly from the class Clostridia e.g. *Clostridium thermocellum* [92]), produce a range of cellulolytic and hemicellulolytic enzymes in a multienzyme complex known as cellulosome. The first anaerobic gut fungi able to break down ingested lignocellulosic residues were identified in 1975 by Orpin [93] and since then 6 genera and 18 species have been identified some of which are shown in Table 2. The cellulosome, however, was initially discovered in anaerobic bacteria (*Clostridium thermocellum*) in 1983 [94], and then first described in anaerobic fungi in 1992 (*Neocallimastix frontalis*) [95]. In anaerobic bacteria, the cellulosome is usually comprised of 20 or more different cellulolytic/hemicellulolytic enzymes. However, in anaerobic fungi such as *N. frontalis* and *Piromyces*, cellulosomes include at least six or ten polypeptides, respectively (cellulosome-type complex) [55,95,96]. All hydrolytic enzymes in the

cellulosome are bound together by noncatalytic scaffolding proteins. In addition to catalytic subunits, all enzymes have noncatalytic subunits known as "fungal dockerin domains" (FDD), which allow binding to cohesin modules of the scaffolding proteins. Interestingly, 50 fungal FDDs have been identified so far which present different amino acid sequences than those found in bacterial dockerins [31,97].

Anaerobic fungi efficiently hydrolyze cellulose and hemicellulose by producing many lignocellulolytic enzymes. Most of the enzymes are associated with the cellulosome; however, some free enzymes also have been identified. In *Piromyces* sp. PC2, a cellulosome-producing anaerobic fungus for example, 17 lignocellulolytic enzyme encoding genes have been isolated including ten cellulases, one β-glucosidases, five hemicellulases and one enzyme facilitating protein folding. Interestingly, FDD has been reported only for 11 of the genes, which indicates that these cellulases are cellulosome-associated. Moreover, CBM has been identified only in three of those 17 genes, including two cellulases and one hemicellulase [31]. Interestingly, the major product of cellulose digestion by fungal cellulosomes is glucose which eliminate the costly addition of β-glucosidase, whereas in the case of bacterial cellulosomes, cellobiose is the major product [98]. Despite many advantages of cellulosomes such as synergistic activity between the components and efficient hydrolytic activity on both cellulose and hemicellulose, fungal cellulosomes are much less well characterized compared to bacterial cellulosomes.

Bioconversion and biotechnological aspects of lignocellulose degradation by microorganisms

The bioconversion of lignocellulosic residues to valuable materials such as ethanol is more complicated than the bioconversion of starch based residues and thus requires four steps of processing, of which the first three are bio-related processes and the fourth is primarily a chemical engineering process that will not be discussed in great detail in this review; i) pretreatment ii) de-polymerization (saccharification) of cellulose and hemicelluloses to soluble monomer sugars (hexoses and pentoses) by a process known as hydrolysis, iii) conversion of these monomeric sugars to valuable products such as

ethanol in a fermentation process and iv) separation and purification of the products (Figure 1). In order to improve the yield, each step in the bioconversion process has to be optimized. In addition, process integration has to be considered in order to minimize process energy demand [22].

Pretreatment

Pretreatment of the lignocellulosic residues is necessary because hydrolysis of non-pretreated materials is slow, and results in low product yield. Some pretreatment methods increase the pore size and reduce the crystallinity of cellulose (Figure 1). Pretreatment also makes cellulose more accessible to the cellulolytic enzymes, which in return reduces enzyme requirements and thus the cost [99]. Many different pretreatment methods have been used, but they can be categorized into three broad groups: chemical (e.g. acid or alkali), physical/ physicochemical (e.g. physical ball milling or physicochemical steam explosion) and biological pretreatment by microorganisms [100]. In the chemical pretreatment method using acid for example, hemicelluloses will be targeted whereas in alkali-catalyzed pretreatment mainly lignin is removed [22]. It has been suggested that there is probably no general pretreatment procedure and that different raw materials will require different pretreatments [22]. Since many fungal cellulolytic enzymes (fungal-derived cellulases and β-glucosidases) work at lower pH (usually 4-5) acidic pretreatment seems a preferred option when fungal enzymes are chosen for the hydrolysis [22].

Biological pretreatment uses microorganisms and their enzymes selectively for delignification of lignocellulosic residues and has the advantages of a low-energy demand, minimal waste production and a lack of environmental effects [101]. White-rot basidiomycetes possess the capabilities to attack lignin. *P. chrysosporium*, for example, has been shown to non-selectively attack lignin and carbohydrate [102]. *P. chrysosporium* was successfully used for biological pretreatment of cotton stalks by solid state cultivation (SSC) and results have shown that the fungus facilitates the conversion into ethanol [101]. Other basidiomycetes such as *Phlebia radiata*, *P. floridensis* and *Daedalea flavida*, selectively degrade lignin in wheat straw and are good choices for delignification of lignocellulosic residues [103]. *Ceriporiopsis*

subvermispora, however, lacks cellulases (cellobiohydrolase activity) but produces manganese peroxide and laccase, and selectively delignifies several different wood species [104].
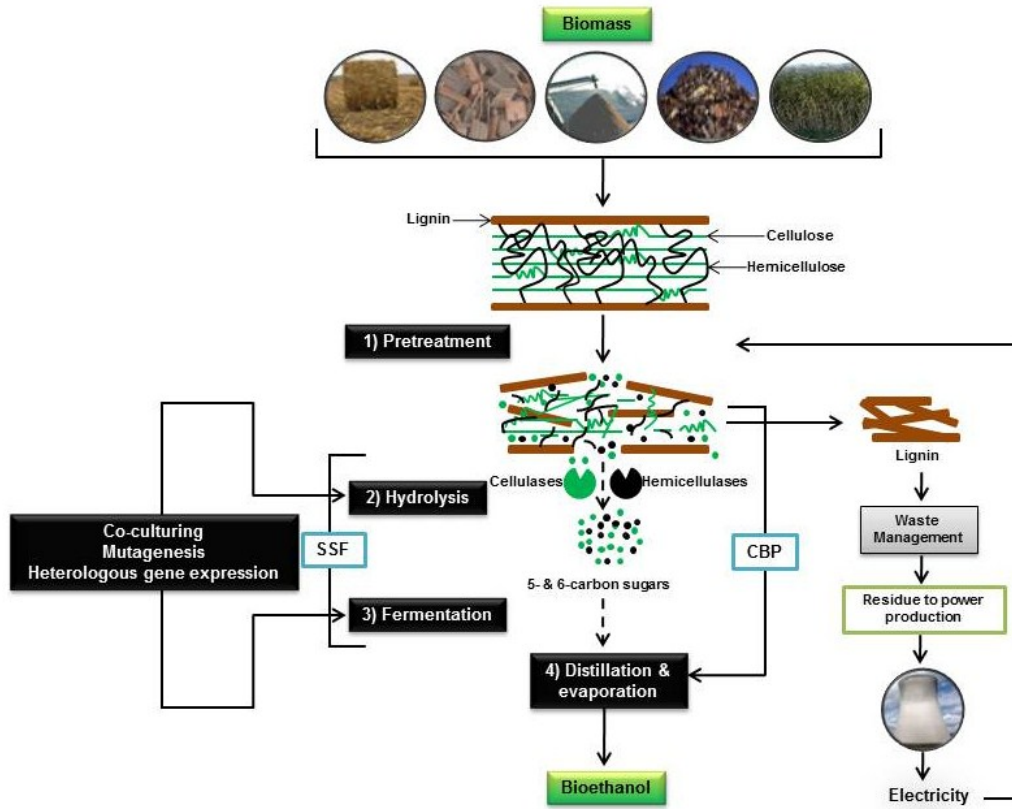
Figure 1. Schematic picture for the conversion of lignocellulosic biomass to ethanol, including the major steps. Hydrolysis and fermentation can be performed separately (SHF, indicated by broken arrows) or as simultaneous saccharification and fermentation (SSF). In consolidated bioprocessing (CBP) however, all bioconversion steps are minimized to one step in a single reactor using one or more microorganisms. Different techniques such as mutagenesis, co-culturing and heterologous gene expression have been used to improve sugars utilization of the microbial biocatalyst as well as activity and/or stability of hydrolytic fungal-derived enzymes in order to improve the overall yields. For reduction of production cost, ethanol production can be integrated with a combined heat and power plant using lignin.

Hydrolysis

After pretreatment, cellulose and hemicelluloses are hydrolyzed to soluble monomeric sugars (hexoses and pentoses) using cellulases and hemicellulases, respectively (Figure 1). As mentioned earlier, many fungal species such as *Trichoderma*, *Penicillium*, *Aspergillus* and *T. emersonii* are able to produce large amounts of extracellular cellulases and hemicellulases. High temperature and low pH tolerant enzymes are preferred for the hydrolysis due to the fact that most current pretreatment strategies rely on acid and heat [105]. In addition, thermostable enzymes have several advantages including higher specific activity and higher stability which improve the overall hydrolytic performance [106]. Ultimately, improvement in catalytic efficiencies of enzymes will reduce the cost of hydrolysis by enabling lower enzyme dosages. Some fungal strains such as *T. emersonii* [107], *T. aurantiacus* [29], *T. terrestris* [30], *S. thermophile* [28], *Chaetomium thermophilum* [108] and *Corynascus thermophilus* [109] can produce thermostable enzymes which are stable and active at elevated temperatures (>60°C) well above their optimum growth temperature (30 to ~55) [110]. Due to the promising thermostability and acidic tolerance of thermophilic fungal enzymes, they have good potential to be used for hydrolysis of lignocellulosic residues at industrial scales.

Fermentation

In the fermentation process, the hydrolytic products including monomeric hexoses (glucose, mannose and galactose) and pentoses (xylose and arabinose) will be fermented to valuable products such as ethanol (Figure 1). Among these hydrolytic products, glucose is normally the most abundant, followed by xylose or mannose and other lower concentration sugars. *Saccharomyces cerevisiae* is the most frequently and traditionally used microorganism for fermenting ethanol from starch-based residues at industrial scales [22]. *S. cerevisiae* has a few advantages such as its wide public acceptance, high fermentation rate and high ethanol tolerance that make it a good candidate for fermentation processes. However, *S. cerevisiae* is unable to efficiently utilize xylose as the sole carbon source or ferment it to

ethanol [111]. To make industrial lignocellulosic bioconversion more economically feasible, it is necessary to choose microorganisms capable of fermenting both glucose and xylose. Therefore, many successful attempts have been made to improve xylose fermentation in *S. cerevisiae* since the first discovery of pentose-fermenting yeasts in 1981 by a Canadian group [112]. These efforts can be classified within two major groups: recombinant (e.g. metabolic engineering of *S. cerevisiae* with genes from other xylose-fermenting yeasts) and non-recombinant (e.g. adaptation) techniques. These improvements have reached the point where the deficient xylose-fermenting *S. cerevisiae* can now convert xylose to ethanol at an efficiency close to its theoretical value of 0.51 g g^{-1} (for extensive review please read Chu and Lee, 2007 [111]).

In addition to xylose, *S. cerevisiae* is also unable to ferment arabinose, unless supplemented with rich media [113]. Therefore, recombinant *S. cerevisiae* harbouring xylose-fermenting genes have been engineered with arabinose-metabolizing genes from other microorganisms. The latest recombinant *S. cerevisiae* (TMB 3400) has been shown to successfully ferment both xylose and arabinose in addition to glucose [114].

During fermentation of lignocellulosic-based biomass, *S. cerevisiae* faces yet another challenge: the presence of inhibiting compounds including low molecular weight organic acids, furan derivatives, phenolics and inorganic compounds. These compounds are released and formed during pretreatment and/or hydrolysis of the lignocellulosic residues [115]. Thus, it is necessary to detoxify hydrolytic products before the fermentation which increases process cost in addition to sugar loss [116]. Interestingly, *S. cerevisiae* is one of the least sensitive microorganisms to the inhibitory effect of lignocellulolytic hydrolysate inhibitors. In a recent study for example, glucose and xylose, the hydrolytic products of steam-pretreated corn stover were efficiently co-fermented to ethanol without detoxification using the recombinant *S. cerevisiae* strain TMB 3400 [117]. It is also possible to adopt recombinant xylose-fermenting *S. cerevisiae* to the hydrolysate inhibitors by continuous cultivation in the presence of the inhibitors [118].

Attempts have been taken to reduce by-product inhibition. In a recent study for example, wheat straw pellets were subjected to wet explosion pretreatment using three different oxidizing agents, H_2O_2, O_2 and air [119]. Interestingly, the pretreatment with O_2 has been shown to be the most efficient in enhancing conversion of the raw material to sugars. Using the method also has minimized the production of furfural as a by-product which improved enzymatic hydrolysis and minimized the enzyme loading to 10 FPU/g with conversion rate of 70 and 68% for cellulose and hemicellulose respectively [119]. Ammonia fiber explosion (AFEX) pretreatment also has been shown to be a good candidate since it does not produce some inhibitory by-products such as furans. However, the disadvantage of the method is that some of the phenolic compounds in lignin may remain on the pretreated material, which then needs to be washed. This creates wastewater, which causes the process to become environmentally unfriendly [100].

The last two steps of bioconversion of pretreated lignocellulolytic residues to ethanol (hydrolysis and fermentation) can be performed separately (SHF) or simultaneously (SSF) (Figure 1). In the separate hydrolysis and fermentation (SHF), the hydrolysate products will be fermented to ethanol in a separate process. The advantage of this method is that both processes can be optimized individually (e.g. optimal temperature is 45-50 °C for hydrolysis, whereas it is 30 °C for fermentation). However, its main drawback is the accumulation of enzyme-inhibiting end-products (cellobiose and glucose) during the hydrolysis. This makes the process inefficient, and the costly addition of β-glucosidase is needed to overcome end-product inhibition [120]. In simultaneous saccharification and fermentation (SSF), however, the end-products will be directly converted to ethanol by the microorganism. Therefore, addition of high amounts of β-glucosidase is not necessary and this reduces the ethanol production costs [121]. However, the main drawback of SSF is the need to compromise processing conditions such that temperature and pH are suboptimal for each individual step. However, the development of recombinant yeast strains (i.e. improved thermotolerance) is expected to enhance the performance of SSF [1]. Further process integration can be achieved by a process known as consolidated bioprocessing (CBP) which aims to minimize all bioconversion steps into one step in a single reactor using one or more microorganisms.

27

CBP operation featuring cellulase production, cellulose/hemicellulose hydrolysis and fermentation of 5- and 6- carbon sugars in one step have shown the potential to provide the lowest cost for biological conversion of cellulosic biomass to fuels, when processes relying on hydrolysis by enzymes and/or microorganisms are used (Figure 1) [122].

Methods used to improve fungal enzyme production, activity and/or stability

In order to increase ethanol yield in the bioconversion process, both cellulose and hemicellulose have to be completely hydrolyzed with minimum sugar degradation. Moreover, all monomeric sugars produced during hydrolysis have to be efficiently fermented to ethanol. Technologies required for bioconversion of lignocelluloses to ethanol and other valuable products are currently available but need to be developed further in order to make biofuels cost competitive compared to other available energy resources such as fossil fuels. Many attempts have been made to improve the overall process yield and cost with a main focus on enzyme production and activity. Not surprisingly, the application of different strains and processes which are selected on the basis of the biomass residues used make comparisons difficult, if not impossible. Nevertheless, the most recent and important improvements in production/activity of fungal enzymes using different techniques such as mutagenesis, co-culturing and heterologous gene expression of cellulases are discussed below and summarized in Table 5.

Mutagenesis

Many fungal strains have been subjected to extensive mutagenesis studies due to their ability to secrete large amounts of cellulose-degrading enzymes. It has been four decades since Mandels and Weber (late 1960s) screened over 100 wild-type strains of *Trichoderma* species to isolate the best cellulolytic strain and came up with *T. reesei* (initially called *T. viride* QM6a) [123]. Cellulolytic activity of *T. reesei* QM6a has been improved by using different mutagenesis techniques including UV-light and chemicals at

the US Army Natick Laboratory, resulting in the mutant QM 9414 with higher filter paper activity (FPA) [124]. Other studies in different laboratories have also made significant contributions to strain improvements using mutagenesis techniques, leading to development of the mutant strains M7, NG14 [125] and RUT-C30 [126]. *T. reesei* RUT-C30 is one of the best known mutants, producing 4-5 times more cellulase than the wild-type strain (QM 6a). A recent study by Kovács and *et al.* (2008) has shown that wild-type *Trichoderma atroviride* (F-1505) produces the most cellulase among 150 wild-type *Trichoderma*. Moreover, *T. atroviride* mutants were created by mutagenesis using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) as well as UV-light. These *T. atroviride* mutants (e.g. *T. atroviride* TUB F-1724) produce high levels of extracellular cellulases as well as β-glucosidase when they are grown on pretreated willow [127].

Cellulase and xylanase activities in *Penicillium verruculosum* 28K mutants were improved about 3-fold using four cycles of UV mutagenesis. The enzyme production was further improved by 2- to 3-fold in a two-stage fermentation process using wheat bran, yeast extract medium and microcrystalline cellulose as the inducer [128]. However, caution has to be taken during strain improvement by mutagenesis. Studies have shown that the best *T. reesei* mutant (RUT-C30) lacks an 85 Kb genomic fragment and is consequently missing 29 genes which include transcription factors, metabolic enzymes and transport proteins. In fact, these genotype changes are correlated with phenotypic changes such as poor growth on α-linked oligo- and polyglucosides or disturbance of osmotic homeostasis [129].

On the other hand, site-directed mutagenesis (SDM) has played a central role in the characterization and improvement of cellulases including their putative catalytic and binding residues. Different site-directed mutagenesis methods such as saturation mutagenesis, error-prone PCR and DNA shuffling have been used to improve specific enzyme properties. For example, by the application of SDM it was found that Glu 116 and 200 are the catalytic nucleophile and acid-base residues in *Hypocrea jecorina* (anamorph *T. reesei*) Cel12A, respectively. In the study, mutant enzymes were produced where Glu was replaced by Asp or Gln at each position (E116D/Q and E200D/Q). The specific activity of these

29

mutants was reduced by more than 98%, suggesting the critical role of these two residues in the catalytic function of the enzyme [130].

In another study, the thermostable endo-1,4-β-xylanase (XynII) mutants from *T. reesei* were further mutated to resist inactivation at high pH by using SDM. All mutants were resistant to thermal inactivation at alkaline pH. For example, thermotolerance for one mutant (P9) at pH 9 was increased approximately 4-5 °C, resulting in better activity in sulphate pulp bleaching compared to the reference [131]. Also, the catalytic efficiency and optimum pH of *T. reesei* endo-β-1,4-glucanase II were improved by saturation mutagenesis followed by random mutagenesis and two rounds of DNA shuffling. The pH optimum of the variant (Q139R/L218H/W276R/N342T) was shifted from 4.8 to 6.2, while the enzyme activity was improved more than 4.5-fold [132]. Moreover, the stability of *T. reesei* endo-1,4-β-xylanases II (XynII) was increased by engineering a disulfide bridge at its N-terminal region. In fact, two amino acids (Thr-2 and Thr-28) in the enzyme were substituted by cysteine (T2C:T28C mutant) resulting in a 15 °C increase in thermostability [133].

Co-culturing

Fungal co-culturing offers a means to improve hydrolysis of lignocellulosic residues, and also enhances product utilization which minimizes the need for additional enzymes in the bioconversion process. In the case of cellulose degradation, for example, all three enzymatic components (EG, CBH and β-glucosidase) have to be present in large amounts. However, none of the fungal strains, including the best mutants, are able to produce high levels of the enzymes at the same time. *T. reesei* for example produces CBH and EG in high quantities whereas its β-glucosidase activity is low [134]. *A. niger* however, produces large amounts of β-glucosidase, but has limited EG components [56]. In addition, hemicellulose hydrolysis must also be considered when lignocellulosic residues are subjected to biomass conversion. However, this will be determined by the pretreatment methods. Specifically in an alkali

pretreatment method, a part of lignin will be removed and thus hemicellulose has to be degraded by the use of hemicellulases, whereas in acid-catalyzed pretreatment, the hemicellulose layer will be hydrolyzed [22]. Again, some fungal strains have been shown to work more efficiently on cellulosic residues whereas others produce more hemicellulolytic enzymes and efficiently hydrolyze hemicellulosic portions [20,135]. Conversion of both cellulosic and hemicellulosic hydrolytic products in a single process can be achieved by co-culturing two or more compatible microorganisms with the ability to utilize the materials. In fact, in nature, lignocellulosic residues are degraded by multiple co-existing lignocellulolytic microorganisms. Co-culturing of two or more fungal strains in mixed culture fermentation is widely used in many biological processes including the production of antibiotics, enzymes and fermented food [136]. Mixed fungal cultures have many advantages compared to their monocultures, including improving productivity, adaptability and substrate utilization. Improving fungal cellulolytic activity of *T. reesei* and *A. niger* by co-culturing was the subject of extensive research including studies done by Maheshwari [137], Ahmad [138] and Juhász [139]. Moreover, other fungal strains have been co-cultured to obtain better cellulolytic activity such as co-culturing of *T. reesei* RUT-C30 and *A. phoenicis* [140] or *A. ellipticus* and *A. fumigatus* (Table 5) [141]. There are a few examples of co-culturing fungal strains for the purpose of combining cellulose and hemicellulose hydrolysis such as co-culturing *T. reesei* D1-6 and *A. wentii* Pt 2804 in a mixed submerged culture [142] or co-culturing *T. reesei* LM-UC4 and *A. phoenicis* QM329 using ammonia-treated bagasse [143]. In the both cases, enzyme activity for cellulases and hemicellulases was significantly increased. The main drawback of co-culturing however is the complexity of growing multiple microorganisms in the same culture [144].

Alternatively to co-culture, microorganisms can be metabolically engineered, which enables one microorganism to complete an entire task from beginning to end. This can be done by altering metabolic flux by blocking undesirable pathway(s) and/or enhancement of desirable pathway(s). For example by application of homologous recombination, the production of *T. reesei* β-glucosidase I was enhanced using xylanase (*xyn3*) and cellulase (*egl3*) promoters which improved β-glucosidase activity to 4.0 and 7.5 fold

31

compared to the parent, respectively. This will permit one fungal strain such as *T. reesei* to be more efficient on hydrolysis of cellulose to glucose which improve the yield and therefore lower the cost [70].

Heterologous expression of cellulases

Heterologous expression is a powerful technique to improve production yield of enzymes, as well as activity. In order to make a robust lignocellulolytic fungal strain, many different fungal cellulases with higher and/or specific activity based on the need for a functional cellulase system in the organism have been cloned and expressed. For example, thermostable β-glucosidase (*cel3a*) from thermophilic fungus *T. emersonii* was expressed in *T. reesei* RUT-C30 using a strong *T. reesei cbh1* promoter. The expressed enzyme has been shown to be highly thermostable (optimum temperature at 71.5 °C) with high specific activity [69]. In the study for the improvement of biofinishing of cotton, *T. reesei* cellobiohydrolase (I & II) were overexpressed using additional copy(s) of the genes cloned under *T. reesei cbh1* promoter.

The results have shown that the expression of CBHI was increased to 1.3- and 1.5-fold with one or two additional copies of the gene, respectively. In the case of CBHII, however, the expression was increased to 3- to 4-fold using just one additional copy of the gene [145]. In addition, chimeric proteins with specific applications have been designed using recombinant DNA technology. For example, an endoglucanase from *Acidothermus cellulolyticus* was fused to *T. reesei* cellobiohydrolase and expressed in *T. reesei*. This bi-functional endo- & exo-acting cellulolytic enzyme has been shown to improve saccharification yields [146]. Moreover, the structural and biochemical information obtained from family GH 12 homologues was used to create a wide range of *H. jecorina* Cel12 A variants which were heterologously expressed as secreted proteins in *A. niger* displaying temperature stability changes ranging from none to an increase of 3.9 °C (the most stable variant, P201C) (Table 5) [36].

Table 5. Some methods which have been used to improve fungal lignocellulolytic activity or stability.

Methods	Fungal strain	Enzyme	Altered feature	Technique	Reference
Mutagenesis	*T. reesei* RUT-C30	Cellulases	Activity	UV treatment followed by 2 rounds of NTG treatment	[126,129]
	T. atroviride TUB F-1724	β-glucosidase	Activity	2 rounds of NTG treatment followed by UV treatment	[127]
	P. verruculosum 28K mutants	Cellulases and xylanases	Activity	Four cycles of UV mutagenesis followed by two-stage fermentation process	[128]
	T. reesei P9	Thermophilic endo-1,4-β-xylanase (XynII)	pH stability (alkalinity), Thermostability	SDM (using PCR and synthetic oligonucleotide primers) (N97R+F93W+H144K)	[131]
	T. reesei (Variants L218H, Q139R/N342T)	Endo-β-1,4-glucanase II	Catalytic efficiency, pH optimum	Saturation mutagenesis followed by random mutagenesis and two rounds of DNA shuffling	[132]
	T. reesei (T2C:T28C mutant)	Endo-1,4-β-xylanases II (XynII)	Thermostability	PCR and synthetic oligonucleotide primers (Engineering a disulfide bridge at N-terminal region)	[133]
Co-culturing	*T. reesei* RUT-C30 and *A. niger* LMA	β-glucosidase	Activity	Fed-batch fermentor on a Cellulose-Yeast extract medium	[138]
	T. reesei RUT-C30 and *A. phoenicis*	β-glucosidase	Activity	Shake flask culture	[140]
	A. ellipticus and *A. fumigatus*	β-glucosidase	Activity	Solid state fermentation using pretreated sugarcane bagasse	[141]
	T. reesei D1-6 and *A. wentii* Pt 2804	Cellulases, xylanases	Activity	Mixed culture medium (M3) supplemented with trace metal & vitamin solutions	[142]
	T. reesei LM-UC4 and *A. phoenicis* QM329	Cellulases, hemicellulases	Activity	Solid state fermentation using ammonia-treated bagasse	[143]
Heterologous gene expression	*T. reesei* RUT-C30	Thermostable β-glucosidase (*cel3a*) from thermophilic fungus *T. emersonii*	Activity	Heterologous gene expression using *T. reesei cbh1* promoter	[69]
	T. reesei	Cellobiohydrolase (I & II)	Activity	Overexpression using *T. reesei cbh1* promoter	[145]
	Acidothermus cellulolyticus and *T. reesei*	Endoglucanase & cellobiohydrolase	Bi-functional endo- & exo-acting cellulase	Chimeric protein, expressed in *T. reesei*	[146]
	H. jecorina (P201C)	Cel12 A	Thermostability	Mutation followed by heterologous expression in *A. niger*	[36]

SDM: site-directed mutagenesis

Concluding remarks

Lignocellulolytic microorganisms, especially fungi, have attracted a great deal of interest as biomass degraders for large-scale applications due to their ability to produce large amounts of extracellular lignocellulolytic enzymes. Many successful attempts have been made to improve fungal

lignocellulolytic activity including recombinant and non-recombinant techniques. Process integration has also been considered for the purpose of decreasing the production cost, which was partly achieved by performing hydrolysis and fermentation in a single reactor (SSF) using one or more microorganisms (co-culturing). Moreover, recombinant *S. cerevisiae* with efficient fermenting activity for both 5- and 6-carbon sugars in the presence of inhibitors contributed to process integration. These laboratory improvements should now be verified in pilot and demonstration plants, such as the projects completed at the Iogen pilot plant (Canada). Scaling up the production of lignocellulosic ethanol, however, requires further reduction of the production cost. Overall, in order to improve the technology and reduce the production cost, two major issues have to be addressed: i) improving technologies to overcome the recalcitrance of cellulosic biomass conversion (pretreatment, hydrolysis and fermentation) and ii) sustainable production of biomass in very large amounts. In the case of large scale biomass production, in addition to forest and crop residues, energy crops such as switchgrass and *Miscanthus* can be grown to meet the needs. On the other hand, biotechnological approaches including systems biology and computational tools are likely good candidates to overcome these issues.

References:

1. Galbe M, Zacchi G: A review of the production of ethanol from softwood. Appl Microbiol Biotechnol 2002;59:618-628.

2. Hoegh-Guldberg O, Mumby PJ, Hooten AJ, Steneck RS, Greenfield P, Gomez E, Harvell CD, Sale PF, Edwards AJ, Caldeira K, Knowlton N, Eakin CM, Iglesias-Prieto R, Muthiga N, Bradbury RH, Dubi A, Hatziolos ME: Coral reefs under rapid climate change and ocean acidification. Science 2007;318:1737-1742.

3. Hill J, Nelson E, Tilman D, Polasky S, Tiffany D: Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. Proc Natl Acad Sci U S A 2006;103:11206-11210.

4. Alonso A, Perez P, Morcuende R, Martinez-Carrasco R: Future CO2 concentrations, though not warmer temperatures, enhance wheat photosynthesis temperature responses. Physiol Plant 2008;132:102-112.

5. United Nations Department of Economic and Social Affairs PD: World Population Prospects, The 2006 Revision, Highlights. 2007:Working Paper No. ESA/P/WP.202.

6. Schiffer H-W: WEC energy policy scenarios to 2050. Energy Policy 2008;36:2464-2470.

7. Sanchez C: Lignocellulosic residues: biodegradation and bioconversion by fungi. Biotechnol Adv 2009;27:185-194.

8. Percival Zhang YH, Himmel ME, Mielenz JR: Outlook for cellulase improvement: screening and selection strategies. Biotechnol Adv 2006;24:452-481.

9. Saha BC: Alpha-L-arabinofuranosidases: biochemistry, molecular biology and application in biotechnology. Biotechnol Adv 2000;18:403-423.

10. Saha BC: Hemicellulose bioconversion. J Ind Microbiol Biotechnol 2003;30:279-291.

11. Himmel ME, Ding SY, Johnson DK, Adney WS, Nimlos MR, Brady JW, Foust TD: Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science 2007;315:804-807.

12. Kim S, Dale BE: Global potential bioethanol production from wasted crops and crop residues. Biomass and Bioenergy 2004;26:361-375.

13. Kalogo Y, Habibi S, MacLean HL, Joshi SV: Environmental implications of municipal solid waste-derived ethanol. Environ Sci Technol 2007;41:35-41.

14. Pokhrel D, Viraraghavan T: Municipal solid waste management in Nepal: practices and challenges. Waste Manag 2005;25:555-562.

15. Statistics Canada: Human Activity and the Environment, Annual Statistics 2005, Solid Waste in Canada. Catalogue no. 16-201-XIE November 2005:pp 8.

16. Champagne P: Feasibility of producing bio-ethanol from waste residues: A Canadian perspective Feasibility of producing bio-ethanol from waste residues in Canada. Resources, Conservation and Recycling 2007;50:211-230.

17. Wen Z, Liao W, Chen S: Hydrolysis of animal manure lignocellulosics for reducing sugar production. Bioresour Technol 2004;91:31-39.

18. Palacios-Orueta A, Chuvieco E, Parra A, Carmona-Moreno C: Biomass burning emissions: a review of models using remote-sensing data. Environ Monit Assess 2005;104:189-209.

19. Levine JS: Biomass burning and global change. In: Levine JS (eds) (vol. 1) Remote sensing and inventory development and biomass burning in Africa. The MIT Press, Cambridge, Massachusetts, USA 1996: pp 35.

20. Howard RL, Abotsi E, Jansen van Rensburg EL, Howard S: Lignocellulose biotechnology: issues of bioconversion and enzyme production. African Journal of Biotechnology 2003;2:602-619.

21. Renewable Fuels Association (RFA), Licht FO: Annual World Ethanol Production by Country Estimates, 2008. http://www.ethanolrfa.org/industry/statistics/ 2008.

22. Hahn-Hagerdal B, Galbe M, Gorwa-Grauslund MF, Liden G, Zacchi G: Bio-ethanol--the fuel of tomorrow from the residues of today. Trends Biotechnol 2006;24:549-556.

23. Mandels M, Sternberg D: Recent advances in cellulase technology. Ferment. Technol. 1976;54:267-286.

24. Zhou S, Ingram LO: Synergistic hydrolysis of carboxymethyl cellulose and acid-swollen cellulose by two endoglucanases (CelZ and CelY) from Erwinia chrysanthemi. J Bacteriol 2000;182:5676-5682.

25. Zhang YH, Ding SY, Mielenz JR, Cui JB, Elander RT, Laser M, Himmel ME, McMillan JR, Lynd LR: Fractionating recalcitrant lignocellulose at modest reaction conditions. Biotechnol Bioeng 2007;97:214-223.

26. Rubin EM: Genomics of cellulosic biofuels. Nature 2008;454:841-845.

27. Palmqvist E, Hahn-Hägerdal B: Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification. Bioresource Technology 2000;74:17-24.

28. Bhat KM, Maheshwari R: Sporotrichum thermophile Growth, Cellulose Degradation, and Cellulase Activity. Appl Environ Microbiol 1987;53:2175-2182.

29. Gomes I, Gomes J, Gomes DJ, Steiner W: Simultaneous production of high activities of thermostable endoglucanase and beta-glucosidase by the wild thermophilic fungus Thermoascus aurantiacus. Appl Microbiol Biotechnol 2000;53:461-468.

30.   Gilbert M, Yaguchi M, Watson DC, Wong KK, Breuil C, Saddler JN: A comparison of two xylanases from the thermophilic fungi Thielavia terrestris and Thermoascus crustaceus. Appl Microbiol Biotechnol 1993;40:508-514.

31.   Ljungdahl LG: The cellulase/hemicellulase system of the anaerobic fungus Orpinomyces PC-2 and aspects of its applied use. Ann N Y Acad Sci 2008;1125:308-321.

32.   Yoon JJ, Cha CJ, Kim YS, Son DW, Kim YK: The brown-rot basidiomycete Fomitopsis palustris has the endo-glucanases capable of degrading microcrystalline cellulose. J Microbiol Biotechnol 2007;17:800-805.

33.   Bayer EA, Chanzy H, Lamed R, Shoham Y: Cellulose, cellulases and cellulosomes. Curr Opin Struct Biol 1998;8:548-557.

34.   Weng JK, Li X, Bonawitz ND, Chapple C: Emerging strategies of lignin engineering and degradation for cellulosic biofuel production. Curr Opin Biotechnol 2008;19:166-172.

35.   Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B: The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. Nucleic Acids Res 2009;37:D233-238.

36.   Sandgren M, Stahlberg J, Mitchinson C: Structural and biochemical studies of GH family 12 cellulases: improved thermal stability, and ligand complexes. Prog Biophys Mol Biol 2005;89:246-291.

37.   Chahal DS: Solid-State Fermentation with Trichoderma reesei for Cellulase Production. Appl Environ Microbiol 1985;49:205-210.

38.   Kurzatkowski W, Torronen A, Filipek J, Mach RL, Herzog P, Sowka S, Kubicek CP: Glucose-induced secretion of Trichoderma reesei xylanases. Appl Environ Microbiol 1996;62:2859-2865.

39.   Sivan A, Elad Y, Chet I: Biological control effects of a new isolate of Trichoderma harzianum on Pythium aphanidermatum. Phytopathology 1984;74:498-501.

40.   Khan MH, Ali S, Fakhru'l-Razi A, Alam Z: Use of fungi for the bioconversion of rice straw into cellulase enzyme. J Environ Sci Health B 2007;42:381-386.

41.   Park YS, Kang SW, Lee JS, Hong SI, Kim SW: Xylanase production in solid state fermentation by Aspergillus niger mutant using statistical experimental designs. Appl Microbiol Biotechnol 2002;58:761-766.

42.   Hao J, Song F, Huang F, Yang C, Zhang Z, Zheng Y, Tian X: Production of laccase by a newly isolated deuteromycete fungus Pestalotiopsis sp. and its decolorization of azo dye. J Ind Microbiol Biotechnol 2007;34:233-240.

43.   Hao JJ, Tian XJ, Song FQ, He XB, Zhang ZJ, Zhang P: Involvement of lignocellulolytic enzymes in the decomposition of leaf litter in a subtropical forest. J Eukaryot Microbiol 2006;53:193-198.

44.   Baldrian P, Valaskova V: Degradation of cellulose by basidiomycetous fungi. FEMS Microbiol Rev 2008;32:501-521.

45.   Abbas A, Koc H, Liu F, Tien M: Fungal degradation of wood: initial proteomic analysis of extracellular proteins of Phanerochaete chrysosporium grown on oak substrate. Curr Genet 2005;47:49-56.

46.   Song BC, Kim KY, Yoon JJ, Sim SH, Lee K, Kim YS, Kim YK, Cha CJ: Functional analysis of a gene encoding endoglucanase that belongs to glycosyl hydrolase family 12 from the brown-rot basidiomycete Fomitopsis palustris. J Microbiol Biotechnol 2008;18:404-409.

47.   Lee SS, Ha JK, Cheng KJ: The effects of sequential inoculation of mixed rumen protozoa on the degradation of orchard grass cell walls by anaerobic fungus Anaeromyces mucronatus 543. Can J Microbiol 2001;47:754-760.

48.   Doi RH: Cellulases of mesophilic microorganisms: cellulosome and noncellulosome producers. Ann N Y Acad Sci 2008;1125:267-279.

49.   Roger V, Grenet E, Jamot J, Bernalier A, Fonty G, Gouet P: Degradation of maize stem by two rumen fungal species, Piromyces communis and Caecomyces communis, in pure cultures or in association with cellulolytic bacteria. Reprod Nutr Dev 1992;32:321-329.

50.   Gerbi C, Bata J, Breton A, Prensier G: Glycoside and polysaccharide hydrolase activity of the rumen anaerobic fungus Caecomyces communis (Sphaeromonas communis SENSU ORPIN) at early and final stages of the developmental cycle. Curr Microbiol 1996;32:256-259.

51.   Ozkose E, Thomas BJ, Davies DR, G. W. Griffith, Theodorou MK: Cyllamyces aberensis gen.nov. sp.nov., a new anaerobic gut fungus with branched sporangiophores isolated from cattle. Can. J. Bot. 2001;79:666-673.

52.   Li XL, Calza RE: Fractionation of cellulases from the ruminal fungus Neocallimastix frontalis EB188. Appl Environ Microbiol 1991;57:3331-3336.

53.   Griffith GW, Ozkose E, Theodoroua MK, Davies DR: Diversity of anaerobic fungal populations in cattle revealed by selective enrichment culture using different carbon sources. Fungal Ecology 2009;2:87-97.

54.   Chen H, Li XL, Blum DL, Ljungdahl LG: Two genes of the anaerobic fungus Orpinomyces sp. strain PC-2 encoding cellulases with endoglucanase activities may have arisen by gene duplication. FEMS Microbiol Lett 1998;159:63-68.

55.   Ali BR, Zhou L, Graves FM, Freedman RB, Black GW, Gilbert HJ, Hazelwood GP: Cellulases and hemicellulases of the anaerobic fungus Piromyces constitute a multiprotein cellulose-binding complex and are encoded by multigene families. FEMS Microbiol Lett 1995;125:15-21.

56.   Kumar R, Singh S, Singh OV: Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. J Ind Microbiol Biotechnol 2008;35:377-391.

57.   Foreman PK, Brown D, Dankmeyer L, Dean R, Diener S, Dunn-Coleman NS, Goedegebuur F, Houfek TD, England GJ, Kelley AS, Meerman HJ, Mitchell T, Mitchinson C, Olivares HA, Teunissen PJ, Yao J, Ward M: Transcriptional regulation of biomass-degrading enzymes in the filamentous fungus Trichoderma reesei. J Biol Chem 2003;278:31988-31997.

58.   Divne C, Stahlberg J, Reinikainen T, Ruohonen L, Pettersson G, Knowles JK, Teeri TT, Jones TA: The three-dimensional crystal structure of the catalytic core of cellobiohydrolase I from Trichoderma reesei. Science 1994;265:524-528.

59.   Rouvinen J, Bergfors T, Teeri T, Knowles JK, Jones TA: Three-dimensional structure of cellobiohydrolase II from Trichoderma reesei. Science 1990;249:380-386.

60.   Divne C, Stahlberg J, Teeri TT, Jones TA: High-resolution crystal structures reveal how a cellulose chain is bound in the 50 A long tunnel of cellobiohydrolase I from Trichoderma reesei. J Mol Biol 1998;275:309-325.

61.   Morais H, Ramos C, Matos N, Forgacs E, Cserhati T, Almeida V, Oliveira J, Darwish Y, Iles Z: Liquid chromatographic and electrophoretic characterisation of extracellular beta-glucosidase of Pleurotus ostreatus grown in organic waste. J Chromatogr B Analyt Technol Biomed Life Sci 2002;770:111-119.

62.   Ishikawa E, Sakai T, Ikemura H, Matsumoto K, Abe H: Identification, cloning, and characterization of a Sporobolomyces singularis beta-galactosidase-like enzyme involved in galacto-oligosaccharide production. J Biosci Bioeng 2005;99:331-339.

63.   Cao WG, Crawford DL: Purification and some properties of beta-glucosidase from the ectomycorrhizal fungus Pisolithus tinctorius Strain Smf. Can J Microbiol 1993;39:125-129.

64.   Henrissat B: A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J 1991;280 (Pt 2):309-316.

65.   Dan S, Marton I, Dekel M, Bravdo BA, He S, Withers SG, Shoseyov O: Cloning, expression, characterization, and nucleophile identification of family 3, Aspergillus niger beta-glucosidase. J Biol Chem 2000;275:4973-4980.

66.   Evans CS: Properties of the beta-D-glucosidase (cellobiase) from the wood-rotting fungus, Coriolus versicolor. Appl Microbiol Biotechnol 1985;22:128-131.

67.   Cai YJ, Chapman SJ, Buswell JA, Chang ST: Production and distribution of endoglucanase, cellobiohydrolase, and beta-glucosidase components of the cellulolytic system of Volvariella volvacea, the edible straw mushroom. Appl Environ Microbiol 1999;65:553-559.

68.   Kubicek CP: Release of carboxymethyl-cellulase and β-glucosidase from cell walls of Trichoderma reesei. Eur J Appl Microbiol Biotechnol 1981;13:226-231.

69. Murray P, Aro N, Collins C, Grassick A, Penttila M, Saloheimo M, Tuohy M: Expression in Trichoderma reesei and characterisation of a thermostable family 3 beta-glucosidase from the moderately thermophilic fungus Talaromyces emersonii. Protein Expr Purif 2004;38:248-257.

70. Rahman Z, Shida Y, Furukawa T, Suzuki Y, Okada H, Ogasawara W, Morikawa Y: Application of Trichoderma reesei cellulase and xylanase promoters through homologous recombination for enhanced production of extracellular beta-glucosidase I. Biosci Biotechnol Biochem 2009;73:1083-1089.

71. Shallom D, Shoham Y: Microbial hemicellulases. Curr Opin Microbiol 2003;6:219-228.

72. Bourne Y, Henrissat B: Glycoside hydrolases and glycosyltransferases: families and functional modules. Curr Opin Struct Biol 2001;11:593-600.

73. Wang L, Yan W, Chen J, Huang F, Gao P: Function of the iron-binding chelator produced by Coriolus versicolor in lignin biodegradation. Sci China C Life Sci 2008;51:214-221.

74. Martinez AT, Speranza M, Ruiz-Duenas FJ, Ferreira P, Camarero S, Guillen F, Martinez MJ, Gutierrez A, del Rio JC: Biodegradation of lignocellulosics: microbial, chemical, and enzymatic aspects of the fungal attack of lignin. Int Microbiol 2005;8:195-204.

75. Moredo N, Lorenzo M, Domínguez A, Moldes D, Cameselle C, Sanroman A: Enhanced ligninolytic enzyme production and degrading capability of Phanerochaete chrysosporium and Trametes versicolor. World J Microb Biotechnol 2003;19:665–669.

76. Srebotnik E, Messner K, Foisner R: Penetrability of White Rot-Degraded Pine Wood by the Lignin Peroxidase of Phanerochaete chrysosporium. Appl Environ Microbiol 1988;54:2608-2614.

77. Tanaka H, Itakura S, Enoki A: Hydroxyl radical generation by an extracellular low-molecular-weight substance and phenol oxidase activity during wood degradation by the white-rot basidiomycete Phanerochaete chrysosporium. Holzforschung 1999;53:21–28.

78. Suzuki MR, Hunt CG, Houtman CJ, Dalebroux ZD, Hammel KE: Fungal hydroquinones contribute to brown rot of wood. Environ Microbiol 2006;8:2214-2223.

79. Guillen F, Martinez AT, Martinez MJ: Substrate specificity and properties of the aryl-alcohol oxidase from the ligninolytic fungus Pleurotus eryngii. Eur J Biochem 1992;209:603-611.

80. Call HP, Mücke I: History, overview and applications of mediated lignolytic systems, especially laccase-mediator-systems (Lignozyme®-process). J Biotechnol 1997;53:163-202.

81. Wang W, Huang F, Mei Lu X, Ji Gao P: Lignin degradation by a novel peptide, Gt factor, from brown rot fungus Gloeophyllum trabeum. Biotechnol J 2006;1:447-453.

82. Renganathan V, Usha SN, Lindenburg F: Cellobiose oxidizing enzymes from the lignocellulose-degrading basidiomycete Phanerochaete chrysosporium - interaction with microcrystalline cellulose. Appl Microbiol Biotechnol 1990;32:609-613.

83.   Henriksson G, Johansson G, Pettersson G: A critical review of cellobiose dehydrogenases. J Biotechnol 2000;78:93-113.

84.   Baminger U, Subramaniam SS, Renganathan V, Haltrich D: Purification and characterization of cellobiose dehydrogenase from the plant pathogen Sclerotium (Athelia) rolfsii. Appl Environ Microbiol 2001;67:1766-1774.

85.   Yang W, Liu J, Wang W, Zhang Y, Gao P: Function of a low molecular peptide generated by cellulolytic fungi for the degradation of native cellulose. Biotechnol Lett 2004;26:1799-1802.

86.   Enoki A, Hirano T, Tanaka H: Extracellular substance from the brown rot basidiomycete Gloeophyllum trabeum that produces and reduces hydrogen peroxide. Mater Organism 1992;27:247–261.

87.   Tanaka H, Yoshida G, Baba Y, Matsumura K, Wasada H, Murata J, Agawa M, Itakura S, Enoki A: Characterization of a hydroxyl-radical-producing glycoprotein and its presumptive genes from the white-rot basidiomycete Phanerochaete chrysosporium. J Biotechnol 2007;128:500-511.

88.   Kurek B, Gaudard F: Oxidation of spruce wood sawdust by $MnO(2)$ plus oxalate: a biochemical investigation. J Agric Food Chem 2000;48:3058-3062.

89.   Makela MR, Hilden K, Hatakka A, Lundell TK: Oxalate decarboxylase of the white rot fungus Dichomitus squalens demonstrates a novel enzyme primary structure and non-induced expression on wood and in liquid cultures. Microbiology 2009.

90.   Brock BJ, Rieble S, Gold MH: Purification and Characterization of a 1,4-Benzoquinone Reductase from the Basidiomycete Phanerochaete chrysosporium. Appl Environ Microbiol 1995;61:3076-3081.

91.   Enoki A, Tanaka H, Itakura S: Physical and chemical characteristics of glycopeptide from wood decay fungi. Wood Deterioration and Preservation (Goodell B, Nicholas DD & Schultz TP, eds). 2003:pp. 140-153. Oxford University Press, Washington, DC.

92.   Raman B, Pan C, Hurst GB, Rodriguez M, Jr., McKeown CK, Lankford PK, Samatova NF, Mielenz JR: Impact of pretreated Switchgrass and biomass carbohydrates on Clostridium thermocellum ATCC 27405 cellulosome composition: a quantitative proteomic analysis. PLoS ONE 2009;4:e5271.

93.   Orpin CG: Studies on the rumen flagellate Neocallimastix frontalis. J Gen Microbiol 1975;91:249-262.

94.   Lamed R, Setter E, Bayer EA: Characterization of a cellulose-binding, cellulase-containing complex in Clostridium thermocellum. J Bacteriol 1983;156:828-836.

95.   Wilson CA, Wood TM: The anaerobic fungus Neocallimastix frontalis: isolation and properties of a cellulosome-type enzyme fraction with the capacity to solubilize hydrogen-bond-ordered cellulose. Appl. Microbiol. Biotechnol. 1992;37:125-129.

96.   Bayer EA, Belaich JP, Shoham Y, Lamed R: The cellulosomes: multienzyme machines for degradation of plant cell wall polysaccharides. Annu Rev Microbiol 2004;58:521-554.

97.   Nagy T, Tunnicliffe RB, Higgins LD, Walters C, Gilbert HJ, Williamson MP: Characterization of a double dockerin from the cellulosome of the anaerobic fungus Piromyces equi. J Mol Biol 2007;373:612-622.

98.   Dijkerman R, Op den Camp HJ, Van der Drift C, Vogels GD: The role of the cellulolytic high molecular mass (HMM) complex of the anaerobic fungus Piromyces sp. strain E2 in the hydrolysis of microcrystalline cellulose. Arch Microbiol 1997;167:137-142.

99.   Mosier N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch M: Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour Technol 2005;96:673-686.

100.   Taherzadeh MJ, Karimi K: Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review. Int J Mol Sci 2008;9:1621-1651.

101.   Shi J, Chinn MS, Sharma-Shivappa RR: Microbial pretreatment of cotton stalks by solid state cultivation of Phanerochaete chrysosporium. Bioresour Technol 2008;99:6556-6564.

102.   Anderson WF, Akin DE: Structural and chemical properties of grass lignocelluloses related to conversion for biofuels. J Ind Microbiol Biotechnol 2008;35:355-366.

103.   Arora DS, Chander M, K. GP: Involvement of lignin peroxidase, manganese peroxidase and laccase in degradation and selective ligninolysis of wheat straw. Int. Bioterior. Biodegrad. 2002;50:115-120.

104.   A. Ferraz, Ana M. Córdova, A. Machuca: Wood biodegradation and enzyme production by Ceriporiopsis subvermispora during solid-state fermentation of Eucalyptus grandis. Enzyme and Microbial Technology 2003;32:59–65.

105.   Turner P, Mamo G, Karlsson EN: Potential and utilization of thermophiles and thermostable enzymes in biorefining. Microb Cell Fact 2007;6:9.

106.   Viikari L, Alapuranen M, Puranen T, Vehmaanpera J, Siika-Aho M: Thermostable enzymes in lignocellulose hydrolysis. Adv Biochem Eng Biotechnol 2007;108:121-145.

107.   Grassick A, Murray PG, Thompson R, Collins CM, Byrnes L, Birrane G, Higgins TM, Tuohy MG: Three-dimensional structure of a thermostable native cellobiohydrolase, CBH IB, and molecular characterization of the cel7 gene from the filamentous fungus, Talaromyces emersonii. Eur J Biochem 2004;271:4495-4506.

108.   Li YL, Li DC, Teng FC: [Purification and characterization of a cellobiohydrolase from the thermophilic fungus Chaetomium thermophilus CT2]. Wei Sheng Wu Xue Bao 2006;46:143-146.

109. Rosgaard L, Pedersen S, Cherry JR, Harris P, Meyer AS: Efficiency of new fungal cellulase systems in boosting enzymatic degradation of barley straw lignocellulose. Biotechnol Prog 2006;22:493-498.

110. Maheshwari R, Bharadwaj G, Bhat MK: Thermophilic fungi: their physiology and enzymes. Microbiol Mol Biol Rev 2000;64:461-488.

111. Chu BC, Lee H: Genetic improvement of Saccharomyces cerevisiae for xylose fermentation. Biotechnol Adv 2007;25:425-441.

112. Schneider H, Wang PY, Chan YK, Maleszka R: Conversion of D-xylose into ethanol by the yeast Pachysolen tannophilus. Biotechnol Lett 1981;3:89-92.

113. McMillan JD, Boynton BL: Arbinose utilization by xylose-fermenting yeasts and fungi. Appl Biochem Biotechnol 1994;45-46:569-584.

114. Karhumaa K, Wiedemann B, Hahn-Hagerdal B, Boles E, Gorwa-Grauslund MF: Co-utilization of L-arabinose and D-xylose by laboratory and industrial Saccharomyces cerevisiae strains. Microb Cell Fact 2006;5:18.

115. Larsson S, Quintana-Sainz A, Reimann A, Nilvebrant NO, Jonsson LJ: Influence of lignocellulose-derived aromatic compounds on oxygen-limited growth and ethanolic fermentation by Saccharomyces cerevisiae. Appl Biochem Biotechnol 2000;84-86:617-632.

116. Nilvebrant NO, Persson P, Reimann A, De Sousa F, Gorton L, Jonsson LJ: Limits for alkaline detoxification of dilute-acid lignocellulose hydrolysates. Appl Biochem Biotechnol 2003;105 - 108:615-628.

117. Ohgren K, Bengtsson O, Gorwa-Grauslund MF, Galbe M, Hahn-Hagerdal B, Zacchi G: Simultaneous saccharification and co-fermentation of glucose and xylose in steam-pretreated corn stover at high fiber content with Saccharomyces cerevisiae TMB3400. J Biotechnol 2006;126:488-498.

118. Martin C, Marcet M, Almazan O, Jonsson LJ: Adaptation of a recombinant xylose-utilizing Saccharomyces cerevisiae strain to a sugarcane bagasse hydrolysate with high content of fermentation inhibitors. Bioresour Technol 2007;98:1767-1773.

119. Georgieva TI, Hou X, Hilstrom T, Ahring BK: Enzymatic hydrolysis and ethanol fermentation of high dry matter wet-exploded wheat straw at low enzyme loading. Appl Biochem Biotechnol 2008;148:35-44.

120. Philippidis GP, Smith TK, Wyman CE: Study of the enzymatic hydrolysis of cellulose for production of fuel ethanol by the simultaneous saccharification and fermentation process. Biotechnol Bioeng 1993;41:846-853.

121.   Stenberg K, Bollok M, Reczey K, Galbe M, Zacchi G: Effect of substrate and cellulase concentration on simultaneous saccharification and fermentation of steam-pretreated softwood for ethanol production. Biotechnol Bioeng 2000;68:204-210.

122.   Lynd LR, van Zyl WH, McBride JE, Laser M: Consolidated bioprocessing of cellulosic biomass: an update. Curr Opin Biotechnol 2005;16:577-583.

123.   Mandels M, Weber J: The production of cellulases. In Cellulases and their applications. Advan. Chem. Ser. 1969;95:391-414.

124.   Mandels M, Weber J, Parizek R: Enhanced cellulase production by a mutant of Trichoderma viride. Appl Microbiol 1971;21:152-154.

125.   Montenecourt BS, Eveleigh DE: Preparation of mutants of Trichoderma reesei with enhanced cellulase production. Appl Environ Microbiol 1977;34:777-782.

126.   Montenecourt BS, Eveleigh DE: Selective screening methods for the isolation of high yielding cellulase mutants of Trichoderma reesei. Adv. Chem. Ser. 1979;181:289-301.

127.   Kovács K, Megyeri L, Szakacsa G, Kubicekc CP, Galbeb M, Zacchi G: Trichoderma atroviride mutants with enhanced production of cellulase and β-glucosidase on pretreated willow. Enzyme and Microbial Technology 2008;43 48–55.

128.   Solov'eva IV, Okunev ON, Vel'kov VV, Koshelev AV, Bubnova TV, Kondrat'eva EG, Skomarovskii AA, Sinitsyn AP: The selection and properties of Penicillium verruculosum mutants with enhanced production of cellulases and xylanases. Mikrobiologiia 2005;74:172-178.

129.   Seidl V, Gamauf C, Druzhinina IS, Seiboth B, Hartl L, Kubicek CP: The Hypocrea jecorina (Trichoderma reesei) hypercellulolytic mutant RUT C30 lacks a 85 kb (29 gene-encoding) region of the wild-type genome. BMC Genomics 2008;9:327.

130.   Okada H, Mori K, Tada K, Nogawa M, Morikawa Y: Identification of active site carboxylic residues in Trichoderma reesei endoglucanase Cel12A by site-directed mutagenesis. J. Mol. Catal. B 2000:249–255.

131.   Fenel F, Zitting AJ, Kantelinen A: Increased alkali stability in Trichoderma reesei endo-1, 4-beta-xylanase II by site directed mutagenesis. J Biotechnol 2006;121:102-107.

132.   Qin Y, Wei X, Song X, Qu Y: Engineering endoglucanase II from Trichoderma reesei to improve the catalytic efficiency at a higher pH optimum. Journal of Biotechnology 2008;135:190-195.

133.   Fenel F, Leisola M, Janis J, Turunen O: A de novo designed N-terminal disulphide bridge stabilizes the Trichoderma reesei endo-1,4-beta-xylanase II. J Biotechnol 2004;108:137-143.

134.   Stockton BC, Mitchell DJ, Grohmann K, Himmel ME: Optimum β-D-glucosidase supplementation of cellulase for efficient conversion of cellulose to glucose. Biotechnol. Lett. 1991;13 57-62.

135. Johnson KG: Exocellular β-mannanases from hemicellulolytic fungi World Journal of Microbiology and Biotechnology 1990;6:209-217.

136. Gutierrez-Correa M, Tengerdy RP: Cellulolytic enzyme production by fungal mixed culture solid substrate fermentation. Biotechnol. Lett. 1998;20:45–47.

137. Maheshwari DK, Gohade S, Paul J, Verma A: A paper mill sludge as a potential source for cellulase production by Trichoderma reesei QM9123 and Aspergillus niger using mixed cultivation. Carbohydr Polym 1994; 23:161–163.

138. Ahamed A, Vermette P: Enhanced enzyme production from mixed cultures of Trichoderma reesei RUT-C30 and Aspergillus niger LMA grown as fed batch in a stirred tank bioreactor. Biochemical Engineering Journal 2008;42:41–46.

139. Juhász T, Kozma K, Szengyel Z, Réczey K: Production of β-glucosidase in mixed culture of Aspergillus niger BKMF 1305 and Trichoderma reesei RUT C30, Food. Technol. Biotechnol. 2003;41:49–53.

140. Duff SJB: Cellulase and beta-glucosidase production by mixed culture of Trichoderma reesei Rut C30 and Aspergillus phoenicis. Biotechnol. Lett. 1985;7:185-190.

141. Gupte A, Madamwar D: Solid state fermentation of lignocellulosic waste for cellulose and β-glucosidase production by co-cultivation by Aspergillus ellipticus and Aspergillus fumigatus. Biotechnol Prog 1997;13:166–169.

142. Panda T, Bisaria VS, Ghose TK: Studies on mixed fungal culture for cellulase and hemicellulase production. Part 1. Optimization of medium for the mixed culture of Trichoderma reesei Dl-6 and Aspergillus wentii Pt 2804. Biotechnology Letters 1983;5:767-772.

143. Dueñas R, Tengerdy RP, Gutierrez-Correa M: Cellulase production by mixed fungi in solid-substrate fermentation of bagasse. World Journal of Microbiology & Botechnology 1995;11:333-337.

144. Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS: Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev 2002;66:506-577, table of contents.

145. Miettinen-Oinonen A, Paloheimo M, Lantto R, Suominen P: Enhanced production of cellobiohydrolases in Trichoderma reesei and evaluation of the new preparations in biofinishing of cotton. J Biotechnol 2005;116:305-317.

146. Bower B, Larenas E, Mitchinson C: Exo-endo cellulase fusion protein. Patent. WO2005093073 2005.

1.3 Fungal biodegradation and enzymatic modification of lignin

Adapted from: Mehdi Dashtban[1,2], Heidi Schraft[2], Tarannum A. Syed[3] and Wensheng Qin*[1,2]

[1] Biorefining Research Institute
Lakehead University
955 Oliver Road
Thunder Bay, ON P7B 5E1 Canada

[2] Department of Biology
Lakehead University
955 Oliver Road
Thunder Bay, ON P7B 5E1 Canada

[3] Department of Biology
Trent University
1600 West Bank Dr.
Peterborough, ON K9J 7B8 Canada

*Corresponding author: Wensheng Qin

Abstract

Lignin, the most abundant aromatic biopolymer on Earth, is extremely recalcitrant to degradation. By linking to both hemicellulose and cellulose, it creates a barrier to any solutions or enzymes and prevents the penetration of lignocellulolytic enzymes into the interior lignocellulosic structure. Some basidiomycetes white-rot fungi are able to degrade lignin efficiently using a combination of extracellular ligninolytic enzymes, organic acids, mediators and accessory enzymes. This review describes ligninolytic enzyme families produced by these fungi that are involved in wood decay processes, their molecular structures, biochemical properties and the mechanisms of action which render them attractive candidates in biotechnological applications. These enzymes include phenol oxidase (laccase) and heme peroxidases [lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP)]. Accessory enzymes such as H_2O_2-generating oxidases and degradation mechanisms of plant cell-wall components in a non-enzymatic manner by production of free hydroxyl radicals ($^\cdot OH$) are also discussed.

Lignocellulosic materials

Lignocellulose is a renewable organic material and is the major structural component of all plants. Lignocellulosic wastes are produced in large amounts by many industries including those of forestry, pulp and paper, agriculture, and food. Such wastes are also present in municipal solid waste (MSW), and animal wastes [1-6]. These potentially valuable materials were treated as waste in many countries in the past, and still are today in some developing counties, which raises many environmental concerns [7,8].

Lignocellulose consists of three major components: cellulose, hemicellulose and lignin. In addition, small amounts of other materials such as ash, proteins and pectin can be found in lignocellulosic residues in varying degrees based on the source [9]. Cellulose, the major constituent of all plant material

and the most abundant organic molecule on Earth, is a linear biopolymer consisting of anhydroglucopyranose-molecules (glucose) connected by β-1,4-glycosidic bonds. Coupling of adjacent cellulose chains via hydrogen bonds, hydrophobic interactions and Van der Waal's forces results in the parallel alignment of crystalline structures known as microfibrils [10]. Unlike cellulose, hemicelluloses are heterogeneous polymers of pentoses (including xylose and arabinose), hexoses (mainly mannose, less glucose and galactose) and sugar acids. The highly variable composition of hemicelluloses is dependent on its plant source [11,12]. Lignin, the second-most abundant biopolymer on Earth and a heterogeneous polymer in lignocellulosic residues, is the only naturally synthesised polymer with an aromatic backbone. It generally contains three precursor aromatic alcohols including coniferyl alcohol, sinapyl and *p*-coumaryl [13]. These precursors form the guaiacyl- (G), syringyl- (S) and *p*-hydroxyphenyl (H) subunits in the lignin molecule, respectively [14]. The subunits ratio, and consequently, the lignin composition, varies between different plant groups. Oxidative coupling of these lignin aromatic alcohol monomers creates a complex structure in lignin which is highly recalcitrant to degradation [15]. By linking to both hemicelluloses and cellulose, lignin acts as a barrier to any solutions or enzymes and prevents penetration of lignocellulolytic enzymes to the interior lignocellulosic structure. Not surprisingly, of the components of lignocellulosic material, lignin is the most resistant to degradation [9,16]. Although lignin resists attack by most microorganisms, basidiomycetes white-rot fungi, are able to degrade lignin efficiently [15,17].

Lignocellulolytic enzyme-producing fungi

Lignocellulolytic enzymes-producing fungi are widespread, and include species from the ascomycetes (e.g. *Trichoderma reesei*) and basidiomycetes phyla such as white-rot (e.g. *Phanerochaete chrysosporium)* and brown-rot fungi (e.g. *Fomitopsis palustris*). In addition, a few anaerobic species (e.g. *Orpinomyces sp.*) are found to be able to degrade cellulose in the gastrointestinal tracts of ruminant animals [18,19]. Biomass degradation by these fungi is performed by complex mixtures of cellulases [20],

hemicellulases [18] and ligninases [9,21], reflecting the complexity of the materials. In nature, degradation of cellulosic biomass is performed by mixtures of hydrolytic enzymes collectively known as cellulases. Cellulases include endo-acting (endoglucanases) and exo-acting (cellobiohydrolases) enzymes, which behave in a synergistic manner in biomass-degrading microbes. Many microorganisms, including fungi and bacteria, have been found to be capable of degrading cellulose and other plant cell wall fibres. By 1976, over 14,000 fungal species expressing this ability had been isolated, but only a few of them were subjected to in-depth studies [22]. Most fungal strains produce and secrete various lignocellulolytic synergistically-acting enzymes into the environment, thus contributing significantly to the decay of lignocellulosic residues in nature. The breakdown of lignocellulosic biomass involves the formation of long-chain polysaccharides, mainly cellulose and hemicellulose, and the subsequent hydrolysis of these polysaccharides into their component 5- and 6-carbon chain sugars. In biofuel production, these sugars can be further converted to bioethanol through fermentation processes [23,24].

In nature, efficient lignin degradation during the process of wood decay became possible mainly by basidiomycetes white-rot fungi. Many white-rot fungi simultaneously attack lignin, hemicellulose and cellulose whereas some other white-rot fungi preferentially work on lignin in a selective manner. For example, while *Ceriporiopsis subvermispora* [25], *Phlebia spp*. [26,27], *Physisporinus rivulosus* [28] and *Dichomitus squalens* [27] selectively attack lignin, *Trametes versicolor* [29], *Heterobasidium annosum* [30] , *P. chrysosporium* [9] and *Irpex lacteus* [31] simultaneously degrade all cell wall components. Selective lignin degraders may have significant potential biotechnological applications when the removal of lignin is required to obtain intact cellulose such as in biopulping processes and also in procedures where the main objective is to provide an unprotected carbohydrate for subsequent use (e.g. animal feed and/or biofuel substrate) [32,33]. For example *C. subvermispora,* which lacks cellulase, has been selected for biopulping processes as a selective lignin degrader [32,34].

In contrast to white-rot fungi, brown-rot fungi, such as *Postia placenta*, *Laetiporus portentosus*, *Piptoporus betulinus* and *Gloeophyllum trabeum*, can degrade wood carbohydrates, but not oxidized

lignin. As a result, brown-colored rot ensues [14,15]. Ascomycetes are mostly able to degrade cellulose and hemicellulose, while their ability to convert lignin is limited [14]. Plant pathogenic fungi such as *Fusarium solani* f. sp. *glycines* are able to degrade lignin by production of laccase and lignin peroxidase. Lignin degradation by the fungi is suggested to play a role in sudden death syndrome (SDS) in soybean [35].

Fungal extracellular ligninases

Fungi degrade lignin by secreting enzymes collectively termed "ligninases". Ligninases can be classified as either phenol oxidases (laccase) or heme peroxidases [lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP)] (Table 1) [14]. In general, laccases use molecular oxygen as electron acceptors while peroxidases use hydrogen peroxide as a co-substrate [33]. White-rot fungi variously secrete one or more of the lignin-modifying enzymes (LMEs) in addition to other compounds necessary for effective lignin degradation (discussed in section 2.2.) [36]. It has been shown that *P. chrysosporium* produces several LiP and MnP isoenzymes but no laccase [37]. Correspondingly, the genome of *P. chrysosporium* contains ten LiP and five MnP genes [38]. In addition, H_2O_2-generating enzyme, glyoxal oxidase (GLOX) has been found in *P. chrysosporium* cultures [39]. White-rot basidiomycetes, such as *Coriolus versicolor* [40], *P. chrysosporium* and *T. versicolor* [41], have been found to be the most efficient lignin-degrading microorganisms studied. Although LiP is able to oxidize the non-phenolic part of lignin (which forms 80-90% of lignin composition), it is absent from many lignin degrading fungi [40]. In addition, electron microscopy studies of the early stages of the fungal degradation of wood have shown that oxidative ligninolytic enzymes are too large to penetrate into the wood cell wall micropores [42]. Thus, it has been suggested that prior to the enzymatic attack, low-molecular weight diffusible reactive oxidative compounds must initiate changes to the lignin structure (as discussed below) [42,43]. Figure 1 summarized the major steps and enzymes involved in lignin degradation by basidiomycetes white-rot fungi.

Table 1. The features of the main two groups of fungal ligninolytic enzymes.

Type of enzymes	Reaction[1]	Cofactor	Metals or ions[2]	Mediators	Subunits & molecular mass (kDa)	Range of optimum temperature (°C)	Range of pH optimum	Localization	Glycosylation	References
Phenol oxidase (laccase)	4 benzenediol + O_2 = 4 benzosemiquinone + 2 H_2O	N/A	Ca^{2+} Cd^{2+} Cu^{2+} H_2O_2 Imidazole K^+ K_2SO_4 Mn^{2+} Na_2SO_4 $(NH_4)_2SO_4$	Phenols, aniline, 3-HAA, NHA, syringaldehyde, hydroxybenzotriazole and ABTS	Monomeric (43-100), dimeric, trimeric & oligomeric (e.g. tetramers with ~390 KDa)	20-80	2-10	Mostly extracellular[3]	Yes (N-glycosylated)[4]	[36,44, 45,68,84,131]
Peroxidases: a) Lignin peroxidase (LiP)	1,2-bis(3,4-dimethoxyphenyl)propane-1,3-diol + H_2O_2 = 3,4-dimethoxybenzaldehyde + 1-(3,4-dimethoxyphenyl)ethane-1,2-diol + H_2O	Heme	Iron	Veratryl alcohol	Monomeric (37-50)	35-55	1-5	extracellular	Yes (N-glycosylated)[4]	[36,68,84,132]
b) Manganese peroxidase (MnP)	2Mn(II) + 2H^+ + H_2O_2 = 2Mn(III) + 2H_2O	Heme	Ca^{2+} Cd^{2+} Mn^{2+} Sm^{3+}	Organic acid as chelators, thiols, unsaturated fatty acids	Monomeric (32-62.5)	30-60	2.5-6.8	extracellular	Yes (N-glycosylated)[4]	[36,68,84,133,134]
c) Versatile peroxidase (VP)	donor + H_2O_2 = oxidized donor + 2H_2O (e.g. reactive black 5 + H_2O_2 = oxidized reactive black 5 + 2H_2O)	Heme	Mn^{2+} Ca^{2+} Cu^{2+} Iron	Veratryl alcohol, compounds similar to LiP and MnP mediators	Monomeric	Not known	3-5	extracellular	Yes	[68,101,135]

[1]General reactions. [2]Different enzymes from different species need different metal(s) or ion(s). [3]Fungal laccases are mostly extracellular enzymes but cytoplacmic or intracellular laccases were also found especially in plants and bacteria [45]. [4]Glycosylation degree varies between different fungal ligninolytic enzymes [68]. N/A: not applicable, 3-HAA: 3-hydroxyanthranilic acid, NHA: *N*-hydroxyacetanilide and ABTS: 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonate).

Phenol oxidases (laccases)(benzenediol:oxygen oxidoreductases, EC 1.10.3.2)

Laccases are glycosylated blue multi-copper oxidoreductases (BMCO) that use molecular oxygen to oxidize various aromatic and non-aromatic compounds through a radical-catalyzed reaction mechanism

(Table 1) [44,45]. Laccases couple the electron reduction of dioxygen into two molecules of water with the oxidation of a vast variety of substrates, such as phenols, arylamines, anilines, thiols and lignins (Figure 1, a) [46]. Four copper ions in their catalytic center mediate the redox process. These are classified as being type-1 (T1), type-2 (T2) and two type-3 (T3 and T3́), based on the copper's coordination and spectroscopic properties [47]. The oxidation reactions catalyzed by laccases lead to the formation of free radicals which act as intermediate substrates for the enzymes (Figure 1, b) [48]. These mediators can leave the enzyme site and react with a broad range of high-redox potential substrates and thus create non enzymatic routes of oxidative polymerizing or depolymerizing reactions (Figure 1, c). Ultimately, laccase-mediator system (LMS) becomes involved in a range of physiological functions such as lignolysis (Figure 1, d), lignin synthesis, morphogenesis, pathogenesis and detoxification [49].

Initially discovered in the Japanese lacquer tree *Rhus vernicifera* [50], laccases have since been found in many other plants and insects [44]. However, for the most part, laccases have been found and studied in white-rot fungi, such as *Lentinus tigrinus* [48], *Pleurotus ostreatus* D1 [51], *Cerrena unicolor* strain 137 [52], *T. versicolor* [53], *Trametes* sp. strain AH28-2 [54], *Trametes pubescens* [55] and *Cyathus bulleri* [56]. Laccase production using a liquid culture has also been reported in brown-rot fungi, including *Coniophora puteana* [57]. Also, ascomycetes such as *Melanocarpus albomyces* [58], *Chaetomium thermophile* [59], *Magnaporthe grisea* [60], *Myrothecium verrucaria* 24G-4 [61] and *Neurospora crassa* [62] are able to produce laccases. In addition by application of gene-specific PCR primers, laccase genes were detected in a few different fungal species including *Pycnoporus cinnabarinus*, *Pycnoporus coccineus*, *Pycnoporus sanguineus*, *Cyathus* sp. and also in xylariaceous ascomycetes *Xylaria* sp. and *Hypoxylon* sp. [63]. Interestingly, laccases were also detected in some bacteria such as *Bacillus subtilis* [64], while other bacteria, like *Streptomyces griseus* [65], produce a laccase-like phenol oxidase. Also, laccase genes were detected in bacteria such as *Bacillus licheniformis* [66] and *B. subtilis* [67]. For extensive information please refer to the following website http://www.brenda-enzymes.org [68].

As laccases work efficiently on a broad range of substrates without cofactors, they may have significant value in many biotechnological applications, such as pulp bio-bleaching [69], biosensors [70], food industries [71], textile industries [72], soil bioremediation [73] and in the production of complex polymers in synthetic chemistry [74]. However, commercial application of laccases face major obstacles, such as the lack of sufficient enzyme stocks and the cost of redox mediators [75]. Heterologous expression of the enzymes with protein engineering allows for the cost-effective creation of more robust and active enzymes. In addition, an improvement in immobilization methods would result in greater stability of laccases with long life times [76].

Heme Peroxidases

Lignin peroxidases (LiP)(1,2-bis(3,4-dimethoxyphenyl)propane-1,3-diol:hydrogen-peroxide oxidoreductases, EC 1.11.1.14)

LiPs are heme-containing glycoproteins and play a central role in the biodegradation of the cell wall constituent, lignin [77]. LiPs catalyze the H_2O_2-dependent oxidative depolymerization of a variety of non-phenolic lignin compounds (diarylpropane), β-O-4 non-phenolic lignin model compounds and a wide range of phenolic compounds (e.g. guaiacol, vanillyl alcohol, catechol, syringic acid, acteosyringone) with redox potentials up to 1.4 V (Table 1) [15]. LiPs oxidize the substrates in multi-step electron transfers and form intermediate radicals, such as phenoxy radicals and veratryl alcohol radical cations (Figure 1, e). These intermediate radicals undergo non-enzymatic reactions such as radical coupling and polymerization, side-chain cleavage, demethylation and intramolecular addition and rearrangement (Figure 1, f) [15]. Unlike the other peroxidises, like MnP, LiP is able to oxidize non-phenolic aromatic substrates and does not require the participation of mediators due to its unusually high redox potential (Figure 1, g) [15,40]. The crystal structure of the first LiP has shown that the heme group is buried in the interior of the protein and has access to the outer medium through a channel. Although the size of the

channel is not sufficient to allow the large polymer lignin to access the heme group, small molecule substrates can find a suitable binding site [77].

Since the discovery of LiP in *P. chrysosporium* [78] in 1983, more LiPs have been found in different *P. chrysosporium* strains [79,80] and other white-rot fungi, such as *T. versicolor* [81]. In addition, LiP genes were detected in a few different fungal species including *Panus* sp., *P. coccineus*, *P. sanguineus* and *Perenniporia medulla-panis* [63]. LiP was also detected in some bacteria, such as *Acinetobacter calcoaceticus* NCIM 2890 [82] and *Streptomyces viridosporus* T7A [83].
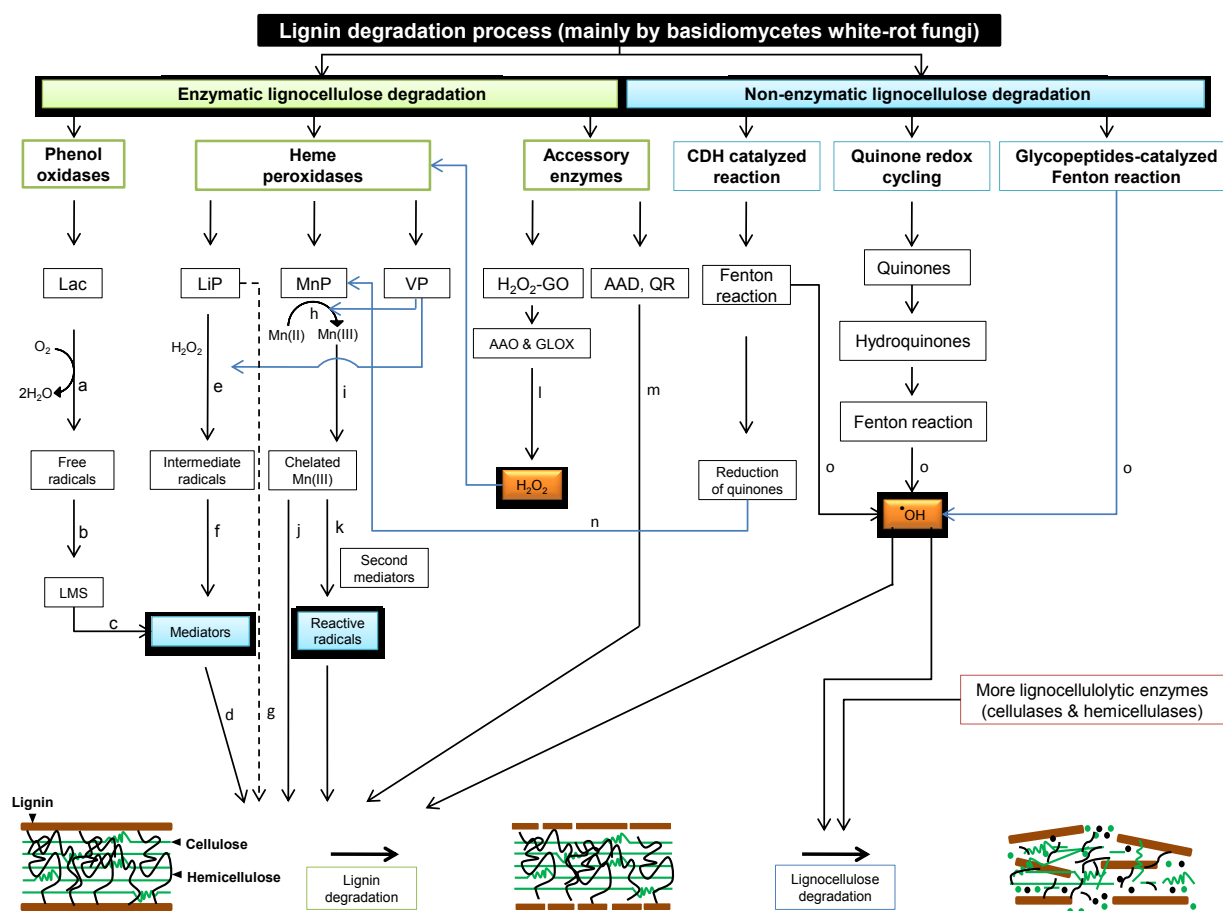
Figure 1. Schematic diagram of lignin degradation by basidiomycetes white-rot fungi: the major steps and enzymes involved (refer to text).

Lac: laccase, LMS: laccase-mediator system, LiP: lignin peroxidase, MnP: manganese peroxidase, VP: versatile peroxidase, H_2O_2-GO: H_2O_2-generating oxidases, AAO: aryl-alcohol oxidase, GLOX: glyoxal oxidase, H_2O_2: hydrogen peroxide AAD: aryl-alcohol dehydrogenases, QR: quinone reductases and $^•OH$: free hydroxyl radicals.

Manganese peroxidases (MnP)(Mn(II):hydrogen-peroxide oxidoreductases, EC 1.11.1.13)

MnPs are extracellular glycoproteins and are secreted in multiple isoforms which contain one molecule of heme as iron protoporphyrin IX [84]. MnP catalyzes the peroxide dependent oxidation of Mn(II) (as the reducing substrate) to Mn(III) (Figure 1, h), which is then released from the enzyme surface in complex with oxalate or with other chelators (Figure 1, i). Chelated Mn(III) complex acts as a reactive low molecular weight, diffusible redox-mediator (Figure 1, j) of phenolic substrates including simple phenols, amines, dyes, phenolic lignin substructures and dimers (Table 1) [15,36,84]. The oxidation potential of Mn(III) chelator is only limited to phenolic lignin structures. However, for the oxidation of non-phenolic substrates by Mn(III), reactive radicals must be formed in the presence of a second mediator (Figure 1, k). Organic acids, such as oxalate and malonate, are the primary compounds that act as second mediators in the production of reactive radicals like carbon-centered radicals (acetic acid radicals, $COOH-C^•H_2$), peroxyl radicals ($COOH-CH_2OO^•$), superoxide ($O_2^{•-}$) and formate radicals ($CO2^{•-}$) [15,36,84]. In the absence of H_2O_2 (e.g. in fungi lacking H_2O_2-generating oxidases), these radicals can be used by MnP as a source of peroxides and increase the lignin-degrading efficiency of the fungi [15,36,85].

Since the discovery of MnP in *P. chrysosporium* in 1985 [86], more MnPs have been found in other basidiomycetes, such as *Panus tigrinus* [87], *Lenzites betulinus* [88], *Phanerochaete flavido-alba* [89], *Agaricus bisporus* [90], *Bjerkandera* sp. [91] and *Nematoloma frowardii b19* [92].

MnPs may be capable of rivalling the potential applications of laccases in biotechnology. This is evident in studies which illustrate that the presence of MnP can increase the degree of dye decolorization. One study in particular found that MnP was the main enzyme involved in dye decolorization by *P. chrysosporium* [93]. Also, in another study, MnP produced by *P. chrysosporium* was used for the decolonization of sulfonphthalein (SP) dyes [94]. Complete decolorization took place at pH 4.0. In addition, MnP from white-rot fungi is considered the primary enzyme responsible for biobleaching of kraft pulps. The main drawback in commercial applications of MnP is the unavailability of the enzyme in large quantities; this can be resolved with the use of DNA recombinant technology [95]. For example, wild-type MnP from white-rot fungi [96] and recombinant MnP (rMnP) expressed in *Pichia pastoris* [97] have been used to remove lignin from cellulose fibers in pulp bleaching experiments [95]. The rMnP used in the study was found to be effective in lignin degradation and removal in both hardwood and softwood unbleached kraft pulps [95].

Versatile peroxidases (VP) (EC 1.11.1.16)

VPs are glycoproteins with hybrid properties capable of oxidizing typical substrates of other basidiomycetes peroxidases including Mn(II) and also veratryl alcohol (VA), MnP and the typical LiP substrate, respectively (Figure 1) [36,84,98]. VPs form an attractive ligninolytic enzyme group due to their dual oxidative ability to oxidize Mn(II) and also phenolic and nonphenolic aromatic compounds (Table 1) [36]. It has been found that VPs can also efficiently oxidize high redox-potential compounds such as dye Reactive Black 5 (RB5) as well as a wide variety of phenols, including hydroquinones [99,100]. It has been suggested that VPs can oxidize substrates spanning a wide range of potentials, including low- and high-redox potentials. This is a result of their hybrid molecular structures which provide multiple binding sites for the substrates [101]. This makes VPs superior to both LiPs and MnPs, which are not able to efficiently oxidize phenolic compounds in the absence of VA or oxidize phenols in

the absence of Mn(II), respectively [98]. Similar to the MnP mechanism, Mn(III) is released from VPs and acts as a diffusible oxidizer of phenolic lignin and free phenol substrates (Figure 1, h, i and j). Like other members of heme peroxidases, heme is buried in the interior of the protein and has access to the outer medium through two channels [100,101]. The function of the first channel is similar to that described for LiP and is conserved among all heme peroxidases. Conversely, the second channel is found to be specific to VP and MnP and is where the oxidation of Mn(II) to Mn(III) takes place [98].

Since the discovery of VP in 1999 in members of the genus *Pleurotus,* such as *P. eryngii* [101,102] and *P. ostreatus* [103], more VPs have been found in other basidiomycetes such as *Bjerkandera adusta* [100,104], *Bjerkandera* sp. strain BOS55 [105], *Bjerkandera* sp. (B33/3) [106], *Bjerkandera fumosa* [107] and *Pleurotus pulmonarius* [108]. Although *P. chrysosporium* did not show any VP activity, a putative extracellular peroxidase related to *Pleurotus* VP has been identified in its genome [109]. In addition, VP was detected in Polyporales basidiomycetes, including species from the genera *Panus* (e.g. *P. tigrinus* 8/18) [110].

Among basidiomycetes peroxidases, VPs have attracted the greatest biotechnological attention due to their catalytic versatility, which includes the degradation of compounds that other peroxidases are not able to oxidize directly. This unique feature allows VP to oxidize not only Mn(II) but also VA, phenolic, non-phenolic and high molecular weight compounds, including dyes in Mn-independent reactions [15,84]. Like MnP, VP's primary disadvantage in commercial applications is its limited availability in large quantities, which can be resolved with the use of DNA recombinant technology [95,98]. Efforts have been made to produce VP using heterologous expression systems. For example, in an experiment in which VP from *Pleurotus eryngii* was expressed under control of the alcohol dehydrogenase (alcA) promoter of *Aspergillus nidulans,* and lowering the growing temperature further improved the expression level [111]. Alternatively, other basidiomycete peroxidases such as LiP or MnP can be engineered to create peroxidases with new functions that emulate those of natural occurring VPs. For example, the Mn(II) binding site was introduced into a *P. chrysosporium* LiP by site-directed

mutagenesis and the engineered enzyme also showed MnP activity while retaining its ability to oxidize VA (LiP activity) [112]. In another experiment, when a tryptophan residue analogous to the essential one in LiP was introduced to *P. chrysosporium* MnP by site-directed mutagenesis (single mutation, S168W), MnP with LiP activity was created while full MnP activity was maintained [113].

Other lignin degrading enzymes and accessory enzymes

In addition to ligninases, other fungal extracellular enzymes which act as accessory enzymes have been found to be involved in lignin degradation. These include oxidases generating H_2O_2, which provide the hydrogen peroxide required by peroxidases, and mycelium-associated dehydrogenases, which reduce lignin-derived compounds (Figure 1, l) [14]. Oxidases generating H_2O_2 include aryl-alcohol oxidase (AAO) (EC 1.1.3.7) found in various fungi, such as *P. eryngii,* and glyoxal oxidase (GLOX, a copper-radical protein) found in *P. chrysosporium* [39,114]. In addition, aryl-alcohol dehydrogenases (AAD) (a flavoprotein) and quinone reductases (QR) are also involved in lignin degradation by fungi (Figure 1, m) [115]. Moreover, it has been shown that cellobiose dehydrogenase (CDH), which is produced by many different fungi under cellulolytic conditions, is also involved in lignin degradation in the presence of H_2O_2 and chelated Fe ions [116]. It is proposed that the effect of CDH on lignin degradation is through the reduction of quinones, which can be used by ligninolytic enzymes or the support of a Mn-peroxidase reaction (Figure 1, n) (for detailed information please refer to the review by Henriksson et al. 2000 [117]).

Oxidative (non-lignocellulolytic) lignocellulose-degradation mechanisms in higher fungi

Over the last few decades, there has been emerging evidence in support of the involvement of non-enzymatic mechanisms in plant cell-wall polysaccharide degradation. These mechanisms are mostly assisted by oxidation through the production of free hydroxyl radicals ($^{\bullet}OH$). Many white and brown-rot

fungi have been shown to produce hydrogen peroxide (H_2O_2) which enters the Fenton reaction and results in release of $^{\bullet}OH$ (Figure 1, o) [114,118]. By attacking polysaccharides and lignin in plant cell walls in a non-specific manner, these radicals create a number of cleavages which facilitate the penetration of the cell wall by lignocellulolytic enzymes [119,120]. The pathways by which fungi generate free $^{\bullet}OH$ radicals are: cellobiose dehydrogenase (CDH) catalyzed reactions, low molecular weight peptides/quinone redox cycling and glycopeptide-catalyzed Fenton reactions (Figure 1 and Table 2) [121].

Table 2. Mechanisms and enzymes involved in the production of $^{\bullet}OH$ in different fungi

Fungi	Mechanisms	Other enzymes involved/their function	References
White-rot fungi (e.g. *D. squalens*)	CDH catalyzed reaction	Oxalate decarboxylase/regulation of oxalate concentration	[136,137]
Brown and white-rot fungi (e.g. *C. puteana, P. chrysosporium*)	Quinone redox cycling	Benzoquinone reductases, CDH, sugar dehydrogenases/convert quinones to hydroquinones	[117,138]
Brown and white-rot fungi (e.g. *F. palustris, P. chrysosporium*)	Glycopeptides-catalyzed Fenton reaction	Cell wall-associated reductase/reduction of glycopeptides	[139]

CDH, an extracellular monomeric protein with some glycosylation, has been identified in a number of wood- and cellulose-degrading fungi, including basidiomycetes (mostly white-rot fungi) and ascomycetes, growing on cellulosic medium. This enzyme is able to oxidize cellobiose, higher cellodextrins and other disaccharides or oligosaccharides with β-1,4 linkages. In addition, CDH has been found with cellulose binding module (CBM) (in ascomycetes) and without CBM (in basidiomycetes). In the absence of CBM, CDH is able to bind to cellulose through hydrophobic interactions [122]. In some fungi, under cellulolytic conditions, CDH production increases, which in turn helps cellulases and hemicellulases [117,123]. It is now widely accepted that CDH is able to degrade and modify all three major components of the lignocellulosic residues (cellulose, hemicelluloses and lignin) by producing free

hydroxyl radicals in a Fenton-type reaction (Figure 1) (for detailed information please refer to the review by Baldrin and Valaskova, 2008 [121]).

It has been shown that white and brown-rot fungi produce low molecular weight chelators which are able to penetrate into the cell wall. For example, *G. trabeum* produces a low molecular weight peptide (known as short fiber generating factor, SFGF) which can degrade cellulose into short fibers by an oxidative reaction [120,124]. It has also been reported that some of these low molecular weight compounds are quinones, which must first be converted to hydroquinones by particular fungal enzymes (Table 2) before free hydroxyl radicals can be produced through the Fenton reaction (Figure 1, o) [40].

Glycopeptides of varying molecular weights (ranging from 1.5 to 12 kDa) have been found in many brown-rot fungi, such as *G. trabeum* [125] and white-rot fungi, such as *P. chrysosporium* [43,126]. Similar to other mechanisms, glycopeptides are able to catalyze redox reactions and thus produce free hydroxyl radicals (Figure 1, o).

Concluding remarks

Lignocellulolytic microorganisms, especially fungi, have attracted a great deal of interest as potential biomass degraders for large-scale applications due to their ability to produce vast amounts of extracellular lignocellulolytic enzymes. Lignin, the most recalcitrant component of lignocellulosic material, acts as a barrier for any solutions or enzymes by linking to both hemicelluloses and celluloses and prevents penetration of lignocellulolytic enzymes to the interior lignocellulosic structure. It is primarily basidiomycetes white-rot fungi that are responsible for efficient lignin degradation in wood decay processes. Their production of extracellular enzymes known as lignin-modifying enzymes (LMEs) facilitates the degradation process. Based on their activity on lignin and other lignocellulosic materials, white-rot fungi are categorized into two groups, simultaneous and selective degraders. Selective lignin degraders white-rot fungi are most attractive for their potential biotechnological applications in removing

lignin, as in biopulping processes, and for providing an unprotected carbohydrate for subsequent use, as in animal feed and/or biofuel substrate. LMEs include mainly two ligninolytic enzyme families; i) phenol oxidase (laccase) and ii) heme peroxidases (LiP, MnP and VP). LMEs, especially VPs require more research to understand the efficiency of the enzymes on lignin oligomers and the mechanisms of their action. In addition, accessory enzymes, such as oxidases that generate the H_2O_2 required by peroxidases, have been found to be involved in lignin degradation. Hydrogen peroxide is used by many white and brown-rot fungi to produce $^\bullet$OH in the Fenton reaction. Ultimately, these free radicals attack polysaccharides as well as lignin in plant cell walls in a non-specific manner creating some cleavage sites which allow for easier penetration by lignocellulolytic enzymes.

Genome sequencing projects of 62 fungal species, including six basidiomycetes and 27 ascomycetes, have been completed thus far [127]. Availability of the full genome sequence of white-rot fungi, such as that of *P. chrysosporium*, now allows for the creation of proteomic methods to identify all enzymes involved in lignin degradation. In turn, such methods may lead to the discovery of new enzymes involved in the degradation [17]. In order to achieve this, small sample sizes were used to identify the proteins released by *P. chrysosporium* grown in a solid-substrate culture (red oak wood chips). Traditional two-dimensional (2-D) gel electrophoresis was coupled to advanced instrumentation, such as matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) or capillary liquid chromatography-nanoelectrospray ionization-tandem MS (CapLC-ESI-MS/MS). Using this combination, 16 extracellular proteins were identified from over 40 proteins spotted on 2-D gel [17]. However, it was predicted that *P. chrysosporium*, which was found to have a 30-Mb genome with more than 10,000 gene models (based on computational analysis), could potentially release about 790 proteins into the culture medium [128]. The goal of this and other on-going projects is to identify the extracellular proteome (proteomic secretome) of *P. chrysosporium* or other basidiomycetes white-rot fungi when grown on a solid substrate. More recently, the first genome-level transcriptome study of *P. chrysosporium* was performed to determine all the gene products involved in wood degradation using red oak as a carbon

source. The results have shown that in addition to other lignocellulolytic enzymes, lignin peroxidase and alcohol oxidase (H_2O_2-generating enzyme) are highly expressed during lignin degradation [129]. Further investigation is needed to identify the novel proteins involved in fungal lignin degradation and their mechanisms of action. In addition, the suppression of lignin biosynthesis enzymes via plant genetic engineering may be of potential use in overcoming some of the problems related to the recalcitrance of lignin [130].

References:

[1]   Kim S and Dale BE. Global potential bioethanol production from wasted crops and crop residues. Biomass and Bioenergy 2004; 26: 361-375.

[2]   Kalogo Y, Habibi S, MacLean HL and Joshi SV. Environmental implications of municipal solid waste-derived ethanol. Environ Sci Technol 2007; 41: 35-41.

[3]   Pokhrel D and Viraraghavan T. Municipal solid waste management in Nepal: practices and challenges. Waste Manag 2005; 25: 555-562.

[4]   Statistics Canada. Human Activity and the Environment, Annual Statistics 2005, Solid Waste in Canada. Catalogue no. 16-201-XIE November 2005; pp 8.

[5]   Champagne P. Feasibility of producing bio-ethanol from waste residues: A Canadian perspective Feasibility of producing bio-ethanol from waste residues in Canada. Resources, Conservation and Recycling 2007; 50: 211-230.

[6]   Wen Z, Liao W and Chen S. Hydrolysis of animal manure lignocellulosics for reducing sugar production. Bioresour Technol 2004; 91: 31-39.

[7]   Palacios-Orueta A, Chuvieco E, Parra A and Carmona-Moreno C. Biomass burning emissions: a review of models using remote-sensing data. Environ Monit Assess 2005; 104: 189-209.

[8]   Levine JS. Biomass burning and global change. In: Levine JS (eds) (vol. 1) Remote sensing and inventory development and biomass burning in Africa. The MIT Press, Cambridge, Massachusetts, USA 1996; pp 35.

[9]   Sanchez C. Lignocellulosic residues: biodegradation and bioconversion by fungi. Biotechnol Adv 2009; 27: 185-194.

[10]   Percival Zhang YH, Himmel ME and Mielenz JR. Outlook for cellulase improvement: screening and selection strategies. Biotechnol Adv 2006; 24: 452-481.

[11]   Saha BC. Alpha-L-arabinofuranosidases: biochemistry, molecular biology and application in biotechnology. Biotechnol Adv 2000; 18: 403-423.

[12]   Saha BC. Hemicellulose bioconversion. J Ind Microbiol Biotechnol 2003; 30: 279-291.

[13]   Wei H, Xu Q, Taylor LE, 2nd, Baker JO, Tucker MP and Ding SY. Natural paradigms of plant cell wall degradation. Curr Opin Biotechnol 2009; 20: 330-338.

[14]   Martinez AT, Speranza M, Ruiz-Duenas FJ, Ferreira P, Camarero S, Guillen F, Martinez MJ, Gutierrez A and del Rio JC. Biodegradation of lignocellulosics: microbial, chemical, and enzymatic aspects of the fungal attack of lignin. Int Microbiol 2005; 8: 195-204.

[15]   Wong DW. Structure and action mechanism of ligninolytic enzymes. Appl Biochem Biotechnol 2009; 157: 174-209.

[16]   Himmel ME, Ding SY, Johnson DK, Adney WS, Nimlos MR, Brady JW and Foust TD. Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science 2007; 315: 804-807.

[17]   Abbas A, Koc H, Liu F and Tien M. Fungal degradation of wood: initial proteomic analysis of extracellular proteins of Phanerochaete chrysosporium grown on oak substrate. Curr Genet 2005; 47: 49-56.

[18]   Ljungdahl LG. The cellulase/hemicellulase system of the anaerobic fungus Orpinomyces PC-2 and aspects of its applied use. Ann N Y Acad Sci 2008; 1125: 308-321.

[19]   Yoon JJ, Cha CJ, Kim YS, Son DW and Kim YK. The brown-rot basidiomycete Fomitopsis palustris has the endo-glucanases capable of degrading microcrystalline cellulose. J Microbiol Biotechnol 2007; 17: 800-805.

[20]   Bayer EA, Chanzy H, Lamed R and Shoham Y. Cellulose, cellulases and cellulosomes. Curr Opin Struct Biol 1998; 8: 548-557.

[21]   Weng JK, Li X, Bonawitz ND and Chapple C. Emerging strategies of lignin engineering and degradation for cellulosic biofuel production. Curr Opin Biotechnol 2008; 19: 166-172.

[22]   Mandels M and Sternberg D. Recent advances in cellulase technology. Ferment. Technol. 1976; 54: 267-286.

[23]   Zhou S and Ingram LO. Synergistic hydrolysis of carboxymethyl cellulose and acid-swollen cellulose by two endoglucanases (CelZ and CelY) from Erwinia chrysanthemi. J Bacteriol 2000; 182: 5676-5682.

[24]   Dashtban M, Schraft H and Qin W. Fungal bioconversion of lignocellulosic residues; opportunities & perspectives. Int J Biol Sci 2009; 5: 578-595.

[25]   Guerra A, Mendonca R, Ferraz A, Lu F and Ralph J. Structural characterization of lignin during Pinus taeda wood treatment with Ceriporiopsis subvermispora. Appl Environ Microbiol 2004; 70: 4073-4078.

[26]   Arora DS and Sharma RK. Enhancement in in vitro digestibility of wheat straw obtained from different geographical regions during solid state fermentation by white rot fungi. BioResour 2009; 4: 909-920.

[27]   Fackler K, Gradinger C, Hinterstoisser B, Messner K and Schwanninger M. Lignin degradation by white rot fungi on spruce wood shavings during short-time solid-state fermentations monitored by near infrared spectroscopy. Enzyme and Microbial Technology 2006; 39 1476-1483.

[28]   Hilden K, Hakala TK, Maijala P, Lundell TK and Hatakka A. Novel thermotolerant laccases produced by the white-rot fungus Physisporinus rivulosus. Appl Microbiol Biotechnol 2007; 77: 301-309.

[29]   Tanaka H, Itakura S and Enoki A. Hydroxyl radical generation by an extracellular low-molecular-weight substance and phenol oxidase activity during wood degradation by the white-rot basidiomycete Trametes versicolor. J Biotechnol 1999; 75: 57-70.

[30]   Daniel G, Asiegbu F and Johansson M. The saprotrophic wood-degrading abilities of Heterobasidium annosum intersterility groups P and S. Mycol Res 1998; 102: 991-997.

[31]   Xu C, Ma F and Zhang X. Lignocellulose degradation and enzyme production by Irpex lacteus CD2 during solid-state fermentation of corn stover. J Biosci Bioeng 2009; 108: 372-375.

[32]   Anderson WF and Akin DE. Structural and chemical properties of grass lignocelluloses related to conversion for biofuels. J Ind Microbiol Biotechnol 2008; 35: 355-366.

[33]   Mai C, Kues U and Militz H. Biotechnology in the wood industry. Appl Microbiol Biotechnol 2004; 63: 477-494.

[34]   Blanchette RA, Burnes TA, Eerdmans MM and Akhtar M. Evaluating Isolates of Phanerochaete chrysosporium and Ceriporiopsis subvermispora for Use in Biological Pulping Processes. Holzforschung 1992; 46: 109-115.

[35]   Lozovaya VV, Lygin AV, Zernova OV, Ulanov AV, Li S, Hartman GL and Widholm JM. Modification of phenolic metabolism in soybean hairy roots through down regulation of chalcone synthase or isoflavone synthase. Planta 2007; 225: 665-679.

[36]   Wesenberg D, Kyriakides I and Agathos SN. White-rot fungi and their enzymes for the treatment of industrial dye effluents. Biotechnol Adv 2003; 22: 161-187.

[37]   Singh D and Chen S. The white-rot fungus Phanerochaete chrysosporium: conditions for the production of lignin-degrading enzymes. Appl Microbiol Biotechnol 2008; 81: 399-417.

[38]   Martinez D, Larrondo LF, Putnam N, Gelpke MD, Huang K, Chapman J, Helfenbein KG, Ramaiya P, Detter JC, Larimer F, Coutinho PM, Henrissat B, Berka R, Cullen D and Rokhsar D. Genome sequence of the lignocellulose degrading fungus Phanerochaete chrysosporium strain RP78. Nat Biotechnol 2004; 22: 695-700.

[39]   Kersten PJ. Glyoxal oxidase of Phanerochaete chrysosporium: its characterization and activation by lignin peroxidase. Proc Natl Acad Sci U S A 1990; 87: 2936-2940.

[40]   Wang L, Yan W, Chen J, Huang F and Gao P. Function of the iron-binding chelator produced by Coriolus versicolor in lignin biodegradation. Sci China C Life Sci 2008; 51: 214-221.

[41]   Moredo N, Lorenzo M, Domínguez A, Moldes D, Cameselle C and Sanroman A. Enhanced ligninolytic enzyme production and degrading capability of Phanerochaete chrysosporium and Trametes versicolor. World J Microb Biotechnol 2003; 19: 665–669.

[42]   Srebotnik E, Messner K and Foisner R. Penetrability of White Rot-Degraded Pine Wood by the Lignin Peroxidase of Phanerochaete chrysosporium. Appl Environ Microbiol 1988; 54: 2608-2614.

[43]   Tanaka H, Itakura S and Enoki A. Hydroxyl radical generation by an extracellular low-molecular-weight substance and phenol oxidase activity during wood degradation by the white-rot basidiomycete Phanerochaete chrysosporium. Holzforschung 1999; 53: 21–28.

[44]   Claus H. Laccases: structure, reactions, distribution. Micron 2004; 35: 93-96.

[45]   Baldrian P. Fungal laccases - occurrence and properties. FEMS Microbiol Rev 2006; 30: 215-242.

[46]   Thurston CF. The structure and function of fungal laccases. Microbiol (UK) 1994; 140: 19-26.

[47]   Messerschmidt A and Huber R. The blue oxidases, ascorbate oxidase, laccase and ceruloplasmin. Modelling and structural relationships. Eur J Biochem 1990; 187: 341-352.

[48] Ferraroni M, Myasoedova NM, Schmatchenko V, Leontievsky AA, Golovleva LA, Scozzafava A and Briganti F. Crystal structure of a blue laccase from Lentinus tigrinus: evidences for intermediates in the molecular oxygen reductive splitting by multicopper oxidases. BMC Struct Biol 2007; 7: 60.

[49] Mayer AM and Staples RC. Laccase: new functions for an old enzyme. Phytochemistry 2002; 60: 551-565.

[50] Yoshida H. Chemistry of lacquer (urushi). J. Chem. Soc 1883; 43: 472-486.

[51] Pozdniakova NN, Turkovskaia OV, Iudina EN and Rodakiewicz-Nowak Y. [Yellow laccase from the fungus Pleurotus ostreatus D1: purification and characterization]. Prikl Biokhim Mikrobiol 2006; 42: 63-69.

[52] Michniewicz A, Ullrich R, Ledakowicz S and Hofrichter M. The white-rot fungus Cerrena unicolor strain 137 produces two laccase isoforms with different physico-chemical and catalytic properties. Appl Microbiol Biotechnol 2006; 69: 682-688.

[53] Necochea R, Valderrama B, Diaz-Sandoval S, Folch-Mallol JL, Vazquez-Duhalt R and Iturriaga G. Phylogenetic and biochemical characterisation of a recombinant laccase from Trametes versicolor. FEMS Microbiol Lett 2005; 244: 235-241.

[54] Xiao YZ, Tu XM, Wang J, Zhang M, Cheng Q, Zeng WY and Shi YY. Purification, molecular characterization and reactivity with aromatic compounds of a laccase from basidiomycete Trametes sp. strain AH28-2. Appl Microbiol Biotechnol 2003; 60: 700-707.

[55] Shleev S, Nikitina O, Christenson A, Reimann CT, Yaropolov AI, Ruzgas T and Gorton L. Characterization of two new multiforms of Trametes pubescens laccase. Bioorg Chem 2007; 35: 35-49.

[56] Salony, Mishra S and Bisaria VS. Production and characterization of laccase from Cyathus bulleri and its use in decolourization of recalcitrant textile dyes. Appl Microbiol Biotechnol 2006; 71: 646-653.

[57] Lee KH, Wi SG, Singh AP and Kim YS. Micromorphological characteristics of decayed wood and laccase produced by the brown-rot fungus Coniophora puteana. J Wood Sci 2004; 50: 281-284.

[58] Hakulinen N, Kruus K, Koivula A and Rouvinen J. A crystallographic and spectroscopic study on the effect of X-ray radiation on the crystal structure of Melanocarpus albomyces laccase. Biochem Biophys Res Commun 2006; 350: 929-934.

[59] Ishigami T and Yamada Y. Purification and properties of polyphenol oxidase from Chaetomium thermophile, a thermophilic fungus. Journal of General and Applied Microbiology 1986; 32: 293-301.

[60] Iyer G and Chattoo BB. Purification and characterization of laccase from the rice blast fungus, Magnaporthe grisea. FEMS Microbiol Lett 2003; 227: 121-126.

[61] Sulistyaningdyah WT, Ogawa J, Tanaka H, Maeda C and Shimizu S. Characterization of alkaliphilic laccase activity in the culture supernatant of Myrothecium verrucaria 24G-4 in comparison with bilirubin oxidase. FEMS Microbiol Lett 2004; 230: 209-214.

[62]   Germann UA, Muller G, Hunziker PE and Lerch K. Characterization of two allelic forms of Neurospora crassa laccase. Amino- and carboxyl-terminal processing of a precursor. J Biol Chem 1988; 263: 885-896.

[63]   Pointing SB, Pelling AL, Smith GJ, Hyde KD and Reddy CA. Screening of basidiomycetes and xylariaceous fungi for lignin peroxidase and laccase gene-specific sequences. Mycol Res 2005; 109: 115-124.

[64]   Durao P, Bento I, Fernandes AT, Melo EP, Lindley PF and Martins LO. Perturbations of the T1 copper site in the CotA laccase from Bacillus subtilis: structural, biochemical, enzymatic and stability studies. J Biol Inorg Chem 2006; 11: 514-526.

[65]   Endo K, Hayashi Y, Hibi T, Hosono K, Beppu T and Ueda K. Enzymological characterization of EpoA, a laccase-like phenol oxidase produced by Streptomyces griseus. J Biochem 2003; 133: 671-677.

[66]   Koschorreck K, Richter SM, Ene AB, Roduner E, Schmid RD and Urlacher VB. Cloning and characterization of a new laccase from Bacillus licheniformis catalyzing dimerization of phenolic acids. Appl Microbiol Biotechnol 2008; 79: 217-224.

[67]   Martins LO, Soares CM, Pereira MM, Teixeira M, Costa T, Jones GH and Henriques AO. Molecular and biochemical characterization of a highly stable bacterial laccase that occurs as a structural component of the Bacillus subtilis endospore coat. J Biol Chem 2002; 277: 18849-18859.

[68]   Chang A, Scheer M, Grote A, Schomburg I and Schomburg D. BRENDA, AMENDA and FRENDA the enzyme information system: new content and tools in 2009. Nucleic Acids Res 2009; 37: D588-592.

[69]   Balakshin M, Chen C-L, Gratzla JS, Kirkmana AG and Jakob H. Biobleaching of pulp with dioxygen in laccase-mediator system—effect of variables on the reaction kinetics. Journal of Molecular Catalysis B: Enzymatic 2001; 16: 205-215.

[70]   Kulys J and Vidziunaite R. Amperometric biosensors based on recombinant laccases for phenols determination. Biosens Bioelectron 2003; 18: 319-325.

[71]   Selinheimo E, Autio K, Kruus K and Buchert J. Elucidating the mechanism of laccase and tyrosinase in wheat bread making. J Agric Food Chem 2007; 55: 6357-6365.

[72]   Rodríguez Couto S, Hofer D, Sanromán MA and Gübitz GM. Production of laccase by Trametes hirsuta grown in an immersion bioreactor. Application to decolourisation of dyes from a leather factory. Eng Life Sci 2004; 4: 233-238.

[73]   Nyanhongo GS, Couto SR and Guebitz GM. Coupling of 2,4,6-trinitrotoluene (TNT) metabolites onto humic monomers by a new laccase from Trametes modesta. Chemosphere 2006; 64: 359-370.

[74]   Mustafa R, Muniglia L, Rovel B and Girardin M. Phenolic colorants obtained by enzymatic synthesis using a fungal laccase in a hydroorganic biphasic system. Food Res Int 2005; 38: 995-1000.

[75]   Rodriguez Couto S and Toca Herrera JL. Industrial and biotechnological applications of laccases: a review. Biotechnol Adv 2006; 24: 500-513.

[76]   Lu L, Zhao M and Wang Y. Immobilization of laccase by alginate-chitosan microcapsules and its use in dye decolorization. World J Microbiol Biotechnol 2007; 23: 159-166.

[77]   Piontek K, Smith AT and Blodig W. Lignin peroxidase structure and function. Biochem Soc Trans 2001; 29: 111-116.

[78]   Glenn JK, Morgan MA, Mayfield MB, Kuwahara M and Gold MH. An extracellular H2O2-requiring enzyme preparation involved in lignin biodegradation by the white rot basidiomycete Phanerochaete chrysosporium. Biochem Biophys Res Commun 1983; 114: 1077-1083.

[79]   Renganathan V, Miki K and Gold MH. Multiple molecular forms of diarylpropane oxygenase, an H2O2-requiring, lignin-degrading enzyme from Phanerochaete chrysosporium. Arch Biochem Biophys 1985; 241: 304-314.

[80]   Tien M and Kirk TK. Lignin-degrading enzyme from Phanerochaete chrysosporium: Purification, characterization, and catalytic properties of a unique H(2)O(2)-requiring oxygenase. Proc Natl Acad Sci U S A 1984; 81: 2280-2284.

[81]   Johansson T, Welinder KG and Nyman PO. Isozymes of lignin peroxidase and manganese(II) peroxidase from the white-rot basidiomycete Trametes versicolor. II. Partial sequences, peptide maps, and amino acid and carbohydrate compositions. Arch Biochem Biophys 1993; 300: 57-62.

[82]   Ghodake GS, Kalme SD, Jadhav JP and Govindwar SP. Purification and partial characterization of lignin peroxidase from Acinetobacter calcoaceticus NCIM 2890 and its application in decolorization of textile dyes. Appl Biochem Biotechnol 2009; 152: 6-14.

[83]   Gottschalk LM, Bon EP and Nobrega R. Lignin peroxidase from Streptomyces viridosporus T7A: enzyme concentration using ultrafiltration. Appl Biochem Biotechnol 2008; 147: 23-32.

[84]   Asgher M, Bhatti HN, Ashraf M and Legge RL. Recent developments in biodegradation of industrial pollutants by white rot fungi and their enzyme system. Biodegradation 2008; 19: 771-783.

[85]   Hofrichter M, Ziegenhagen D, Vares T, Friedrich M, Jager MG, Fritsche W and Hatakka A. Oxidative decomposition of malonic acid as basis for the action of manganese peroxidase in the absence of hydrogen peroxide. FEBS Lett 1998; 434: 362-366.

[86]   Glenn JK and Gold MH. Purification and characterization of an extracellular Mn(II)-dependent peroxidase from the lignin-degrading basidiomycete, Phanerochaete chrysosporium. Arch Biochem Biophys 1985; 242: 329-341.

[87]   Lisov AV, Leontievsky AA and Golovleva LA. Hybrid Mn-peroxidase from the ligninolytic fungus Panus tigrinus 8/18. Isolation, substrate specificity, and catalytic cycle. Biochemistry (Mosc) 2003; 68: 1027-1035.

[88]   Hoshino F, Kajino T, Sugiyama H, Asami O and Takahashi H. Thermally stable and hydrogen peroxide tolerant manganese peroxidase (MnP) from Lenzites betulinus. FEBS Lett 2002; 530: 249-252.

[89]   de la Rubia T, Linares A, Perez J, Munoz-Dorado J, Romera J and Martinez J. Characterization of manganese-dependent peroxidase isoenzymes from the ligninolytic fungus Phanerochaete flavido-alba. Res Microbiol 2002; 153: 547-554.

[90]   Lankinen VP, Bonnen AM, Anton LH, Wood DA, Kalkkinen N, Hatakka A and Thurston CF. Characteristics and N-terminal amino acid sequence of manganese peroxidase from solid substrate cultures of Agaricus bisporus. Appl Microbiol Biotechnol 2001; 55: 170-176.

[91]   Palma C, Martinez AT, Lema JM and Martinez MJ. Different fungal manganese-oxidizing peroxidases: a comparison between Bjerkandera sp. and Phanerochaete chrysosporium. J Biotechnol 2000; 77: 235-245.

[92]   Hilden KS, Bortfeldt R, Hofrichter M, Hatakka A and Lundell TK. Molecular characterization of the basidiomycete isolate Nematoloma frowardii b19 and its manganese peroxidase places the fungus in the corticioid genus Phlebia. Microbiology 2008; 154: 2371-2379.

[93]   Chagas EP and Durrant LR. Decolorization of azo dyes by Phanerochaete chrysosporium and Pleurotus sajorcaju. Enzyme Microb Technol 2001; 29: 473-477.

[94]   Christian VV, Shrivastava R, Novotny C and Vyas BR. Decolorization of sulfonphthalein dyes by manganese peroxidase activity of the white-rot fungus Phanerochaete chrysosporium. Folia Microbiol (Praha) 2003; 48: 771-774.

[95]   Xu H, Scott GM, Jiang F and Kelly C. Recombinant manganese peroxidase (rMnP) from Pichia pastoris. Part 1: Kraft pulp delignification. Holzforschung 2010; 64: 137–143.

[96]   Harazono K, Kondo R and Sakai K. Bleaching of Hardwood Kraft Pulp with Manganese Peroxidase from Phanerochaete sordida YK-624 without Addition of MnSO(inf4). Appl Environ Microbiol 1996; 62: 913-917.

[97]   Gu L, Lajoie C and Kelly C. Expression of a Phanerochaete chrysosporium manganese peroxidase gene in the yeast Pichia pastoris. Biotechnol Prog 2003; 19: 1403-1409.

[98]   Ruiz-Duenas FJ, Morales M, Garcia E, Miki Y, Martinez MJ and Martinez AT. Substrate oxidation sites in versatile peroxidase and other basidiomycete peroxidases. J Exp Bot 2009; 60: 441-452.

[99]   Gomez-Toribio V, Martinez AT, Martinez MJ and Guillen F. Oxidation of hydroquinones by the versatile ligninolytic peroxidase from Pleurotus eryngii. H_2O_2 generation and the influence of Mn^{2+}. Eur J Biochem 2001; 268: 4787-4793.

[100]   Heinfling A, Ruiz-Duenas FJ, Martinez MJ, Bergbauer M, Szewzyk U and Martinez AT. A study on reducing substrates of manganese-oxidizing peroxidases from Pleurotus eryngii and Bjerkandera adusta. FEBS Lett 1998; 428: 141-146.

[101]   Camarero S, Sarkar S, Ruiz-Duenas FJ, Martinez MJ and Martinez AT. Description of a versatile peroxidase involved in the natural degradation of lignin that has both manganese peroxidase and lignin peroxidase substrate interaction sites. J Biol Chem 1999; 274: 10324-10330.

[102]   Ruiz-Duenas FJ, Guillen F, Camarero S, Perez-Boada M, Martinez MJ and Martinez AT. Regulation of peroxidase transcript levels in liquid cultures of the ligninolytic fungus Pleurotus eryngii. Appl Environ Microbiol 1999; 65: 4458-4463.

[103]   Cohen R, Hadar Y and Yarden O. Transcript and activity levels of different Pleurotus ostreatus peroxidases are differentially affected by Mn2+. Environ Microbiol 2001; 3: 312-322.

[104]   Wang Y, Vazquez-Duhalt R and Pickard MA. Manganese-lignin peroxidase hybrid from Bjerkandera adusta oxidizes polycyclic aromatic hydrocarbons more actively in the absence of manganese. Can J Microbiol 2003; 49: 675-682.

[105]   Mester T and Field JA. Characterization of a novel manganese peroxidase-lignin peroxidase hybrid isozyme produced by Bjerkandera species strain BOS55 in the absence of manganese. J Biol Chem 1998; 273: 15412-15417.

[106]   Moreira PR, Almeida-Vara E, Sena-Martins G, Polonia I, Malcata FX and Cardoso Duarte J. Decolourisation of Remazol Brilliant Blue R via a novel Bjerkandera sp. strain. J Biotechnol 2001; 89: 107-111.

[107]   Rodakiewicz-Nowak J, Jarosz-Wilkolazka A and Luterek J. Catalytic activity of versatile peroxidase from Bjerkandera fumosa in aqueous solutions of water-miscible organic solvents. Applied Catalysis A: General 2006; 308: 56-61.

[108]   Camarero S, Bockle B, Martinez MJ and Martinez AT. Manganese-Mediated Lignin Degradation by Pleurotus pulmonarius. Appl Environ Microbiol 1996; 62: 1070-1072.

[109]   Larrondo L, Gonzalez A, Perez Acle T, Cullen D and Vicuna R. The nop gene from Phanerochaete chrysosporium encodes a peroxidase with novel structural features. Biophys Chem 2005; 116: 167-173.

[110]   Lisov AV, Leontievsky AA and Golovleva LA. Hybrid Mn-peroxidases from basidiomycetes. Applied Biochemistry and Microbiology 2007; 43: 536-543.

[111]   Eibes GM, Lu-Chau TA, Ruiz-Duenas FJ, Feijoo G, Martinez MJ, Martinez AT and Lema JM. Effect of culture temperature on the heterologous expression of Pleurotus eryngii versatile peroxidase in Aspergillus hosts. Bioprocess Biosyst Eng 2009; 32: 129-134.

[112]   Mester T and Tien M. Engineering of a manganese-binding site in lignin peroxidase isozyme H8 from Phanerochaete chrysosporium. Biochem Biophys Res Commun 2001; 284: 723-728.

[113]   Timofeevski SL, Nie G, Reading NS and Aust SD. Addition of veratryl alcohol oxidase activity to manganese peroxidase by site-directed mutagenesis. Biochem Biophys Res Commun 1999; 256: 500-504.

[114]   Guillen F, Martinez AT and Martinez MJ. Substrate specificity and properties of the aryl-alcohol oxidase from the ligninolytic fungus Pleurotus eryngii. Eur J Biochem 1992; 209: 603-611.

[115]   Guillen F, Martinez MJ, Munoz C and Martinez AT. Quinone redox cycling in the ligninolytic fungus Pleurotus eryngii leading to extracellular production of superoxide anion radical. Arch Biochem Biophys 1997; 339: 190-199.

[116]   Henriksson G, Ander P, Pettersson B and Pettersson G. Cellobiose dehydrogenase (cellobiose oxidase) from Phanerochaete chrysosporium as a wood degrading enzyme-studies on cellulose, xylan and synthetic lignin. Appl Microbiol Biotechnol 1995; 42: 790-796.

[117]   Henriksson G, Johansson G and Pettersson G. A critical review of cellobiose dehydrogenases. J Biotechnol 2000; 78: 93-113.

[118]   Suzuki MR, Hunt CG, Houtman CJ, Dalebroux ZD and Hammel KE. Fungal hydroquinones contribute to brown rot of wood. Environ Microbiol 2006; 8: 2214-2223.

[119]   Call HP and Mücke I. History, overview and applications of mediated lignolytic systems, especially laccase-mediator-systems (Lignozyme®-process). J Biotechnol 1997; 53: 163-202.

[120]   Wang W, Huang F, Mei Lu X and Ji Gao P. Lignin degradation by a novel peptide, Gt factor, from brown rot fungus Gloeophyllum trabeum. Biotechnol J 2006; 1: 447-453.

[121]   Baldrian P and Valaskova V. Degradation of cellulose by basidiomycetous fungi. FEMS Microbiol Rev 2008; 32: 501-521.

[122]   Renganathan V, Usha SN and Lindenburg F. Cellobiose oxidizing enzymes from the lignocellulose-degrading basidiomycete Phanerochaete chrysosporium - interaction with microcrystalline cellulose. Appl Microbiol Biotechnol 1990; 32: 609-613.

[123]   Baminger U, Subramaniam SS, Renganathan V and Haltrich D. Purification and characterization of cellobiose dehydrogenase from the plant pathogen Sclerotium (Athelia) rolfsii. Appl Environ Microbiol 2001; 67: 1766-1774.

[124]   Yang W, Liu J, Wang W, Zhang Y and Gao P. Function of a low molecular peptide generated by cellulolytic fungi for the degradation of native cellulose. Biotechnol Lett 2004; 26: 1799-1802.

[125]   Enoki A, Hirano T and Tanaka H. Extracellular substance from the brown rot basidiomycete Gloeophyllum trabeum that produces and reduces hydrogen peroxide. Mater Organism 1992; 27: 247–261.

[126]   Tanaka H, Yoshida G, Baba Y, Matsumura K, Wasada H, Murata J, Agawa M, Itakura S and Enoki A. Characterization of a hydroxyl-radical-producing glycoprotein and its presumptive genes from the white-rot basidiomycete Phanerochaete chrysosporium. J Biotechnol 2007; 128: 500-511.

[127]   Lundell TK, Makela MR and Hilden K. Lignin-modifying enzymes in filamentous basidiomycetes--ecological, functional and phylogenetic review. J Basic Microbiol 50: 5-20.

[128]   Bouws H, Wattenberg A and Zorn H. Fungal secretomes--nature's toolbox for white biotechnology. Appl Microbiol Biotechnol 2008; 80: 381-388.

[129]   Sato S, Feltus FA, Iyer P and Tien M. The first genome-level transcriptome of the wood-degrading fungus Phanerochaete chrysosporium grown on red oak. Curr Genet 2009; 55: 273-286.

[130]   Sticklen MB. Plant genetic engineering for biofuel production: towards affordable cellulosic ethanol. Nat Rev Genet 2008; 9: 433-443.

[131]   Hilden K, Hakala TK and Lundell T. Thermotolerant and thermostable laccases. Biotechnol Lett 2009; 31: 1117-1128.

[132]   Hammel KE and Cullen D. Role of fungal peroxidases in biological ligninolysis. Curr Opin Plant Biol 2008; 11: 349-355.

[133]   Sundaramoorthy M, Youngs HL, Gold MH and Poulos TL. High-resolution crystal structure of manganese peroxidase: substrate and inhibitor complexes. Biochemistry 2005; 44: 6463-6470.

[134]   Baborova P, Moder M, Baldrian P, Cajthamlova K and Cajthaml T. Purification of a new manganese peroxidase of the white-rot fungus Irpex lacteus, and degradation of polycyclic aromatic hydrocarbons by the enzyme. Res Microbiol 2006; 157: 248-253.

[135]   Davila-Vazqueza G, Tinoco R, Pickard MA and Vazquez-Duhalt R. Transformation of halogenated pesticides by versatile peroxidase from Bjerkandera adusta. Enzyme and Microbial Technology 2005; 36 223-231.

[136]   Kurek B and Gaudard F. Oxidation of spruce wood sawdust by MnO(2) plus oxalate: a biochemical investigation. J Agric Food Chem 2000; 48: 3058-3062.

[137]   Makela MR, Hilden K, Hatakka A and Lundell TK. Oxalate decarboxylase of the white rot fungus Dichomitus squalens demonstrates a novel enzyme primary structure and non-induced expression on wood and in liquid cultures. Microbiology 2009; 155, 2726–2738.

[138]   Brock BJ, Rieble S and Gold MH. Purification and Characterization of a 1,4-Benzoquinone Reductase from the Basidiomycete Phanerochaete chrysosporium. Appl Environ Microbiol 1995; 61: 3076-3081.

[139]   Enoki A, Tanaka H and Itakura S. Physical and chemical characteristics of glycopeptide from wood decay fungi. Wood Deterioration and Preservation (Goodell B, Nicholas DD & Schultz TP, eds). 2003; pp. 140-153. Oxford University Press, Washington, DC.

1.4 Cellulase activities in biomass conversion: measurement methods and comparison

Adapted from: **Mehdi Dashtban[1,2], **Miranda Maki[1,2], Kam Tin Leung[2], Canquan Mao[3] and ****Wensheng Qin[1,2]

[1] Biorefining Research Institute
Lakehead University
955 Oliver Road
Thunder Bay, ON P7B 5E1 Canada

[2] Department of Biology
Lakehead University
955 Oliver Road
Thunder Bay, ON P7B 5E1 Canada

[3] School of Life Science and Engineering
Southwest Jiaotong University
Chengdu 610031 China

* Authors with equal contributions

**Corresponding author: Wensheng Qin

Abstract

Cellulose, the major constituent of all plant materials and the most abundant organic molecule on the Earth, is a linear biopolymer of glucose molecules, connected by β-1,4-glycosidic bonds. Enzymatic hydrolysis of cellulose requires mixtures of hydrolytic enzymes including endoglucanases, exoglucanases (cellobiohydrolases) and β-glucosidases acting in a synergistic manner. In biopolymer hydrolysis studies, enzyme assay is an indispensable part. The most commonly used assays for the individual enzymes as well as total cellulase activity measurements, including their advantages and limitations are summarized in this review article. In addition, some novel approaches recently used for enzyme assays are summarized.

Keywords: Biofuel, Biomass, Bioconversion, Cellulase, and Cellulase Assays

Introduction

Many microorganisms including fungi and bacteria had been found to degrade cellulose and other plant cell wall fibres. In nature, degradation of cellulosic biomass is performed by mixtures of hydrolytic enzymes collectively known as cellulases. The cellulases include endo-acting (endoglucanases) and exo-acting (cellobiohydrolases, CBH) enzymes, which act in a synergistic manner in biomass-degrading microbes. The cellobiose and cellodextran products of exoglucanases and cellobiohydrolases are inhibitory to their activity. Thus, efficient cellulose hydrolysis requires the presence of β-glucosidases to cleave the final glycosidic bonds of cellobiose producing glucose (Dashtban et al. 2009; Maki et al. 2009).

Assays for determining cellulase activity have been classified differently over years of cellulase research. Sharrock (1988) grouped cellulase assays into two basic approaches: 1) determining the activities of individual cellulases (endoglucanases, exoglucanases, and β-glucosidases), and 2) measuring the total saccharifying activity of a crude cellulase system (Sharrock 1988). Whereas Zhang et al. (2006) classified all cellulase activity assays into three main groups: 1) assays in which the accumulation of

74

products after hydrolysis were targeted, 2) assays in which the reduction in substrate quantity were monitored, and 3) assays in which the change in the physical properties of the substrate were measured (Zhang et al. 2006). Due to the complexity of cellulose-cellulase systems and differences between kinetic characteristics of initial hydrolysis reaction and the extended time, cellulase activity assays are either expressed based on the initial hydrolysis rate or using the end-point hydrolysis. The first one is preferred when measuring an individual cellulase activity in a short time; however, the last one is a method of choice for the total enzyme activity assay within a given time (Wu et al. 2006; Zhang et al. 2006).

Cellulase activity is mainly evaluated using a reducing sugar assay to measure the end products of cellulase hydrolysis activities. Thus, the results of such an assay are typically expressed as the hydrolysis capacity of the enzymes. There are several issues with this work: it cannot be easily expressed in a quantitative manner, lacks theoretical basis, and does not consider all effective factors, such as concentration of cellulose and cellulase, the hydrolysis time, the ratio of crystalline and amorphous cellulose, and the proportion between different individual components in the enzyme preparations (Wu et al. 2006). Researchers have mainly focused on improving methods for measurement of cellulase activity which have already been widely used. Developing new sufficient cellulase assays is hampered by the physical heterogeneity and limited enzyme-accessibility of cellulosic materials, and the complexity of cellulase enzyme systems (synergy and/or competition) (Zhang et al. 2006). Thus, an accurate and reproducible assay for the measurement of cellulase hydrolysis rate is still required (Wu et al. 2006).

In this review article, total cellulase activity by application of filter paper (filter paper assay, FPA) will be explained and then individual cellulase activities including endoglucanases, exoglucanases and β-glucosidases will be discussed. Moreover, we will also summarize some novel approaches such as (1) quartz crystal microbalance, (2) miniaturized colorimetric assay, (3) automated FPA for the measurement of cellulase activity, (4) fluorescent microfibrils, and (5) amperometric cellobiose dehydrogenase biosensor. Figure 1 recaps the different cellulase assays discussed in the article. This

review paper summarizes and compares past and present cellulase assaying techniques and suggests future directions important for the ever growing field of biofuel research.

1. **Filter paper assay (FPase activity): total cellulase activity**

To compare the efficacy of cellulase activity between microorganisms or their secreted enzymes, techniques for measuring total cellulase activity are required. The filter paper assay (FPA) is the key method for analysis of total cellulase activity. In 1976, the filter paper assay was developed by Mandels et al. (Mandels et al. 1976). The filter paper assay became widely used since 1984, when the Commission on Biotechnology of the International Union of Pure and Applied Chemistry (IUPAC) proposed a number of standard procedures for the measurement of cellulase activity. Traditionally, the filter paper assay uses a 1×6-cm strip of Whatman no. 1 filter paper, as the standard substrate because it is readily available and inexpensive (Coward-Kelly et al. 2003). This standard filter paper method has been reviewed by Ghose (Ghose 1987). The International Unit (IU) of filter paper activity (FPase) (FPU) is defined as the micromoles of glucose equivalent liberated per minute of culture filtrate under assay conditions such as specific pH and temperature values that depend largely on the properties of the enzyme, varying widely between cellulases and microorganisms. Reducing sugar is estimated as glucose by the Miller method. This assay is performed so that 0.5 mL of diluted enzymes releases about 2.0 mg of glucose equivalents in 60 min, as determined by the dinitrosalicylic acid (DNS) assay (Miller 1959; Wood and Bhat 1988).

The DNS reagent is used as a colorimetric method for the determination of reducing sugars, such as glucose. It contains sodium potassium tartrate, which decreases the tendency to dissolve oxygen by increasing the ion concentration in the solution. Phenol increases the amount of color produced during the color developing reaction. Sodium bisulphite stabilizes the color obtained and reacts with any oxygen present in the buffer. Finally, an alkaline buffer is required for the redox reaction between DNS and glucose, or other reducing sugars. DNS will be added at the last step of the enzyme assay to stop the reaction. To promote full color development, samples have to be boiled vigorously and the absorbance of

diluted samples will be read at 540 nm (Zhang et al. 2009). One disadvantage of using such a dye for quantification is that, some of the reducing sugars are degraded while the analysis is performed (Miller 1959).

There are several more concerns associated with using the filter paper assay to quantify total cellulase activity. Although the FPA is commonly used, it is also known for being non-reproducible. Difficulties arise from the preparation of the DNS reagent which is a tedious task requiring optimal mixing ratios of the different components. Additionally, DNS reagent requires appropriate temperature control to allow for proper colour development and colour stability (Miller 1959). Furthermore, it is known that the decomposition of sugars in the alkaline solution recommended by the IUPAC method causes an increase of (measured) enzyme activity to values higher than the actual ones (Gilman 1943). To summarize, it is time-consuming, labor-intensive and requires large quantities of reagents. It is also difficult to obtain adequate sensitivity and reproducibility when characterizing newly isolated cellulases using this method. Factors that affect sensitivity and reproducibility often result from the fact that most natural cellulase complexes tend to have a shortage of β-glucosidase activity (Breuil et al. 1986; Coward-Kelly et al. 2003).

Several methods have been developed to improve the filter paper assay for the evaluation of total cellulase activity. Nordmark et al. (2007) designed a modified method for the filter paper assay which requires the use of protein stabilizers. This method allows the sensitive measurement of cellulase activity below the level required for the detection of reducing sugars using the traditional filter paper assay. The traditional filter paper assay requires a fixed degree of conversion of substrate, i.e. a fixed amount (2 mg) of glucose (based on reducing sugars measured by the DNS assay) released from 50 mg of filter paper within a fixed time (60 min). Because of the heterogeneous (amorphous/crystalline) nature of filter paper, reducing sugar yield during hydrolysis is not a linear function of the quantity of cellulase enzyme in the assay mixture (Zhang et al. 2009). To overcome this limitation, researchers usually measure two enzyme activities (slightly less than and slightly greater than 2.0 mg of Reducing Sugar Equivalents (RSE) in 1h).

It is difficult to measure activities greater than 2.0 mg RSE in 1 h for all cellulases because cellulases preparations typically have lower cellulase activity due to lower concentration. Protein stabilizers (such as bovine serum albumin) extended the enzyme reaction time thereby allowing a proportionate calculation of cellulase activities on natural cellulosic substrates to those obtained in the IUPAC assay (Nordmark et al. 2007).

Similarly, Coward-Kelly et al. (2003) found that the filter paper assay could be improved by adding supplemental β-glucosidase. If an organism or enzyme complex has low β-glucosidase activity a high amount of cellobiose will be produced resulting in a lowered or 'false' absorbance reading for the DNS assay because it is not a reducing sugar. Adding supplemental β-glucosidase can help to overcome this issue. In this study, supplemental β-glucosidase increased the assay reading by 56%. They also tested the hypothesis that extended boiling time will improve the filter paper assay but failed to find any such benefit. A 5-min boiling time is sufficient; however, they suggest that the water bath boil vigorously to eliminate temperature excursions (Coward-Kelly et al. 2003).

Finally, downsizing the filter paper assay has also been developed as an improvement to the assay, allowing researchers to assay a large number of samples simultaneously. This has been achieved by reducing the volume of the reagents and substrate so the assay can be done in a 96-well microtitre plate. The overall enzymatic reaction volume was reduced from the IUPAC 1.5 mL standard to 60 µL. An office hole puncher was used to create small disks of filter paper substrate to fit perfectly in the wells. No significant difference was observed between the activities measured using the IUPAC filter paper assay compared to the minimized reactions in the microtitre plate (Xiao et al. 2004).

2. **Endoglucanases activity: carboxymethyl cellulase activity (CMCase)**

Endoglucanases (EG) can randomly hydrolyze internal glycosidic bonds in cellulose chains. EGs activities can be measured using a soluble cellulose derivative with a high degree of polymerization (DP) such as carboxymethyl cellulose (CMC). Carboxymethyl cellulase (CMCase) is mainly evaluated based

on the procedure described by Mandels et al. (1976). In this method, CMCase activity is measured by determining reducing sugars released after 5 min of enzyme reaction with 0.5% CMC at pH 4.8 and 50 °C (Mandels et al. 1976). Also, one unit (IU) of EG is defined as the amount of enzyme that liberates 1 μmol of glucose per minute under assay conditions. Reducing sugar can be estimated by application of different methods such as high performance liquid chromatography (HPLC) (Fujita et al. 2002) or glucose oxidase/peroxidase reagent (Trinder 1969) or a colorimetric method such as the Somogyi-Nelson method which uses alkaline copper as an inorganic oxidant. Cupric ions (Cu (II)) accept electrons from the donating aldehyde groups of reducing sugars and reduce to Cu (I). In the second step, reduced Cu (I) ions will be oxidized back to Cu (II) using a chromogenic compound. The reduced chromogenic compound produces color which can be measured using a colorimeter and compared to standards prepared from reacting sugar solutions of known concentration, to determine the amount of reducing sugar present (Nelson 1944; Somogyi 1952).

Although CMC is commonly used as a substrate to quantify EG activity, there are several concerns associated with using CMC. It is known for being non-reproducible which is only linear to about 12% conversion (hydrolysis of CMC to glucose) due to interference by substituents. In this case, substituted glucose units available in different CMCs are also accessible to cellulase which caused non-reproducibility. In addition, the quantity of reducing sugars produced and thus the unit values, will be highly affected by the particular type of CMC used in the assay (Eveleigh et al. 2009; Mandels et al. 1976). These difficulties arise from two important variable physical parameters of CMC: 1) the degree of substitution (DS), and 2) the degree of polymerization (DP) which will affect its solubility and viscosity, respectively. It is recommended that a reducing sugar assay or viscosity assay should be limited to the first 2% hydrolysis of substrate when CMC is used as the substrate with DS=0.7, this is to ensure that only nonsubstituted glucose units are accessible to EG (Zhang et al. 2006). Additionally, the DP of CMC has an important role in determination of viscosity reduction. Therefore, to minimize the influence of some conditions such as pH and ionic strength on DP and thus viscosity, some substituted CMC

substrates such as ionic CMC have to be avoided for determining EG activity. Whereas non-ionic substituted cellulose such as hydroxyethyl cellulose (HEC) is preferred (Guignard and Pilet 1976; Zhang et al. 2009).

EGs activities can be measured using dye, either by adding dye to soluble cellulose derivatives or by adding it to solid agar plates known as "zymograms". Remazol Brilliant Blue R and Ruthenium Red are two examples of dyes that have been used in CMC assays. Recently in a zymogram assay, Gram's iodine has been used for a fast and easy detection of endoglucanase activity which makes a sharp and distinct zone around the cellulase producing microbial colonies in a bluish-black background within a short time (3-4 min) (Kasana et al. 2008). This method and other zymogram methods are applicable for screening of a large number of colonies. However, they do not provide a quantitative result for the enzyme activity due to the lacking of a linear relationship between halo zones and enzyme activity. Moreover, EGs activities can be measured using some other dyes by adding them to insoluble cellulose derivatives or substituting insoluble cellulose derivatives chemically to produce chromogenic CMC. Examples of these are Cibacron Blue 3GA (Ten et al. 2004) and chromogenic trinitrophenyl CMC (TNP-CMC) (Huang and Tang 1976), respectively.

3. Exoglucanases activity: Avicellulases

Cellobiohydrolases (exoglucanases) are classified as exo-acting based on the assumption that they all cleave β-1,4-glycosidic bonds from chain ends releasing cellobiose and some glucose molecules. Commercial Avicel (also called microcrystalline cellulose or hydrocellulose) is used for measuring exoglucanase activity because it has a low degree of polymerisation of cellulose and it is relatively inaccessible to attack by endoglucanases despite some amorphous regions.

Enzymes which show relatively high activity on Avicel and little activity on CMC are identified as exoglucanases (Maki et al. 2009). However, Avicel contains some amorphous cellulose and soluble

cellodextrans which can act as substrates for both exo- and endo-glucanases. There is no highly specific

substrate to test exoglucanase activity in cellulase mixtures (Sharrock 1988; Wood and Bhat 1988).

Different assays have been reported for selection of exoglucanase activity, nevertheless all of

these assays have some sort of limitations. Van Tilbeurgh and Claeyssens (1985) found that 4-

methylumbelliferyl-β-D-lactoside was an effective substrate for assaying CBHI of *Trichoderma reesei*,

where hydrolysis of this substrate yields lactose, phenol and 4-methylumbelliferone (a fluorescent signal

molecule) as products. However, this substrate could not be used to determine CBHII activity of *T. reesei*

thus it is not an effective representation of true exoglucanase activity for this strain (van Tilbeurgh et al.

1982; van Tilbeurgh et al. 1985).

Similarly, Deshpande et al. (1984) developed an assay for quantification of exoglucanase activity

in the presence of endoglucanases and β-glucosidases (Deshpande et al. 1984). This assay is based on the

following: exoglucanases specifically hydrolyze the aglyconic bond of *p*-nitrophenyl-β-D-cellobioside to

yield cellobiose and *p*-nitrophenol; β-glucosidase activity is inhibited by adding D-glucono-1,5-δ-lactone

(Holtzapple et al. 1990); and, the influence of exoglucanase hydrolysis activities must be quantified in the

assay procedure in the presence of added purified endoglucanases. The limitations for this assay are that:

(1) the CBHII activity cannot be measured using *p*-nitrophenyl-β-D-cellobioside, (2) the specific activity

of the available purified endoglucanases may not be representative for all existing endoglucanases in the

mixture, and (3) the product ratio from endoglucanase actions may be influenced by the presence of

exoglucanases (Zhang et al. 2006).

Other less commonly used substrates for measuring or detecting exoglucanase activity for both

bacteria and fungi include the following: PNP-*p*-D-cellobioside (Kohring et al. 1990), bacterial

microcrystalline cellulose (BMCC) (Caspi et al. 2008), and MU-β-D-cellobioside (MU-C) (Courty et al.

2005). Limitations of these substrates are not clearly defined.

4. β-glucosidases assay

β-glucosidase activity can be measured using various chromogenic and nonchromogenic substrates and are mainly evaluated based on the procedure of Kubicek (Kubicek 1982). In one chromogenic method, *p*-nitrophenol-β-glucoside (*p*NPG) is used as the substrate. The liberated *p*-nitrophenol will be measured in order to determine the hydrolysis rate in optimal temperature and pH. Reaction conditions such as temperature and pH of different β-glucosidases vary based on the enzyme (Table 1). *p*NPG as the substrate at the optimal concentration (usually 1-5 mM) will be added to an appropriate buffer with optimal pH, containing the enzyme and incubated at the optimal temperature. After 10-min incubation, the reaction will be stopped by adding 3 volumes of sodium tetraborate saturated solution, and then the absorbance will be read at 405 nm. One unit of β-glucosidase is defined as the amount of enzyme that liberates 1 μmol of *p*-nitrophenol per minute (Chandra et al. 2009). However, in the case of nonchromogenic substrates different methods can be used depending on the substrates. For example when oligo- or di-saccharides (such as cellobiose) are used as the substrates, the liberated glucose can be evaluated by the glucose oxidase (GOD) method with a commercial kit. Nevertheless, when the substrate is a polysaccharide, reducing sugars liberated will be measured by the 3,5-dinitrosalicylic acid (DNS) method. Using polysaccharides as the substrate, the enzyme unit will be determined as the amount of enzyme required for the liberation of one micromole of glucose or reducing sugar per minute. Moreover, substrate specificity of enzymes can be determined using different substrates listed in Table 1 and applying the above mentioned methods.

β-glucosidase activity measurement using chromogenic substrates such as *p*NPG is a common technique used in many different studies (Bhatia et al. 2005; Daroit et al. 2008; Joo et al. 2009; Karnchanatat et al. 2007; Korotkova et al. 2009; Murray et al. 2004; Tsukada et al. 2008; Yang et al. 2008; Yoon et al. 2008). However, correlation between β-glucosidase activity on the analog substrates (e.g. *p*NPG) and the natural substrate (e.g. cellobiose) is not clear. As a natural substrate, cellobiose has been used in β-glucosidase screening experiments using 96-well microtitre plates (McCarthy et al. 2004).

However, this method is not preferred for screening of a large library of enzyme producing microorganisms due to its disadvantages such as being time-consuming and costly (Liu et al. 2009). Recently, thermostable β-glucosidase mutants (BGLA) from *Paenibacillus polymyxa* have been identified using novel and fast combinatorial selection/screening approach. In this study a big mutant library including 100,000 clones were generated using error-prone PCR and cloned and expressed in *E. coli*. Thermostable β-glucosidase mutants have been identified in a two-step process using a natural substrate (cellobiose): 1) selection for mutants with adequate β-glucosidase activity; 2) screening for improved thermostability. In the first step, cells were grown on selection plates containing minimal growth medium plus cellobiose as the sole carbon source and thus, only cells expressing active β-glucosidase could grow on the medium. Colonies on the selection plate were duplicated using a nylon membrane and then incubated at 60 °C for 10 min to break the cells and release intracellular β-glucosidase. Also, heat treatment deactivated most of the β-glucosidase mutants and only thermostable β-glucosidase mutants will remain active and will be able to hydrolyse cellobiose to glucose on the screening plate. In the second step, the membrane was overlaid on the soft agar screening plate containing minimal medium with cellobiose as the sole carbon source. In addition to that, the medium contained an indicator strain of *E. coli* which was enabled to utilize glucose only (but not cellobiose). After incubation the growth of the indicator strain on the screening plate was used as an indicator to detect the clones expressing thermostable BGLA mutants. This screening method enabled scientists to screen larger libraries within a shorter time. In this case, a thermotolerant mutant with 11-folds improved thermotolerance compared to the wild-type has been selected (Liu et al. 2009).

Table 1. Different β-glucosidases have different optimal temperature, pH, and substrate specificity.

β-Glucosidase	Source	Optimum temperature/pH	Substrate specificity	References
	Aspergillus japonicas	65/4.5–5	High toward cellobiose and *p*NPG and low toward β-glucan from barley	Korotkova *et al.* (2009)
	Daldinia eschscholzii (Ehrenb.:Fr.) Rehm	50/5	*p*NPG, cellobiose, sophorose, laminaribiose, and gentiobiose	Karnchanatat *et al.* (2007)
	Fomitopsis palustris	70/4.5	*p*NPG and cellobiose	Yoon *et al.* (2008)
	Fomitopsis pinicola KMJ812	50/4.5	*p*NPG and cellobiose	Joo *et al.* (2009)
	Monascus purpureus NRRL1992	50/5.5	*p*NPG, cellobiose, salicin, *n*-octyl-β-D-glucopyranoside, and maltose	Daroit *et al.* (2008)
	Paecilomyces thermophila	75/6.2	Very broad, *p*NPG, cellobiose, gentiobiose, sophorose, amygdalin, salicin, daidzin, and genistin	Yang *et al.* (2008)
	Penicillium verruculosum	60/5.5	High toward β-glucan from barley and low toward cellobiose and *p*NPG	Korotkova *et al.* (2009)
BGL1B	*P. chrysosporium*	–/6–6.5	Cellobiose, cellobionolactone, and *p*NPG	Tsukada *et al.* (2008)
BGLII	*Pichia etchellsii*	45/6	*p*NPG, sophorose, gentiobiose, cellobiose, laminaribiose, and salicin	Bhatia *et al.* (2005)
Cel3a	*Talaromyces emersonii*	71.5/5	*p*NPG, *p*NPC, salicin, cellobiose, and barley β-glucan	Murray *et al.* (2004)
	T. reesei	70/5	High toward β-glucan from barley and low toward cellobiose and *p*NPG	Korotkova *et al.* (2009)

5. Novel approaches for measurement of cellulase activity: Automated measurement of cellulase activity

Traditionally, enzymatic assays, particularly the widely used FPA, are recognized for their complexity and sensitivity to operator error and are generally deemed tedious and time-consuming. It appears obvious that new and/or improved assays are required; however, there are several difficulties to be addressed when developing assays to quantify the activity of enzymes such as cellulases. Is the assay reproducible, reliable and can it be applied in a time- and cost-efficient manner? The aphorism "You get what you screen for" implies that the screening method/assay is crucial for accurate, reproducible and high quality results. Thus, researchers have focused on developing a wide variety of novel techniques for the measurement of cellulase activity in hopes of addressing some of these difficulties with currently used assays.

There are a variety of different techniques used to evaluate cellulase activity as previously mentioned, such as colorimetric detection of reducing groups, chromogenic, fluorogenic group release, chromatographic substrates and viscometric detection methods, to highly sophisticated mainly research-based methods such as ion, liquid, and anion exchange chromatography and high-performance liquid

chromatography (Schwald et al. 1988). Recently a few novel assays with ease of operation and high reproducibility have been developed. Table 2 summarizes some cellulase assays using novel techniques.

One of the most recent novel assay methods uses a quartz crystal microbalance (QCM) piezoelectric-sensing technique, for measuring cellulase activity, and relates crystallinity of different substrates to the cellulase activity (Hu et al. 2009). The piezoelectric property of quartz crystal allows the production of an ultrasensitive mass balance. Changes in frequency of a quartz crystal can be used to measure viscosity and density changes in a solution used to incubate a given cellulose substrate, after enzymatic hydrolysis. The results can be used to quantify the enzyme activity. Here, the quantification of cellulase activity using QCM was closer to those results obtained by measuring the actual reducing sugars (IUPAC assay). QCM is advantageous to use because it is easier to implement by eliminating the need for colour development during the standard redox methods. It also allows for flexibility in the properties of substrates used.

Also recently, a miniaturized assay for the determination of total enzyme activity based on the colorimetric DNS method has been developed (King et al. 2009). In this study, the mini-assay proved useful for high-throughput bioprospecting of novel enzymes for biofuel production. Reducing sugar released from filter paper, Avicel, corn stalk, switchgrass, carboxymethylcellulose, and arabinoxylan were measured for a variety of fungal isolates and cellulase activities comparable or greater than activities of the widely used wild-type *T. reesei* were observed. The enzyme extracts from biomass/substrate treated samples were aliquoted to 96-well microtitre plates and DNS reagents were reduced producing the miniaturized assay (King et al. 2009). This miniaturized assay can be used not only for bioprospecting novel enzymes but also can be used to replace the traditional colorimetric cellulase assays to measure and compare the activity of known cellulases. It is advantageous because it allows operators to reduce reagents, thereby reducing costs, aliquoting errors and ultimately the time for quantification.

85

Similarly, the possibility of complete automation of a cellulase assay was fully exemplified by Decker et al (2003). This group created an automated version of the traditional filter paper assay using a Cyberlabs C400 robotics deck equipped with customized incubation, reagent storage, and plate reading capabilities. The goal of such an automated assay was to reduce operator error during determination of cellulase activity and to reduce the amount of reagent usage as well as lower reagent disposal costs, while allowing for a high throughput of samples to be assayed. The maximum throughput of samples of the automated procedure is 84 enzymes per day. After the initial cost associated with the purchase of such a piece of equipment the high efficiency and low reagent usage will allow this technology to be successful, however at its current stage this automated assay is not sufficiently comparable to the traditional FPA (Decker et al. 2003).

Furthermore, a more sensitive cellulase assay was developed using fluorescent microfibrils from bacterial cellulose prepared using DTAF (5-(4,6-dichlorotriazinyl) aminofluorescein) as a grafting agent. Fluorescent dyes such as DTAF which bear dichlorotriazinyl groups are known to react with hydroxyl groups of polysaccharides making DTAF a good candidate. A protocol to graft microfibrils with DTAF was developed which does not modify the physical integrity of the substrate. This grafted DTAF-cellulose was created by dissolving 10-70 mg of DTAF into 10 mL of a suspension containing 100 mg of cellulose microfibrils in 0.1 N NaOH. These mixtures were stirred at room temperature for 24 h. Cellulose digestion resulted in the release of fluorescent cellodextrins and reducing sugars. This method allowed for a comparison between the amount of released fluorescence and that of released reducing sugar from which one could differentiate between processive exo- and endo-cellulase activities. This research group also casted films of DTAF-grafted microfibrils to the bottom of microwell titre plates producing sensitive cellulase detection and allowing for possible automation. Sensitivity of detection can be increased by optimization of the grafting conditions which maximizes the quantity of soluble products. The main advantages for using fluorescent microfibrils is it allows for measurement of minute amounts of cellulase activity and it reduces the dependency on using substrates such as carboxymethyl cellulose

which are far different from native cellulosic substrates. Cellulose microfibrils produced by algae and bacteria have been well characterized and shown to contain most of the structural and morphological characteristics of "real" cellulose materials. Being in a dispersed state, these cellulose microfibrils reduce cellulase-substrate accessibility problems (Helbert et al. 2003).

Moreover, Hildén et al. (2001), set out to create a faster, more convenient, yet equally reliable method for determining cellulase activities of a series of samples. They achieved this by using an amperometric redox polymer-based biosensor to determine the total concentration of soluble oligosaccharides. The biosensor was produced based on cellobiose dehydrogenase from *Phanerochaete chrysosporium* wired by a redox polymer. This newly applied method of measuring cellulase activity provides several advantages over traditional methods. Firstly, it is rapid, allowing analysis of a maximum 30 samples in an hour. In addition, the biosensor can be readily used without prior planning because it can be stored in water in flow injection analysis. Furthermore, the enzyme solution may be recovered after passing the electrode due to its non-destructive nature. Not to mention, no harmful chemicals, boiling or cooling is required with this method simplifying implementation. Finally, the precision of the method is equivalent to traditional methods such as the Somogyi-Nelson technique with high sensitivity detection to the same order of magnitude for cellobiose, cellotriose, and cellotetraose (Hilden et al. 2001).

Despite the newly emerging cellulase activity assays, the filter paper assay is still the most widely used method. Perhaps automation of the FPA will help researchers achieve reproducibility while reducing costs. However, biosensors are becoming more popular and may offer a similar promising solution to the evaluation of cellulase activity which will give results comparable to the direct measurement of reducing sugars via FPA.
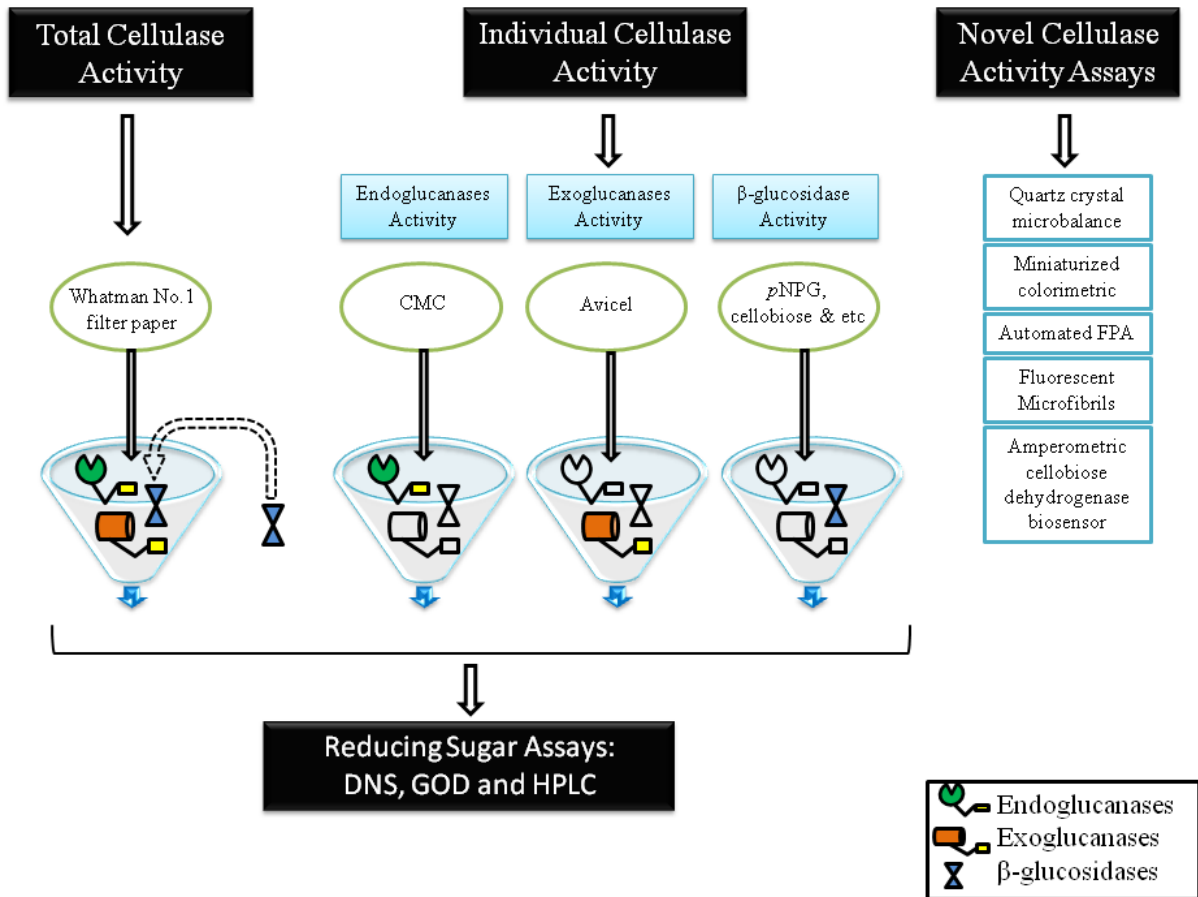
Figure 1. Different cellulase assays which are classified within two groups: 1) total cellulase activity, and 2) individual cellulase activity including endo-, exoglucanases and β-glucosidases. Filter paper assay can be improved by adding supplemental β-glucosidase which is indicated by the broken arrow. Released reducing sugars can be measured using different reducing sugar assay methods such as DNS (dinitrosalicylic acid), GOD (glucose oxidase), and HPLC. Recently a few novel assays with ease of operation and high reproducibility have been developed.

Table 2. Some cellulase assays using novel techniques.

Cellulase assay	Substrate	Advantages	References
Quartz crystal microbalance	FP, Avicel, CMC	Various substrates, Easy to implement. *Comparable results	Hu *et al.* (2009)
Miniaturized colorimetric	FP, Avicel, corn stalk, switchgrass, CMC, arabinoxylan	High-throughput, comparable results, various substrates	King *et al.* (2009)
Automated FPA	FP	Reproducible, high-throughput, reduced reagent usage	Decker *et al.* (2003)
Fluorescent microfibrils	Bacterial cellulose	Possible automation, native cellulose, sensitive cellulose detection	Helbert *et al.* (2003)
Amperometric cellobiose dehydrogenase biosensor	Avicel	Rapid, readily implemented, nondestructive, comparable precision	Hilden *et al.* (2001)

*Comparable results—results of assay are comparable with traditional methods.

6. Concluding remarks

Many different cellulase activity assays have been used and developed over the last few decades. However, only a few of them have been used consistently and they are discussed in this article. Among these assays, total cellulase activity by application of filter paper and methods for measuring individual cellulase activities for endoglucanases, exoglucanases and β-glucosidases are the major cellulase assays. Some of the major obstacles for cellulase assays are heterogeneity of insoluble cellulose, complicated synergy/competition among endo- and exo-glucanases and changes of enzyme/substrate ratio (Zhang et al. 2009). More recently, some novel approaches such as quartz crystal microbalance, miniaturized colorimetric assay, automated FPA, fluorescent microfibrils, and amperometric cellobiose dehydrogenase biosensors have been developed for the measurement of cellulase activity. Among these novel approaches, biosensors are recently attracting more attention.

Amperometric biosensors measure the changes of current of a working electrode resulting from biochemical and electrochemical reactions. In amperometric biosensors the potential at the electrode is held constant while the current is measured. The overall performance of the biosensor will mainly depend on, the properties of biosensing (enzyme) membrane and to a little extent on the instrumentation system used to acquire the signal generated by biochemical reaction at the biosensing membrane (Baronus et al. 2003; Chaubey and Malhotra 2002; Gopel and Heiduschka 1995). The combination of modern electrochemical techniques with enzymatic biosensors may potentially increase demands for investigation

89

on cellulase assays to design high performance biosensing systems in terms of selectivity, sensitivity, reliability, durability, and low cost. An example of an amperometric biosensor with potential application in cellulase assays is the glucose-oxidase biosensor. The enzyme glucose oxidase is incorporated in the membrane of the electrode to detect glucose and ultimately relay glucose concentration. The glucose oxidase biosensor cannot detect small oligosaccharides such as cellobiose and cellotetraose which may be products of cellulase activity relating to endo- and exo- glucanases. However, the previously discussed cellobiose dehydrogenase containing amperometric biosensor is capable of measuring such products. For an accurate analysis of total cellulolytic activity we propose the production of a mixed enzyme membrane for biosensor detection. Combining glucose oxidase with an additional enzyme such as cellobiose dehydrogenase would allow the detection of all cellulose hydrolysis products.

References

Baronus, R., E. Ivanauskas and J. Kulys. 2003. "Computer simulation of the response of Amperometric Biosensors in stirred and non stirred solution." Nonlinear Analysis: Modeling and control 8:3-18.

Bhatia, Y., S. Mishra and V. S. Bisaria. 2005. "Purification and characterization of recombinant Escherichia coli-expressed Pichia etchellsii beta-glucosidase II with high hydrolytic activity on sophorose." Appl Microbiol Biotechnol 66(5):527-535.

Breuil, C., P. Mayers and J. N. Saddler. 1986. "Substrate conditions that influence the assays used for determining the beta-glucosidase activity of cellulolytic microorganisms." Biotechnol Bioeng 28(11):1653-1656.

Caspi, J., D. Irwin, R. Lamed, Y. Li, H. P. Fierobe, D. B. Wilson and E. A. Bayer. 2008. "Conversion of Thermobifida fusca free exoglucanases into cellulosomal components: comparative impact on cellulose-degrading activity." J Biotechnol 135(4):351-357.

Chandra, M., A. Kalra, P. K. Sharma and R. S. Sangwan. 2009. "Cellulase production by six Trichoderma spp. fermented on medicinal plant processings." J Ind Microbiol Biotechnol 36(4):605-609.

Chaubey, A. and B. D. Malhotra. 2002. "Mediated biosensors." Biosens Bioelectron 17(6-7):441-456.
Courty, P. E., K. Pritsch, M. Schloter, A. Hartmann and J. Garbaye. 2005. "Activity profiling of ectomycorrhiza communities in two forest soils using multiple enzymatic tests." New Phytol 167(1):309-319.

Coward-Kelly, G., C. Aiello-Mazzari, S. Kim, C. Granda and M. Holtzapple. 2003. "Suggested improvements to the standard filter paper assay used to measure cellulase activity." Biotechnol Bioeng 82(6):745-749.

Daroit, D. J., A. Simonetti, P. F. Hertz and A. Brandelli. 2008. "Purification and characterization of an extracellular beta-glucosidase from Monascus purpureus." J Microbiol Biotechnol 18(5):933-941.

Dashtban, M., H. Schraft and W. Qin. 2009. "Fungal bioconversion of lignocellulosic residues; opportunities & perspectives." Int J Biol Sci 5(6):578-595.

Decker, S. R., W. S. Adney, E. Jennings, T. B. Vinzant and M. E. Himmel. 2003. "Automated filter paper assay for determination of cellulase activity." Appl Biochem Biotechnol 105 -108:689-703.

Deshpande, M. V, K. E. Eriksson and L. G. Pettersson. 1984. "An assay for selective determination of exo-1,4,-beta-glucanases in a mixture of cellulolytic enzymes." Anal Biochem 138:481-487.

Eveleigh, D. E., M. Mandels, R. Andreotti and C. Roche. 2009. "Measurement of saccharifying cellulase." Biotechnol Biofuels 2:21.

Fujita, Y., S. Takahashi, M. Ueda, A. Tanaka, H. Okada, Y. Morikawa, T. Kawaguchi, M. Arai, H. Fukuda and A. Kondo. 2002. "Direct and efficient production of ethanol from cellulosic material with a yeast strain displaying cellulolytic enzymes." Appl Environ Microbiol 68(10):5136-5141.

Ghose, T. K. . 1987. "Measurement of cellulase activities." Pure Appl Chem 59:257-268.

Gilman, H. 1943. "Organic Chemistry-Advanced Treatise.2nd ed." Wiley: New York.

Gopel, W. and P. Heiduschka. 1995. "Interface analysis in biosensor design." Biosens Bioelectron 10(9-10):853-883.

Guignard, R. and P-E. Pilet. 1976. "Viscosimetric determination of cellulase activity: critical analyses." Plant and Cell Physiology Vol. 17(5):899-908.

Helbert, W., H. Chanzy, T. L. Husum, M. Schulein and S. Ernst. 2003. "Fluorescent cellulose microfibrils as substrate for the detection of cellulase activity." Biomacromolecules 4(3):481-487.

Hilden, L., L. Eng, G. Johansson, S. E. Lindqvist and G. Pettersson. 2001. "An amperometric cellobiose dehydrogenase-based biosensor can be used for measurement of cellulase activity." Anal Biochem 290(2):245-250.

Holtzapple, M., M. Cognata, Y. Shu and C. Hendrickson. 1990. "Inhibition of Trichoderma reesei cellulase by sugars and solvents." Biotechnol Bioeng 36(3):275-287.

Hu, G., J. A. Heitmann and O. J. Rojas. 2009. "Quantification of Cellulase Activity Using the Quartz Crystal Microbalance Technique." Anal Chem.

Huang, J. S. and J. Tang. 1976. "Sensitive assay for cellulase and dextranase." Anal Biochem 73(2):369-377.

Joo, A. R., M. Jeya, K. M. Lee, W. I. Sim, J. S. Kim, I. W. Kim, Y. S. Kim, D. K. Oh, P. Gunasekaran and J. K. Lee. 2009. "Purification and characterization of a beta-1,4-glucosidase from a newly isolated strain of Fomitopsis pinicola." Appl Microbiol Biotechnol 83(2):285-294.

Karnchanatat, A., A. Petsom, P. Sangvanich, J. Piaphukiew, A. J. Whalley, C. D. Reynolds and P. Sihanonth. 2007. "Purification and biochemical characterization of an extracellular beta-glucosidase from the wood-decaying fungus Daldinia eschscholzii (Ehrenb.:Fr.) Rehm." FEMS Microbiol Lett 270(1):162-170.

Kasana, R. C., R. Salwan, H. Dhar, S. Dutt and A. Gulati. 2008. "A rapid and easy method for the detection of microbial cellulases on agar plates using gram's iodine." Curr Microbiol 57(5):503-507.

King, B. C., M. K. Donnelly, G. C. Bergstrom, L. P. Walker and D. M. Gibson. 2009. "An optimized microplate assay system for quantitative evaluation of plant cell wall-degrading enzyme activity of fungal culture extracts." Biotechnol Bioeng 102(4):1033-1044.

Kohring, S., J. Wiegel and F. Mayer. 1990. "Subunit Composition and Glycosidic Activities of the Cellulase Complex from Clostridium thermocellum JW20." Appl Environ Microbiol 56(12):3798-3804.

Korotkova, O. G., M. V. Semenova, V. V. Morozova, I. N. Zorov, L. M. Sokolova, T. M. Bubnova, O. N. Okunev and A. P. Sinitsyn. 2009. "Isolation and properties of fungal beta-glucosidases." Biochemistry (Mosc) 74(5):569-577.

Kubicek, C. P. 1982. "beta-Glucosidase excretion by Trichoderma pseudokoningii: correlation with cell wall bound beta-1.3-glucanase activities." Arch Microbiol 132(4):349-354.

Liu, W., J. Hong, D. R. Bevan and Y-H. P. Zhang. 2009. "Fast identification of thermostable beta-glucosidase mutants on cellobiose by a novel combinatorial selection/screening approach." Biotechnol Bioeng 103(6):1087-1094.

Maki, M., K. T. Leung and W. Qin. 2009. "The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass." Int J Biol Sci 5(5):500-516.

Mandels, M., R. Andreotti and C. Roche. 1976. "Measurement of saccharifying cellulase." Biotechnol Bioeng Symp(6):21-33.

McCarthy, J. K., A. Uzelac, D. F. Davis and D. E. Eveleigh. 2004. "Improved catalytic efficiency and active site modification of 1,4-beta-D-glucan glucohydrolase A from Thermotoga neapolitana by directed evolution." J Biol Chem 279(12):11495-11502.

Miller, G. L. 1959. "Use of dinitrosalicylic acid reagent for determination of reducing sugar." Anal Chem 31:426-428.

Murray, P., N. Aro, C. Collins, A. Grassick, M. Penttila, M. Saloheimo and M. Tuohy. 2004. "Expression in Trichoderma reesei and characterisation of a thermostable family 3 beta-glucosidase from the moderately thermophilic fungus Talaromyces emersonii." Protein Expr Purif 38(2):248-257.

Nelson, N. . 1944. "A photometric adaptation of the Somogyi method for the determination of glucose." J Biol Chem 153:375-380.

Nordmark, T. S., A. Bakalinsky and M. H. Penner. 2007. "Measuring cellulase activity: application of the filter paper assay to low-activity enzyme preparations." Appl Biochem Biotechnol 137-140(1-12):131-139.

Schwald, W., M. Chan, C. Breuil and J. N. Saddler. 1988. "Comparison of HPLC and colorimetric methods for measuring cellulolytic activity." Appl Microbiol Biotechnol 28:398-403.

Sharrock, K. R. 1988. "Cellulase assay methods: a review." J Biochem Biophys Methods 17(2):81-105.

Somogyi, M. 1952. "Notes on sugar determination." J Biol Chem 195(1):19-23.

Ten, L. N., W. T. Im, M. K. Kim, M. S. Kang and S. T. Lee. 2004. "Development of a plate technique for screening of polysaccharide-degrading microorganisms by using a mixture of insoluble chromogenic substrates." J Microbiol Methods 56(3):375-382.

Trinder, P. 1969. "Determination of blood glucose using 4-amino phenazone as oxygen acceptor." J Clin Pathol 22(2):246.

Tsukada, T., K. Igarashi, S. Fushinobu and M. Samejima. 2008. "Role of subsite +1 residues in pH dependence and catalytic activity of the glycoside hydrolase family 1 beta-glucosidase BGL1A from the basidiomycete Phanerochaete chrysosporium." Biotechnol Bioeng 99(6):1295-1302.

van Tilbeurgh, H., M. Claeyssens and C. K. de Bruyne. 1982. "The use of 4-methylumbelliferyl and other chromophoric glycosides in the study of cellulolytic enzymes " FEBS Lett. 149:152 156.

van Tilbeurgh, H., G. Pettersson, R. Bhikabhai, H. De Boeck and M. Claeyssens. 1985. "Studies of the cellulolytic system of Trichoderma reesei QM 9414. Reaction specificity and thermodynamics of interactions of small substrates and ligands with the 1,4-beta-glucan cellobiohydrolase II." Eur J Biochem 148(2):329-334.

Wood, T. M. and K. M. Bhat. 1988. "Methods for measuring cellulase activities." Methods Enzymol 160:87-117.

Wu, B., P. J. Gao and Y. Zhao. 2006. "A new approach to measurement of saccharifying capacities of crude cellulase." BioResources 1(2):189-200.

Xiao, Z., R. Storms and A. Tsang. 2004. "Microplate-based filter paper assay to measure total cellulase activity." Biotechnol Bioeng 88(7):832-837.

Yang, S., Z. Jiang, Q. Yan and H. Zhu. 2008. "Characterization of a thermostable extracellular beta-glucosidase with activities of exoglucanase and transglycosylation from Paecilomyces thermophila." J Agric Food Chem 56(2):602-608.

Yoon, J. J., K. Y. Kim and C. J. Cha. 2008. "Purification and characterization of thermostable beta-glucosidase from the brown-rot basidiomycete Fomitopsis palustris grown on microcrystalline cellulose." J Microbiol 46(1):51-55.

Zhang, Y-H. P. , J. Hong and X. Ye. 2009. "Cellulase assays." Methods Mol Biol 581:213-231.

Zhang, Y-H. P., M. E. Himmel and J. R. Mielenz. 2006. "Outlook for cellulase improvement: screening and selection strategies." Biotechnol Adv 24(5):452-481.

Chapter 2: Effect of different carbon sources on cellulase production by *Hypocrea jecorina* (*Trichoderma reesei*) strains

Adapted from: Mehdi Dashtban[1,2], Robert Buchkowski[2] and Wensheng Qin*[1,2]

[1] Biorefining Research Institute
Lakehead University
955 Oliver Road
Thunder Bay, ON P7B 5E1 Canada

[2] Department of Biology
Lakehead University
955 Oliver Road
Thunder Bay, ON P7B 5E1 Canada

*Corresponding author: Wensheng Qin

2.1 Abstract

The ascomycete *Hypocrea jecorina*, an industrial (hemi)cellulase producer, can efficiently degrade plant polysaccharides. At present, the biology underlying cellulase hyperproduction of *T. reesei*, and the conditions for the enzyme induction, are not completely understood. In the current study, three different strains of *T. reesei*, including QM6a (wild-type), and mutants QM9414 and RUT-C30, were grown on 7 soluble and 7 insoluble carbon sources, with the latter group including 4 pure polysaccharides and 3 lignocelluloses. Time course experiments showed that maximum cellulase activity of QM6a and QM9414 strains, for the majority of tested carbon sources, occurred at 120 hrs, while RUT-C30 had the greatest cellulase activity around 72 hrs. Maximum cellulase production was observed to be 0.035, 0.42 and 0.33 µmol glucose equivalents using microcrystalline celluloses for QM6a, QM9414, and RUTC-30, respectively. Increased cellulase production was positively correlated in QM9414 and negatively correlated in RUT-C30 with ability to grow on microcrystalline cellulose.

Keywords: Trichoderma reesei, cellulase, growth characterization, carbon sources

2.2 Introduction

The ascomycete *Hypocrea jecorina* (anamorph *Trichoderma reesei*) is one of the most studied and industrially important cellulolytic fungi. *T. reesei* is a saprobic fungus capable of efficiently degrading plant cell wall polysaccharides such as cellulose and hemicelluloses [1]. It was first isolated in Solomon Islands in 1944 by the US Army [2]. The original *T. reesei* isolate known as *T. reesei* QM6a soon became an area of great interest, because of its high cellulolytic potential. For many years, random mutagenesis has been applied to improve cellulolytic activity of the strain for its industrial application. The creation of different mutant strains with several-fold increase in the amount of secreted cellulolytic enzymes compared to the wild-type strain has been achieved by both academic and industrial research programs [3, 4].

One of the most hypercellulolytic strains, RUT-C30, was originated from *T. reesei* QM6a by three rounds of mutagenesis including a UV-light treatment followed by N-nitrosoguanidine (NTG) and another round of UV-light treatments [5-7]. Two genetic changes were previously shown in this mutant strain. The first one was a truncation of the *cre1* gene encoding CRE1, the carbon catabolite repressor protein that renders the mutant strain carbon catabolite derepressed [8]. The second mutation was a frameshift mutation in the glycoprotein processing β-glucosidase II encoding gene [9]. Recently, massive parallel sequencing of two mutant strains including RUT-C30 and its ancestor NG-14 (originated from *T. reesei* QM6a) have been reported. The studies have identified higher number of mutagenic events including 223 single nucleotides variants (SNVs), 15 small deletions or insertions, and 18 larger deletions, leading to the loss of more than 100 Kb genomic DNA. These led to mutations in 43 genes mainly involved in nuclear transport, mRNA stability, transcription, protein secretion and metabolism [5-7]. These genetic changes altered the phenotype of RUT-C30 and, for example, it is unable to grow on α-linked oligo- and polysaccharides because of the loss of the maltose permease gene. This alteration makes RUT-C30 an ineffective candidate for cellulolytic enzyme production on carbon sources containing starch and other α-linked glycans [5]. Conversely, RUT-C30 shows an enhanced growth rate on a number of simple carbon sources such as glycerol, D-fructose, D-mannitol and D-mannose which act as catabolite repressing carbon source in *T. reesei* [5]. This alteration may be caused by the loss of CRE1 function in the mutant strain. Recent studies using array comparative genomic hybridization (aCGH) identified 17 new mutations in RUT-C30 and its ancestor NG-14 [10].

The other well-known hypercellulolytic strain *T. reesei* QM9414 also originated from *T. reesei* QM6a; in this case, the two rounds of mutations were included by a particle accelerator [5]. However, the nature of the mutations leading to the mutant strain have never been elucidated [11]. The mutant *T. reesei* QM9414 strain created by irradiation mutagenesis produces two to four times more cellulases than QM6a. Electrophoretic karyotyping of several *T. reesei* strains showed that chromosomal rearrangements have occurred in many strains, and in that of QM9414 the size of the smallest chromosome is different from

that of QM6a [10, 12]. Using aCGH, over forty new identified mutations (various types) have been recently reported in QM9414 and/or its ancestor QM9123 [10].

In order to better understand cellulolytic potential of *T. reesei*, the strain QM6a, with a genome of approximately 34 MB, was subjected to the genome sequencing by US Department of Energy Joint Genome Institute [13]. Although *T. reesei* is well-known for its hypercellulolytic potential, based on the sequencing results, it had the fewest cellulases among the analyzed fungal genomes. *T. reesei* produces seven cellulases in total including two exoglucanases and five endoglucanases in addition to a small set of hemicellulases and pectin degrading enzymes [6]. Additionally, *T. reesei* produces two β-glucosidases to hydrolyze cellobiose (end-product of cellulases) to glucose [14, 15].

The regulation of cellulolytic enzymes expression by hypercellulolytic *T. reesei* is a complex process and our knowledge is still incomplete. Expression of the cellulolytic enzymes in *T. reesei* is induced by cellulose and also by some disaccharides such as D-lactose, cellobiose and sophorose whereas the presence of preferred carbon sources such as D-fructose and D-glucose antagonized the induction effect [16, 17]. This is the case because most *T. reesei* cellulases are adaptive enzymes, meaning their transcripts are not formed during growth on monosaccharides, and their full expression requires the presence of an inducer. Although there are many theories regarding the regulation of cellulases, all of them agree that the actions of cellulases lead to the formation of a cellulase inducer [18]. The need for an inducer to stimulate cellulase gene expression represents tight regulation of the respective promoters. So far, three positive transcriptional activators (XYR1, ACE2 and the HAP2/3/5 complex) as well as two repressors (ACE1 and the carbon catabolite repressor CRE1) have been identified to be involved in the complex regulation of cellulases production by *T. reesei* [18].

One approach to better understand the biology underlying cellulase hyperproduction is the analysis of improved *T. reesei* mutant strains as compared to the wild-type *T. reesei* QM6a. Although the factors that regulate the production of cellulase by *T. reesei* have been previously studied, we systematically determined the effect of different carbon sources on cellulase production by three

important *T. reesei* strains [19, 20]. Because all the experimental conditions were identical, our results provide a more meaningful comparison between different strains and different carbon sources. In order to expand our understanding of the conditions for the production and activity of cellulases by *T. reesei*, we investigated the effect of 14 different carbon sources (7 soluble and 7 insoluble including 4 pure polysaccharides and 3 lignocelluloses, table 1) on three different *T. reesei* strains including QM6a (wild-type) and its two mutant strains QM9414 and RUT-C30. We also analyzed the appropriate cultivation time for highest cellulase activity for each strain growing on different carbon source. We further investigated the effect of different concentrations of the selected carbon sources on cellulase production. Furthermore, the effect of different carbon sources on the growth of the different strains is reported in this paper.

2.3 Materials & Methods

2.3.1. Chemicals

All the chemicals and reagents were of analytical grade, and were obtained from Sigma-Aldrich (Sigma-Aldrich Canada Ltd). Microcrystalline cellulose was obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). Paper mill sludge (obtained from St. Marys Paper Corp., Sault Ste Marie, Canada) was grounded in a blender then dried at 70 °C and kept for later use. Grounded pine sample and organosolv pretreated pine samples were kindly provided by Dr. Charles Xu (Lakehead University, Canada).

2.3.2. Strains and culture conditions

Three different strains of *T. reesei* including QM6a (wild-type, ATCC13631, kindly provided by Dr. Monika Schmoll, Vienna University of Technology, Austria), and its two hyperproducing mutants QM9414 (ATCC 26921, kindly provided by Dr. Tianhong Wang, Shandong University, China) and RUT-C30 (ATCC 56765, kindly provided by Dr. Xiaobin Yu, Jiangnan University, China) were used in

cellulase production experiments. The strains were grown and maintained on potato dextrose agar (PDA) containing 15.0 g/L starch, 20.0 g/L D-glucose, and 18.0 g/L agar [21]. Strains were grown in 250 mL flasks, on a rotary shaker (200 rpm) at 30 °C, and in a 50 mL of medium described by Mandel and Andreotti (MA) [22] with the respective carbon source at a final concentration of 1% (w/v). The media containing the respective carbon sources were autoclaved at 121 °C (15 lb psi) for 15 min.

2.3.3. Inoculum preparation

After 14 days of incubation at 30 °C, the greenish conidia were suspended in 5 mL of sterile saline solution (0.9% w/v, NaCl). The spores were separated from the mycelium by gentle filtration through 12 layers of lens paper (Fisher Scientific, Canada), and spores were counted using a Petroff-Hausser cell counter. The isolated spores were added at 1.0×10^6 (final concentration) to 250 mL flasks containing 50 mL of MA-medium with the respective carbon source at a final concentration of 1% (w/v). Three biological replicates were run for each carbon source.

2.3.4. Carbon source for cellulase production

To test the effect of different types of carbon sources on the mycelia growth and cellulase enzyme production, 14 different carbon sources (table 1) were used and incorporated into MA-medium at 1% (w/v). To examine total cellulase activity, a time course trial was conducted similar to that of Cianchetta et al (2010) [23]. Specifically, the flasks were incubated at 30 °C in an Innova 44 (New Brunswick Scientific, USA) incubator for a total of 120 hours. Samples of 500 µL each were taken from each of the flasks every 24 hrs in a 1300 Series A2 biosafety cabinet (Thermo Fisher Scientific, Canada) to maintain sterile conditions. These 500 µL samples were centrifuged at 13000 rpm for 5 min, and the supernatant was used as the source of enzyme [21].

2.3.5 Concentration of the carbon source

For each strain, the carbon source with the highest level of reducing sugar production was selected for further study. The concentration of this carbon source was varied from 0.25 to 2% (0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75 and 2.0%), and both the reducing sugar production (Section 2.3.7), and fungal growth (Section 2.3.6) were recorded at the time point of maximum activity (obtained from carbon source for cellulase production, Section 2.3.4).

2.3.6. Determination of fungal growth

To determine mycelia growth, agar plates were inoculated with a small piece of agar in the center of an 8-cm plate, containing MA-agar medium with the respective carbon source at a final concentration of 1% (w/v). The increase in colony diameter was monitored daily, and pictures were taken after 4 days. To measure growth in submerged cultures, dry biomass was recorded at the end of the study. Mycelia were harvested after the experiment was completed —usually 120 hrs—and washing extensively with distilled water. Then they were dried to constant weight in a 70 °C oven. This procedure was carried out for all three biological replicates.

2.3.7. Filter Paper Assay (FPA) and determination of reducing sugar content

A microplate based filter paper assay, similar to the one described by Xiao et al (2004), was carried out to measure the total cellulase activity [24]. The method is 25-fold scale down of the IUPAC protocol for FPA assay [25, 26]. In short, 20 µL of the undiluted cell free culture supernatant was added to 40 µL of 75 mM citrate buffer for a final volume of 60 µL. As noted by Cianchetta et al. (2010), the 75 mM buffer was diluted to the desired 50 mM by the enzyme solution [23]. The final reaction volume, citrate buffer concentration and pH were 60 µL, 50 mM and 4.8, respectively. Each of these samples was placed in a well containing a 6 mm diameter filter paper disk (Whatman No. 1, with average weight of 3.0 mg each, ThermoFisher Scientific, Canada) cut using a standard office hole punch. Reagent blank containing 60 µL of 50 mM citrate buffer and substrate control containing only the filter paper, and 60 µL

of 50 mM citrate buffer were also run [27]. To exclude the background of reducing sugars found in the enzyme supernatant from the results, a negative control was run with no filter paper. The absorbance of the no filter paper sets and substrate control were subtracted from the absorbance of the activity assay. A glucose standard curve with a range of 0 to 2 mg/mL was run once, and two standards at 0 and 1.5 mg/mL were run in all subsequent trials. All of the samples and the standards were run in triplicate, while the blanks were run in duplicate.

The microplates were sealed with paraffin wax, covered in a Ziploc bag and incubated at 50 °C in water bath for 60 min. To measure the released reducing sugar, 120 μL of 3,5-dinitrosalicylic acid (DNS reagent) was added, and the plate was resealed with paraffin [28]. Boiling in water bath for 5 min developed the color. A 36 μL aliquot was transferred to a new 96-well flat-bottom microplate containing 160 μL of distilled water, and the absorbance at 540 nm was measured using an xMark Microplate Spectrophotometer (Bio-Rad, Canada). Total reducing sugars generated during the assay was estimated as glucose equivalents. To calculate glucose equivalents, the absorbance of the samples was converted into a concentration using the standard curve, once the negative control and the reagent blank were subtracted. This concentration was converted into glucose equivalents using the assay volume and the molar mass of glucose.

2.3.8. Data processing and statistical analysis

All experimental points are the average values of three independent experiments. The data were collected in a Microsoft Excel spreadsheet where the average and standard error of the mean were determined. A two-way analysis of variance (two-way ANOVA) at a confidence level of 95% ($\alpha= 0.05$) was carried out with the software PRISM 5 to test the statistical significance of differences between the growth rates of the three different *T. reesei* strains on each of the carbon sources. A Bonferroni multiple comparisons post hoc test was conducted, also at a 95% confidence level.

2.4 Results and discussion

2.4.1. Effect of carbon sources on the growth and total cellulase activity of T. reesei strains

In the current study three different *T. reesei* strains including QM6a, QM9414 and RUT-C30 were selected to study the effect of carbon sources on cellulases production. Druzhinina et al. (2006) previously examined carbon source utilization of *T. reesei* QM6a, and some of its cellulase-over producing mutants including QM9414 [29]. In this study, carbon sources were divided into two main groups based on their solubility in water: soluble carbon sources and insoluble carbon sources (the latter further divided into two subgroups: pure polysaccharides and lignocelluloses (Table 1) [30]. By measuring the FPA every 24 hrs, the effect of incubation time on cellulolytic enzyme production by the *T. reesei* strains was examined. The effect of each carbon source on the growth of each strain was determined by harvesting the culture, and measuring the dry weight at the end of each experiment.

It was found that the biological variation among the replicated fungal cultures was the main source of the variation observed in FPA studies (Sections 2.4.2. to 2.4.4.) This is in accordance with the other experiments involving *T. reesei* strains in shake flasks or solid fermentation [23, 31]. Three to four days was chosen by other investigators as the end point for shake flask experiments since at this time the enzyme production reached a plateau phase [23, 32]. This was only the case for RUT-C30, which reached its maximum activity earlier than QM9414 and QM6a. Thus, 5 days was defined as the end point for our shake flask experiment.

2.4.2. Soluble carbon sources

The three *T. reesei* strains were grown on D-glucose, D-xylose, D-lactose, cellobiose, malt extract, carboxymethyl cellulose (CMC) and potato dextrose in submerged culture in shake flasks and on agar plates. Growth rate of the strains were determined by measuring the dry weight (Fig. 1A). The carbon source was found to cause a significant variation in the growth rate of the strains (ANOVA, $P < 0.05$). All three strains grew less effectively on MA-medium and CMC, as compared to the other soluble

carbon sources. The slow growth on MA-medium, suggests that the pure MA-medium contributed very little to the growth of the fungi. The mutant strains QM9414 and RUT-C30 grew significantly slower on malt extract compared to the wild-type QM6a ($P < 0.001$). RUT-C30 grew significantly faster than QM6a and QM9414 on potato dextrose ($P < 0.05$). The strains were also grown on the same carbon source on agar plates to visualize the growth (Fig. 1B). The similarities between the relative sizes of the agar culture, and the relative dry weight of the mycelium in the liquid culture, suggest that the effect of carbon source on the growth of the fungi is independent from the state of the medium.

The effect of the soluble carbon sources on cellulolytic enzymes production by the fungi was determined using a FPA of the liquid culture supernatants (Fig. 1C-E). For QM6a strain, the highest total cellulase activity was obtained at 120 hrs using D-lactose as the carbon source with about 0.019 µmol glucose equivalents. This level of activity was significantly larger than the activity induced by any of the other soluble sugars (Fig. 1C). For the two mutant strains including QM9414 and RUT-C30, the highest total cellulase activity was also obtained using D-lactose at 120 or 72 hrs with about 0.055 and 0.14 µmol glucose equivalents, respectively (Fig. 1D and E). Thus, total cellulase activity of QM9414 and RUT-C30 using D-lactose was, approximately, 3-fold and 7-fold higher than total cellulase activity of the wild-type QM6a, respectively. For QM9414, CMC stimulated high cellulase production at 120 hrs, while cellubiose, D-xylose, and malt extract exhibited a lower activity. D-glucose was found to be intermediate between these two groupings (Fig. 1D). In the case of RUT-C30, cellulase production occurred more quickly than in other strains (Fig. 1E). Levels of activity at 48 hrs were found to be within the range of the maximum activity of QM9414, and greater than the activity of QM6a, suggesting that RUT-C30 favours cellulase production.

The background of reducing sugars present in the medium disappeared at different time points for the tested carbon sources. This could be monitored by the amount of the reducing sugars in the no filter paper control. D-xylose and potato dextrose were completely exhausted between 48 to 96 hrs by all the three strains. D-glucose was slowly exhausted from the medium by QM6a and RUT-C30 strains over 120

hrs, and was quickly exhausted by QM9414 after 48 hrs. D-lactose was also slowly exhausted from the medium over 120 hrs by the all three strains. Malt extract and cellobiose were completely exhausted between 48 to 96 hrs by QM6a and QM9414; however, high background sugars were obtained at every time point for RUT-C30. CMC was not exhausted, and the background sugar level stayed the same low level during the experiment which confirmed overall lower *T. reesei* growth rate using the sugar (Fig. 1A and B).

2.4.3. Insoluble, pure polysaccharide carbon sources

The three *T. reesei* strains were grown on xylan (oat spelt), cellulose powder (cotton linters) and two brands of microcrystalline cellulose including microcrystalline cellulose (J.T. Baker) and Avicel PH-101(Sigma Aldrich) in submerged culture in shake flasks and on agar plates. Growth rate of the strains were determined by measuring the dry weight (Fig. 2A). The carbon source and the strain used were both found to have a significant effect on the growth rate of the mycelium (ANOVA, $P< 0.05$). All the three strains grew more effectively on cellulose powder (cotton linters), microcrystalline cellulose and Avicel, and less effectively on xylan. The mutant strain QM9414 grew significantly faster on microcrystalline cellulose compared to the wild-type QM6a and RUT-C30 (ANOVA, $P< 0.0001$). QM6a also produced significantly more biomass than RUT-C30, when grown on microcrystalline cellulose ($P< 0.05$). This is the only case where the growth rate of all three strains was found to be significantly different, and when compared with the high enzyme production of QM9414 on microcrystalline cellulose, this tentatively suggests that microcrystalline cellulose both stimulates the appropriate inducers of cellulase production and provides an effective source of metabolic substrates for QM9414. The strains were also grown on the same carbon source on agar plates to visualize the growth (Fig. 2B).

The effect of the insoluble pure polysaccharide carbon sources on cellulolytic enzymes production by the fungi was determined using a FPA of the liquid culture supernatants (Fig. 2C-E). For QM6a strain, the highest total cellulase activity was obtained at 96 hrs using Avicel and followed by xylan as the carbon source with about 0.033 and 0.031 µmol glucose equivalents (Fig. 2C). For the two

mutant strains including QM9414 and RUT-C30, the highest total cellulase activity was obtained using microcrystalline cellulose and Avicel at 120 or 72 hrs with about 0.41 and 0.31 μmol glucose equivalents, respectively (Fig. 2D and E). Thus, total cellulase activity of QM9414 and RUT-C30 using microcrystalline cellulose or Avicel was, approximately, 12-fold and 9.5-fold higher than total cellulase activity of the wild-type QM6a, respectively. In the case of RUT-C30, cellulase production using the tested carbon sources occurred more quickly than in other strains and reached the peak (around 72 or 96 hrs) and (Fig. 2E). Similar to QM6a, activity of RUT-C30 was decreased at 120 hrs using most of the tested insoluble carbon sources (Fig. 2C and E) whereas QM9414 reached its maximum activity at 120 hrs (Fig. 2D). Except for one type of microcrystalline cellulose, the cellulase activity of RUT-C30 was higher than QM9414 among the tested insoluble pure polysaccharide carbon sources (Fig. 2D and E). For the most of the insoluble carbon sources tested here the background of reducing sugars present in the medium was not significant. The exception was xylan, which was exhausted after 48.

2.4.4. Insoluble, lignocellulosic carbon sources

The three *T. reesei* strains were grown on pine sample, organosolv pretreated pine sample and paper-mill sludge in submerged culture in shake flasks and on agar plates (Fig. 3A-E). The dry weight of the mycelium indicates the growth rate of the fungi, and both the carbon source and the strain were found to lead to a significant difference in this rate (ANOVA, $P < 0.05$) (Fig. 3A). All the three strains grew less effectively on all the lignocellulosic carbon sources compared to the other tested carbon sources (soluble and insoluble pure polysaccharides, Fig. 1A and Fig. 2A). Other studies have found that pre-treated lignocelluloses do not stimulate microbial growth or enzyme production [21]. In the case of the organosolv pretreated pine sample used in this study, the low activity and fungal growth may be as a result of the inhibitory effects of compounds generated during the pretreatment or the presence of the solvents used for the pretreatment [33]. Also, paper-mill sludge generated by the industrial sources contains a large number of ingredients, some of which are toxic for microbial growth [34]. The lack of

microbial growth and enzyme production was also observed using untreated pine sample (Fig 3A-E). This may have been a result of the enzymes inability to access the celluloses found within the wood chips, because of the presence of lignin [35].

The effect of the insoluble lignocellulosic carbon sources on cellulolytic enzymes production by the fungi was determined using a FPA of the liquid culture supernatants (Fig. 3C-E). For QM6a strain, no significant cellulase activity was obtained using all the three different lignocellulosic carbon sources (Fig. 3C). For the two mutant strains including QM9414 and RUT-C30, the highest total cellulase activity was obtained using organosolv pretreated pine sample at 120 hrs with about 0.04 and 0.07 µmol glucose equivalents, respectively (Fig. 3D and E). The greater activity of RUT-C30, when considered in conjunction with the similarity in dry weight between the two strains, indicates that RUT-C30 is more adept at producing cellulases under these conditions. No reducing sugar background was observed in the medium supplemented with the lignocellulosic carbon sources. Figure 4 summarizes cellulase production by the three fungal strains using all 14 different carbon sources as well as MA-medium (as the control) used in this study.

2.4.5. Concentration of carbon source

Based on the carbon source experiments (Fig. 1, 2 and 3), Avicel showed the highest activity for QM6a and RUT-C30, whereas microcrystalline cellulose showed the highest cellulase activity for QM9414. To identify the optimum concentration of carbon source for each *T. reesei* strain, various concentrations (0.25 to 2%) of Avicel or microcrystalline cellulose were used (Fig. 5A-C). The highest cellulase activity for QM6a was obtained using 1% Avicel with all concentrations above this exhibiting repressed cellulase (Fig. 5A). *T. reesei* QM9414 strain showed the highest cellulase activity at about 1.25% microcrystalline cellulose, but stayed almost the same level when the carbon concentration increased up to 2% (Fig 5B). In the case of RUT-C30, cellulase activity using Avicel was gradually increased starting at 0.5%, peaked at about 1.5%, and then decreased when the carbon concentration

increased up to 2% (Fig. 5C). Growth rate of the strains using the selected carbon sources were also determined by measuring the dry weight (Fig. 5A-C). All the three strains showed the same trend with gradually increasing the dry weight when the concentrations of the carbon sources were increased. The maximum dry weight for all the three *T. reesei* strains was obtained at the highest carbon concentration level (2%). This suggests that an increase in enzyme production is directly caused by an increase in mycelium density, and may be a result of complex inducing factors.

2.5 Conclusion

Although the cellulase activity of the *T. reesei* mutant strains QM9414 and RUT-C30 were appreciably higher than the wild-type QM6a, the carbon source utilization profiles of these strains closely resembled each other. For all the three *T. reesei* strains maximum cellulase production was observed using two different types of microcrystalline celluloses. The highest cellulase activity for QM6a was obtained using 1% Avicel while RUT-C30 showed the peak at about 1.5%. QM9414 strain showed the highest cellulase activity at about 1.25% microcrystalline cellulose. All the three strains exhibited the same gradual increase in the dry weight of the mycelium, as the concentrations of carbon sources were elevated. This consistency in mycelium growth suggests that the enzyme production is not directly correlated with the growth of the mycelium, and may be a result of complex inducing factors.

Acknowledgements

Table 1. List of 14 different carbon sources tested for their effect on cellulase production. MA-medium without carbon source was used as the control.

Type	Carbon Source (1%, w/v)	Abbreviation
Soluble	D-Glucose	Glc
	D-Xylose	
	D-Lactose	Xyl
	Cellobiose	
	Malt extract	Lac
	Carboxymethyl cellulose	CB
	Potato dextrose [a]	ME
		CMC
		PD
Insoluble		
Pure polysaccharides	Xylan (oat spelt)	Xylan
	Cellulose powder (cotton linters)	
	Microcrystalline cellulose	CP
	Microcrystalline cellulose (J.T. Baker)	
	Avicel PH-101(Sigma Aldrich)	
		MCC
		Avi
Lignocelluloses	Pine sample	PS
	Organosolv pretreated pine sample	
	Paper-mill sludge	OrS
		Slu

[a] Becomes soluble in water when heated.

109

Figure 1. Effect of the different soluble carbon sources on growth rate and total cellulase activity of the three *T. reesei* strains. (A) Dry weight of the *T. reesei* strains cultured for 5 days at 30 °C. Letters denote a significant difference between the growth rate of the strains for each carbon source (P< 0.05). Carbon sources that exhibited no significant difference between any of the strains do not have letters; (B) growth comparison of the *T. reesei* mutant strains QM9414 and RUT-C30 to the wild-type strain QM6a on the different soluble carbon sources after 4 days; (C) total cellulase activity of *T. reesei* QM6a; (D) QM9414 and (E) RUT-C30 grown on MA-medium supplemented with 1% of the different soluble carbon sources for 24-120 hrs. Error bars denote standard error of the mean.

Figure 2. Effect of the different insoluble pure polysaccharide carbon sources on growth rate and total cellulase activity of the three *T. reesei* strains. (A) Dry weight of the *T. reesei* strains cultured for 5 days at 30 °C. Letters denote a significant difference between the growth rate of the strains for each carbon source ($P < 0.05$). Carbon sources that exhibited no significant difference between any of the strains do not have letters; (B) growth comparison of the *T. reesei* mutant strains QM9414 and RUT-C30 to the wild-type strain QM6a on the different insoluble pure polysaccharide carbon sources after 4 days; (C) total cellulase activity of *T. reesei* QM6a; (D) QM9414 and (E) RUT-C30 grown on MA-medium supplemented with 1% of the different insoluble pure polysaccharide carbon sources for 24-120 hrs. Error bars denote standard error of the mean.
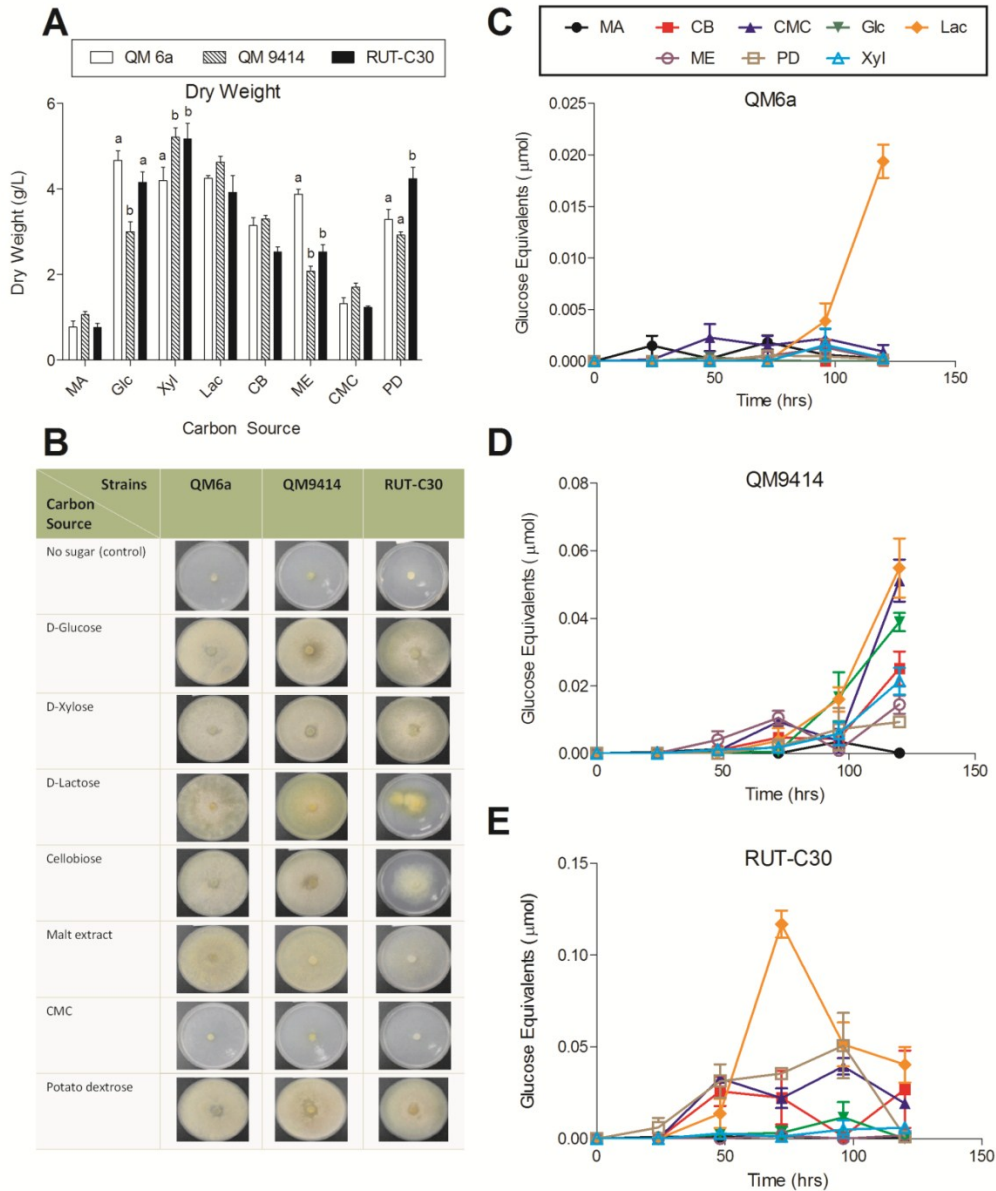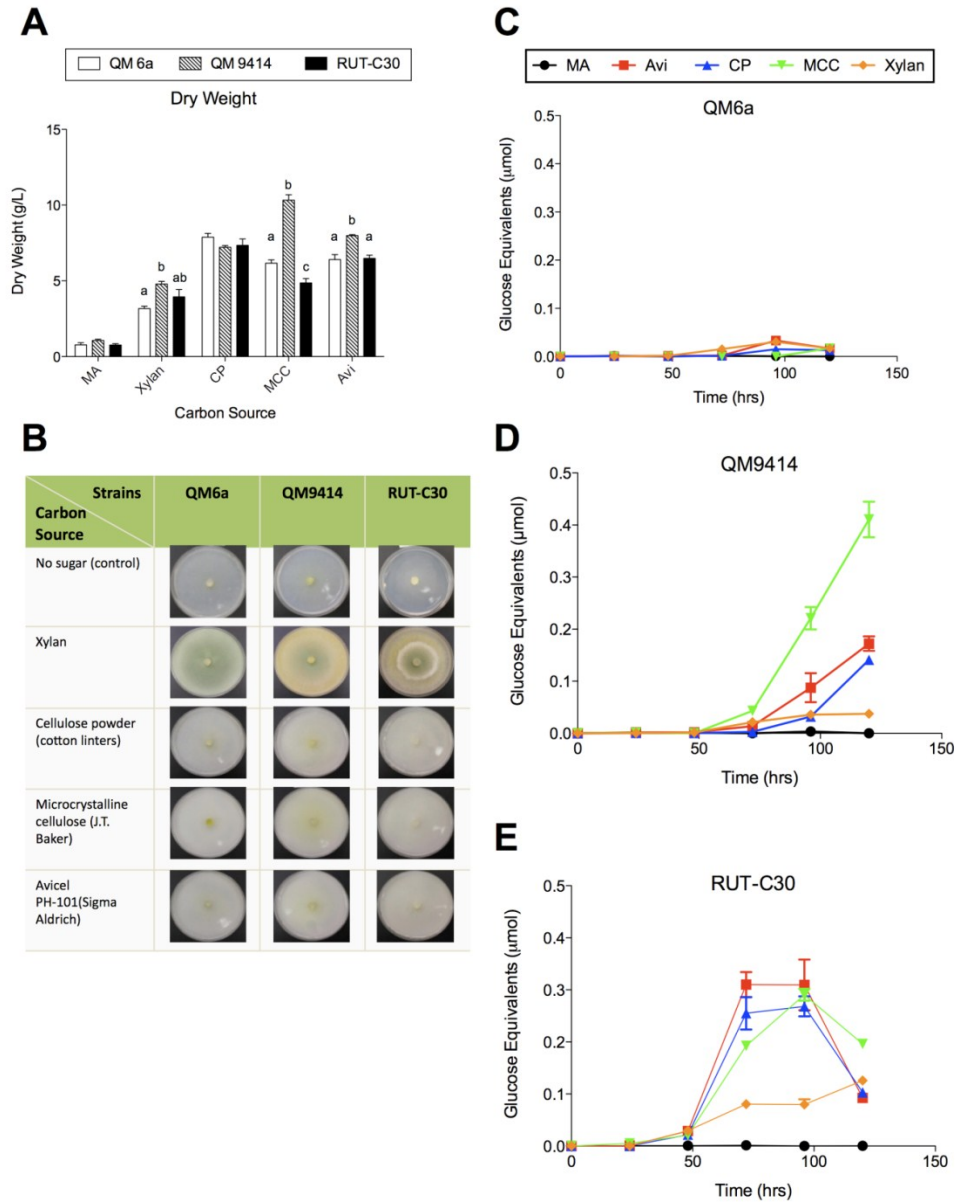
111

Figure 3. Effect of the different insoluble lignocellulosic carbon sources on growth rate and total cellulase activity of the three *T. reesei* strains. (A) Dry weight of the *T. reesei* strains cultured for 5 days at 30 °C. Letters denote a significant difference between the growth rate of the strains for each carbon source ($P< 0.05$). Carbon sources that exhibited no significant difference between any of the strains do not have letters; (B) growth comparison of the *T. reesei* mutant strains QM9414 and RUT-C30 to the wild-type strain QM6a on the different insoluble lignocellulosic carbon sources after 4 days; (C) total cellulase activity of *T. reesei* QM6a; (D) QM9414 and (E) RUT-C30 grown on MA-medium supplemented with 1% of the different insoluble lignocellulosic carbon sources for 24-120 hrs. Error bars denote standard error of the mean.

Figure 4. An outline of cellulase activity profiles of the wild-type QM6a strain of *T. reesei*, and its two hypercellulolytic mutants, QM9414 and RUT-C30 on 14 different carbon sources. The colour scale represents the magnitude of the maximum cellulase activity (µmol glucose equivalents) obtained for the strain when grown on each particular carbon source. The legend contains intermediate values within each colour range.
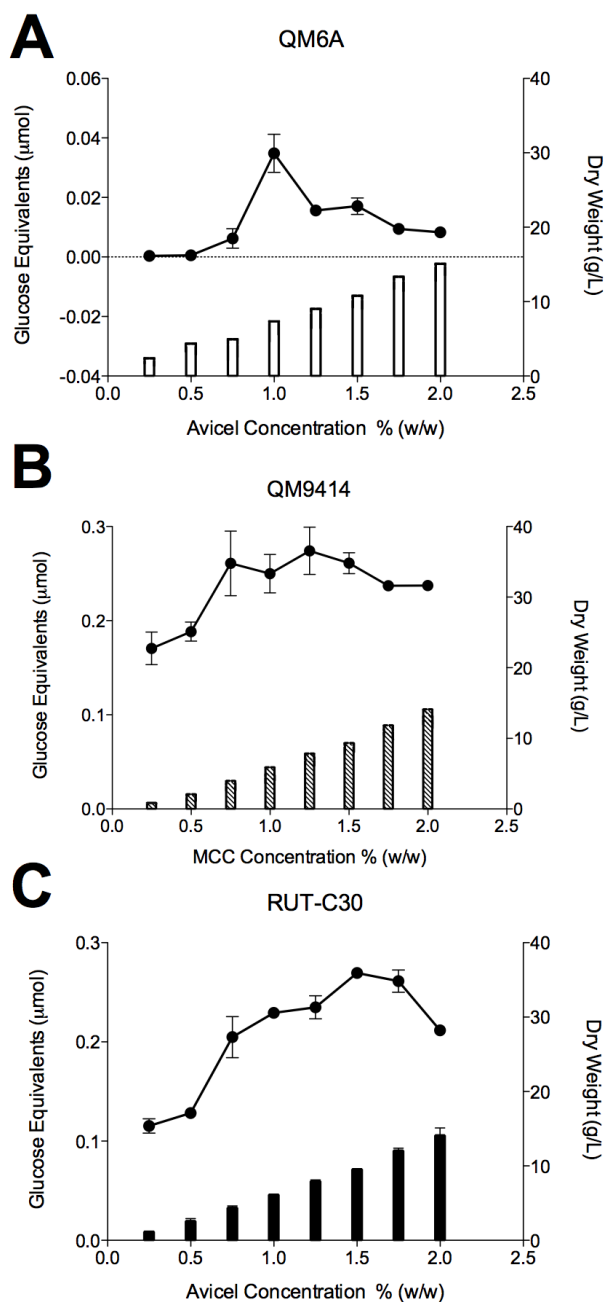
Figure 5. Effect of the selected carbon source concentration (0.25 to 2%) on the growth rate and total cellulase activity of the three *T. reesei* strains (A) QM6a; (B) QM9414 and (C) RUT-C30. *T. reesei* strains QM6a and RUT-C30 were grown on MA-medium supplemented with the different concentration of Avicel for 5 or 3 days, respectively. *T. reesei* strain QM9414 was grown on MA-medium supplemented with the different concentration of microcrystalline cellulose for 4 days. Bars represent dry weight plotted on the right axis, while lines represent glucose equivalents plotted on the left axis. Error bars denote standard error of the mean.

114

References

[1]   Dashtban M, Schraft H and Qin W. Fungal bioconversion of lignocellulosic residues; opportunities & perspectives. Int J Biol Sci 2009; 5: 578-595.

[2]   Reese ET. History of the cellulase program at the US army Natick Development Center. Biotechnol. Bioeng. Symp. 1976; 6:

[3]   Eveleigh DE and Montenecourt BS. Increasing yields of extracellular enzymes. Adv Appl Microbiol 1979; 25: 57-74.

[4]   Durand H, Clanet M and Tiraby G. Genetic improvement of Trichoderma reesei for large scale cellulase production. Enzyme Microbiol 1988; 10:

[5]   Seidl V, Gamauf C, Druzhinina IS, Seiboth B, Hartl L and Kubicek CP. The Hypocrea jecorina (Trichoderma reesei) hypercellulolytic mutant RUT C30 lacks a 85 kb (29 gene-encoding) region of the wild-type genome. BMC Genomics 2008; 9: 327.

[6]   Seidl V and Seiboth B. Trichoderma reesei: genetic approaches to improving strain efficiency. Biofuels 2010; 1(2):343-354.

[7]   Le Crom S, Schackwitz W, Pennacchio L, Magnuson JK, Culley DE, Collett JR, Martin J, Druzhinina IS, Mathis H, Monot F, Seiboth B, Cherry B, Rey M, Berka R, Kubicek CP, Baker SE and Margeot A. Tracking the roots of cellulase hyperproduction by the fungus Trichoderma reesei using massively parallel DNA sequencing. Proc Natl Acad Sci U S A 2009; 106: 16151-16156.

[8]   Ilmen M, Thrane C and Penttila M. The glucose repressor gene cre1 of Trichoderma: isolation and expression of a full-length and a truncated mutant form. Mol Gen Genet 1996; 251: 451-460.

[9]   Geysens S, Pakula T, Uusitalo J, Dewerte I, Penttila M and Contreras R. Cloning and characterization of the glucosidase II alpha subunit gene of Trichoderma reesei: a frameshift mutation results in the aberrant glycosylation profile of the hypercellulolytic strain Rut-C30. Appl Environ Microbiol 2005; 71: 2910-2924.

[10]   Vitikainen M, Arvas M, Pakula T, Oja M, Penttila M and Saloheimo M. Array comparative genomic hybridization analysis of Trichoderma reesei strains with enhanced cellulase production properties. BMC Genomics 2010; 11: 441.

[11]   Morawetz R, Gruber F, Messner R and P. KC. Presence, transcription and translation of cellobiohydrolase genes in several Trichoderma species. Curr Genct 1992; 21:

[12]   Mantyla AL, Rossi KH, Vanhanen SA, Penttila ME, Suominen PL and Nevalainen KM. Electrophoretic karyotyping of wild-type and mutant Trichoderma longibrachiatum (reesei) strains. Curr Genet 1992; 21: 471-477.

[13]   Martinez D, Berka RM, Henrissat B, Saloheimo M, Arvas M, Baker SE, Chapman J, Chertkov O, Coutinho PM, Cullen D, Danchin EG, Grigoriev IV, Harris P, Jackson M, Kubicek CP, Han CS, Ho I, Larrondo LF, de Leon AL, Magnuson JK, Merino S, Misra M, Nelson B, Putnam N, Robbertse B, Salamov AA, Schmoll M, Terry A, Thayer N, Westerholm-Parvinen A, Schoch CL,

Yao J, Barabote R, Nelson MA, Detter C, Bruce D, Kuske CR, Xie G, Richardson P, Rokhsar DS, Lucas SM, Rubin EM, Dunn-Coleman N, Ward M and Brettin TS. Genome sequencing and analysis of the biomass-degrading fungus Trichoderma reesei (syn. Hypocrea jecorina). Nat Biotechnol 2008; 26: 553-560.

[14] Barnett CC, Berka RM and Fowler T. Cloning and amplification of the gene encoding an extracellular beta-glucosidase from Trichoderma reesei: evidence for improved rates of saccharification of cellulosic substrates. Biotechnology (N Y) 1991; 9: 562-567.

[15] Takashima S, Nakamura A, Hidaka M, Masaki H and Uozumi T. Molecular cloning and expression of the novel fungal beta-glucosidase genes from Humicola grisea and Trichoderma reesei. J Biochem 1999; 125: 728-736.

[16] Foreman PK, Brown D, Dankmeyer L, Dean R, Diener S, Dunn-Coleman NS, Goedegebuur F, Houfek TD, England GJ, Kelley AS, Meerman HJ, Mitchell T, Mitchinson C, Olivares HA, Teunissen PJ, Yao J and Ward M. Transcriptional regulation of biomass-degrading enzymes in the filamentous fungus Trichoderma reesei. J Biol Chem 2003; 278: 31988-31997.

[17] Nogawa M, Goto M, Okada H and Morikawa Y. L-Sorbose induces cellulase gene transcription in the cellulolytic fungus Trichoderma reesei. Curr Genet 2001; 38: 329-334.

[18] Kubicek CP, Mikus M, Schuster A, Schmoll M and Seiboth B. Metabolic engineering strategies for the improvement of cellulase production by Hypocrea jecorina. Biotechnol Biofuels 2009; 2: 19.

[19] Tangnu SK, Blanch HW and Wilke CR. Enhanced Production of Cellulase, Hemicellulase, and β-Glucosidase by *Trichoderma reesei* (Rut C-30). Biotechnology and Bioengineering 1981; XXIII: 1837-1849.

[20] Ghosh A, Ghosh BK, Trimino-Vazquez H, Eveleigh DE and Montenecourt BS. Cellulase secretion from a hyper-cellulolytic mutant of Trichoderma reesei Rut-C30. Archives of Microbiology 1984; 140: 126-133.

[21] Benko Z, Drahos E, Szengyel Z, Puranen T, Vehmaanpera J and Reczey K. Thermoascus aurantiacus CBHI/Cel7A production in Trichoderma reesei on alternative carbon sources. Appl Biochem Biotechnol 2007; 137-140: 195-204.

[22] Mandels MM and Andreotti RE. The cellulose to cellulase fermentation. Proc. Biochem 1978; 13: 6-13.

[23] Cianchetta S, Galletti S, Burzi PL and Cerato C. A novel microplate-based screening strategy to assess the cellulolytic potential of Trichoderma strains. Biotechnol Bioeng 2010; 107: 461-468.

[24] Xiao Z, Storms R and Tsang A. Microplate-based filter paper assay to measure total cellulase activity. Biotechnol Bioeng 2004; 88: 832-837.

[25] Ghose TK. Measurement of cellulase activities. Pure Appl Chem 1987; 59: 257-268.

[26] Dashtban M, Maki M, Leung KT, Mao C and Qin W. Cellulase activities in biomass conversion: measurement methods and comparison. Crit Rev Biotechnol 2010; 30: 302-309.

[27]   Zhang Y-HP, Hong J and Ye X. Cellulase assays. Methods Mol Biol 2009; 581: 213-231.

[28]   Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 1959; 31: 426-428.

[29]   Druzhinina IS, Schmoll M, Seiboth B and Kubicek CP. Global carbon utilization profiles of wild-type, mutant, and transformant strains of Hypocrea jecorina. Appl Environ Microbiol 2006; 72: 2126-2133.

[30]   Zhang Y-HP, Himmel ME and Mielenz JR. Outlook for cellulase improvement: screening and selection strategies. Biotechnol Adv 2006; 24: 452-481.

[31]   King BC, Donnelly MK, Bergstrom GC, Walker LP and Gibson DM. An optimized microplate assay system for quantitative evaluation of plant cell wall-degrading enzyme activity of fungal culture extracts. Biotechnol Bioeng 2009; 102: 1033-1044.

[32]   Kovács K, Megyeri L, Szakacsa G, Kubicekc CP, Galbeb M and Zacchi G. Trichoderma atroviride mutants with enhanced production of cellulase and β-glucosidase on pretreated willow. Enzyme and Microbial Technology 2008; 43 48–55.

[33]   Zhao X, Cheng K and Liu D. Organosolv pretreatment of lignocellulosic biomass for enzymatic hydrolysis. Appl Microbiol Biotechnol 2009; 82: 815-827.

[34]   Diez MC, Castillo G, Aguilar L, Vidal G and Mora ML. Operational factors and nutrient effects on activated sludge treatment of Pinus radiata kraft mill wastewater. Bioresour Technol 2002; 83: 131-138.

[35]   Dashtban M, Schraft H, Syed T. A and Qin W. Fungal biodegradation and enzymatic modification of lignin. Int J Biochem Mol Biol 2010; 1: 36-50.

Chapter 3: Overexpression of an exotic thermotolerant β-glucosidase in *Trichoderma reesei* and its significant increase in cellulolytic activity and saccharification of barley straw

Adapted from: Mehdi Dashtban[1,2] and Wensheng Qin*[1,2]

[1] Biorefining Research Institute
Lakehead University
955 Oliver Road
Thunder Bay, ON P7B 5E1 Canada

[2] Department of Biology
Lakehead University
955 Oliver Road
Thunder Bay, ON P7B 5E1 Canada

*Corresponding author: Wensheng Qin

3.1 Abstract

Background: *Trichoderma reesei* is a widely used industrial strain for cellulase production, but its low yield of β-glucosidase has prevented its industrial value. In the hydrolysis process of cellulolytic residues by *T. reesei*, a disaccharide known as cellobiose is produced and accumulates, which inhibits further cellulases production. This problem can be solved by adding β-glucosidase, which hydrolyzes cellobiose to glucose for fermentation. It is, therefore, of high value to construct *T. reesei* strains which can produce sufficient β-glucosidase and other hydrolytic enzymes, especially when those enzymes are capable of tolerating extreme conditions such as high temperature and acidic or alkali pH.

Results: We successfully engineered a thermostable β-glucosidase gene from the fungus *Periconia sp.* into the genome of *T. reesei* QM9414 strain. The engineered *T. reesei* strain showed about 10.5-fold (23.9 IU/mg) higher β-glucosidase activity compared to the parent strain (2.2 IU/mg) after 24 h of incubation. The transformants also showed very high total cellulase activity (about 39.0 FPU/mg) at 24 h of incubation whereas the parent strain almost did not show any total cellulase activity at 24 h of incubation. The recombinant β-glucosidase showed to be thermotolerant and remains fully active after two-hour incubation at temperatures as high as 60 °C. Additionally, it showed to be active at a wide pH range and maintains about 88% of its maximal activity after four-hour incubation at 25 °C in a pH range from 3.0 to 9.0. Enzymatic hydrolysis assay using untreated, NaOH, or Organosolv pretreated barley straw as well as microcrystalline cellulose showed that the transformed *T. reesei* strains released more reducing sugars compared to the parental strain.

Conclusions: The recombinant *T. reesei* overexpressing *Periconia sp.* β-glucosidase in this study showed higher β-glucosidase and total cellulase activities within a shorter incubation time (24 h) as well as higher hydrolysis activity using biomass residues. These features suggest that the transformants can be used for β-glucosidase production as well as improving the biomass conversion using cellulases.

Keywords: *Trichoderma reesei*, genetic engineering, β-glucosidase, *Periconia sp.*

3.2 Introduction

Lignocellulose, a renewable organic material, is the major structural component of plants. It is primarily composed of three major components: cellulose, hemicellulose, and lignin [1]. Large quantities of lignocellulosic wastes produced by different industries, such as the paper-making industry, are released to the environment on a daily basis causing a variety of environmental issues. Bioconversion of biomass is a promising solution to overcome some of the environmental issues associated with the lignocellulosic wastes as well as providing alternative energy resources such as bioethanol. Different microorganisms such as fungi and bacteria primarily initiate the bioconversion of lignocellulosic residues through a process known as hydrolysis. Fungi, however, have received the greatest interest due to their production of high quantities of extracellular cellulolytic enzymes. However, the disadvantage of the fungal system is that many natural fungal strains lack of some of lignocellulolytic enzymes necessary for efficient bioconversion processes [1, 2]. Thus, the initial bioconversion of biomass into sugars remains a key bottleneck in the process of biofuel production. To this end new biotechnological solutions, such as genetic engineering of microorganisms, to improve lignocellulolytic enzymes efficiencies and enzymes able to tolerate harsh conditions are necessary [2].

The ascomycete *Hypocrea jecorina* (anamorph *Trichoderma reesei*) is one of the most studied and industrially important cellulolytic fungi. *T. reesei* is capable of efficiently degrading plant cell wall polysaccharides such as cellulose and hemicelluloses. *T. reesei* also produces a number of cellulases including cellobiohydrolases (exoglucanases) and endoglucanases as well as a set of hemicellulases and pectin degrading enzymes [2, 3]. Additionally, a few different β-glucosidases (BGL) have been identified in *T. reesei*. These three groups of cellulolytic enzymes, i.e., exoglucanases, endoglucanases and β-glucosidases work efficiently on cellulolytic fibers in a synergistic manner. These β-glucosidases were reported to be extracellular [4], cell wall-bound [5], membrane-bound [6], and intracellular [7]. Most of *T. reesei* cellulases are inducible enzymes and their transcripts are not formed in the presence of monosaccharides in the growth medium. This means that an inducer is required in order for *T. reesei* to

produce cellulases [3]. Cellulose polymer acts as a natural inducer despite the fact that it cannot transfer through the cell membrane due to its insolubility. Different investigations into the cellulases gene regulation in *T. reesei* have resulted in different proposed models, however, they all tend to agree that the actions of cellulases lead to the formation of a cellulase inducer [3]. The leading candidate for control of cellulolytic enzymes production in *T. reesei* is β-glucosidase, which is responsible for the production of an inducer, sophorose [3]. This was supported by Mach (1995), who disrupted the gene *cel3a* (*bgl1*) encoding the major extracellular β-glucosidases in *T. reesei* and demonstrated a delay in the induction of the other cellulases genes only by cellulose, but not in the presence of sophorose [3, 8]. Thus, β-glucosidase production remains a key bottleneck in the process of cellulase production by *T. reesei*. Efforts have been made to improve cellulases production in *T. reesei* by homologues or heterologous overexpression of β-glucosidase genes [9-11]. Additionally, β-glucosidase production is important for its potential applications in many different industries such as food [12], winemaking [13] and textile [14].

An endophytic fungus *Periconia sp.* belonging to phylum Ascomycota was selected among 100 studied fungal strains by Harnpicharnchai et al. (2009) due to its highest β-glucosidase activity at elevated temperatures [15]. They ultimately identified the gene encoding β-glucosidase (*bgl1*), cloned and expressed it in *Pichia pastoris*. The purified protein was reported to have high β-glucosidase activity at higher temperatures, and was also active at a wide pH range [15]. Thermostable β-glucosidases with high enzyme activity are especially useful for the bioconversion of lignocellulosic residues at elevated temperature [1].

In the current study, we overexpressed the β-glucosidase gene (*bgl1*) from *Periconia sp.* in *T. reesei* QM9414 under the control of a promoter region of *T. reesei tef1α* (encoding translation elongation factor 1-alpha). The β-glucosidase production and total cellulase activity of the BGLI-overexpressing transformants were significantly increased. Additionally, hydrolysis efficiency of the BGLI-overexpressing transformants was also significantly increased using NaOH- or Organosolv-pretreated barley straw.

3.3 Materials and Methods

3.3.1. Chemicals

All the chemicals and reagents were of analytical grade. Microcrystalline cellulose was obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). Barley straw (obtained from Gammondale Farm, Thunder Bay, Canada) was grounded in a Wiley mill and then sieved to less than 20 mesh and dried in an oven to a constant weight at 70 °C before use.

3.3.2. Pretreatment of barley straw

Organosolv pretreatment of barley straw was done (in Dr. Charles Xu's lab, Lakehead University, Canada) using a 1 L autoclave reactor (Autoclave Engineers, U.S.A.) [24]. Briefly, 50 g of previously grounded barley straw was used as the feedstock. The feedstock/solvent ratio was 1:10 (w/v) and 50% ethanol: water (v/v) was used as the solvent. The pressure was maintained at about 300 psi using nitrogen, and temperature was kept at 190 °C. The reaction was mixed at 130 rpm and maintained for 4 h. Pretreated barley straw was subjected to a wash using 100% acetone. Following this, lignin was removed using filtration and cellulose and hemicellulose were obtained as solid residues. Solid residues (SR) were dried at 105 °C overnight before weighing. The gaseous product inside the reactor was collected into a pre-vacuum fixed-volume (2800 mL) gas-collecting vessel. Liquefied lignin was subjected to a rotary evaporation under reduced pressure at 40 °C to remove acetone and ethanol. The weight of the dry lignin was measured in order to estimate the lignin percentage. Yields of lignin and SR were calculated by the wt% of the mass of each product to the mass of the dry feedstock loaded into the reactor. Using this method, the lignin and SR content of barley straw were 8.77 (17.54%) and 27.46 (54.99%) g/50 g barley straw, respectively. The aqueous and gaseous products of barley straw were 13.77 (27.54%) g/50 g barley straw.

Alkali treatment was done using 2 g (2% w/v) of previously grounded barley straw according to the method described by Deshpande [25]. Briefly, the ground barley straw was subjected to alkali

treatment by soaking in NaOH solution at 2% (w/v) for 48 h at ambient temperature. The material was then washed thrice with water. The water for the fourth wash has 1% phosphoric acid added. The materials were subjected to two more washes with water and then dried to constant weight at 70 °C.

For the estimation of various components of barley straw, sequential fractionation was carried out according to the method described by Arora and Sharma (2009) [26]. Briefly, one g of barley straw was suspended in 100 mL distilled water and kept at 100 °C for 2 h in a water bath. The slurry was filtered and the residue was dried at 80 °C to constant weight. The weight loss was considered as water soluble part (Table 1). To estimate hemicellulose and cellulose in barley straw, they were removed according to the TAPPI T222 protocol with some modifications as described by Sharma and Arora (2010) [27, 28]. Dried sample was briefly treated with 100 mL of 0.5 M H_2SO_4 at 100 °C for 2 h. The content was filtered, dried, weighed and the loss considered as hemicellulose content (Table 1). Following this, the dried sample was treated with 40 mL 72% H_2SO_4 at 30 °C for 1 h at 200 rpm. Subsequently, it was diluted to 3% concentration of H_2SO_4 with distilled water and autoclaved at 121°C for 45 min. The content was filtered, dried, weighed and the loss counted as cellulose content. Acid-soluble lignin was determined by measuring the absorbance of the supernatant at 205 nm according to the TAPPI Useful Method UM 250 [29]. Finally, the acid-insoluble lignin content was measured after the weight of ash was measured by burning the samples in a muffle furnace at 525 °C, according to the TAPPI T211 protocol "Ash in wood, pulp, paper and paperboard: combustion at 525 °C" [28] (Table 1).

3.3.3. Microorganism strains and culture conditions

Escherichia coli JM109 was used for vector construction and propagation. Endophytic fungus *Periconia sp.* (BCC2871, obtained from the BIOTEC Culture Collection, Thailand) was used as the *bgl1* gene provider. Cellulase hyperproducing mutant *T. reesei* QM9414 (ATCC 26921, kindly provided by Dr. Tianhong Wang, Shandong University, China) was used as a host for the overexpression of the *bgl1* gene. The fungal strains were grown and maintained on potato dextrose agar (PDA) containing 15.0 g/L

starch, 20.0 g/L *D*-glucose, and 18.0 g/L agar [30]. PDA medium supplemented with 50 μg/mL

hygromycin was used as a selection marker for screening of *T. reesei* transformants. Strains were grown

in 250 mL flasks, on a rotary shaker (200 rpm) at 30 °C, and in 50 mL of medium described by Mandel

and Andreotti (MA-medium) [31] with the respective carbon source at a final concentration of 1% (w/v).

The media containing the respective carbon sources were autoclaved at 121 °C (15 lb psi) for 15 min.

3.3.4. Construction of bgl1 expression cassette

Periconia sp. total RNA was extracted using Ambion RNA extraction kit (Invitrogen, Canada)

and cDNA was constructed using Fermentas first strand cDNA synthesis kit (Fermentas, Canada). The

2601 bp *bgl1* gene was amplified with PCR using Full-Beta primers (Table 2) designed according to the

cDNA sequence of *Periconia sp bgl1* (Accession No. EU304547) [15]. The *T. reesei* cellobiohydrolases I

(cbh1) terminator region was used as the terminator. The 573 bp *cbh1* terminator was amplified by PCR

using *cbh1* primers (Table 2) and *T. reesei* QM9414 chromosomal DNA as the template. The *bgl1* and

cbh1 PCR products were used as the templates to fuse the *cbh1* terminator to the 3′ end of the *bgl1* gene

(to generate a *bgl1-cbh1* cassette) through fusion PCR using *bgl1-cbh1* fusion primers (Table 2). The

fused PCR product (*bgl1-cbh1* cassette) was gel extracted and used as a template for In-Fusion cloning

(In-Fusion primers, Table 2) into ClaI linearized pPtef1-hph vector (kindly provided by Dr. B. Seiboth,

Vienna University of Technology, Austria) [32] using In-Fusion® Advantage PCR Cloning Kit (Clontech

Laboratories, Inc., USA) to generate pPtef1-*bgl1-cbh1*. The plasmid carrying the *bgl1-cbh1* cassette was

transformed into *E. coli* and ampicillin was used to screen the transformants. The positive transformants

were selected and inserted *bgl1-cbh1* cassette into the plasmid was confirmed using DNA sequencing.

3.3.5. Transformation of T. reesei QM9414 and molecular analysis of the transformants

T. reesei QM9414 protoplasts were prepared according to Szewczyk [33]. The protoplasts were

then transformed with pPtef1-*bgl1-cbh1* containing hygromycin B phosphotransferase (*hph*) expression

cassette as the selection marker, according to the method described by Szewczyk [33]. The transformants

were screened on PDA plates containing 50 μg/mL hygromycin as the selection marker. Single spore separation was done to ensure a pure culture. The integration of pPtef1-*bgl1-cbh1* into the genome of *T. reesei* QM9414 was confirmed using full size *Periconia sp. bgl1* primers (Table 2), with an expected fragment length of 2.6 kb. To identify the gene copy number in the obtained positive transformants, qRT-PCR was carried out using extracted genomic DNA as the template and Real-Time primers (*bgl1* and *tef1a* Real-Time primers, Table 2) according to the method described by Solomon [34]. *Tef1α* (translation elongation factor 1-alpha) was used to represent single copy region within the *T. reesei* genome (2279 bp in scaffold 6, from 764792-767070) confirmed by blasting *tef1α* sequence [GenBank: Z23012.1] [35] against *T. reesei* genome sequence using the *T. reesei* genome database v2.0 [36]. RT-PCRs were performed using a Bio-Rad CFX^{TM} 96 Real-Time PCR Detection System with each well containing the following conditions: 10 μL Sso Fast^{TM} EvaGreen® Supermix (Bio-Rad, Canada), 5.0 μL of appropriately diluted genomic DNA, 1.0 μL of each primer (10 μM) (Table 2) and 3.0 μL of double distilled water with a total well volume of 20 μL. RT-PCR cycling was 120 seconds at 98 °C followed by 40 cycles of 5 seconds at 98 °C and 5 seconds at 58 °C. Three technical replicates were tested for each transformant to ensure consistency and accuracy. To ensure specificity of primers, melt curves were produced for each RT-PCR experiment. All primers were shown to amplify specific sequences and showed only one melting temperature on the melting curve. Serial dilutions of genomic DNA and a temperature gradient were used in RT-PCR in order to determine the efficiencies of all reactions and were found to be between 90-110% efficient. *Tef1a* Real-Time primers were used for the reference gene and data were normalized using tef1α primers.

3.3.6. Inoculum preparation and β-glucosidase production

After 14 days of incubation at 30 °C, the greenish conidia from engineered *T. reesei* carrying pPtef1-*bgl1-cbh1* were suspended in 5 mL of sterile saline solution (0.9% w/v, NaCl). The spores were separated from the mycelia by gentle filtration through 12 layers of lens paper (Fisher Scientific, Canada),

and spores were counted using a Petroff-Hausser cell counter (American Optical, USA). The isolated spores were added at 1.0×10^7 (final concentration) to 250 mL flasks containing 50 mL medium (MA-medium) with 1% glucose (w/v) as the carbon source and incubated at 30 °C for a total of 24 hours. Pre-grown mycelia were washed three times by MA-medium with no carbon source to remove any residual glucose. The mycelia were then transferred into 250 mL flasks containing 50 mL cellulase-inducing medium (MA-medium) in which 1% glucose (w/v) was substituted with 1% microcrystalline cellulose (w/v) [37]. Three biological replicates were done for each transformant. To examine β-glucosidase activity and total cellulase activity, a time course trial was conducted similar to that of Cianchetta et al [38]. Specifically, the flasks were incubated at 30 °C for a total of 144 hours. Samples of 500 µL each were taken from each of the flasks every 24 h. These 500 µL samples were centrifuged at 16060 rcf for 5 min, and the supernatant was used as the source of enzyme [30].

3.3.7. Enzyme assays

Fermentation broth was centrifuged, and aliquots of the supernatant were diluted to assay the enzyme activities. All enzyme activities were expressed as specific activities using international units per mg protein in the supernatant (one unit corresponds to the amount of enzyme required to liberate 1 µmol of product per minute under the standard assay conditions). The protein concentration in the supernatant was measured using the Fermentas Bradford Reagent and also Fermentas bovine serum albumin (BSA) standard set as the standard (Fermentas, Canada).

β-glucosidase activity was determined according to method described by Korotkova using the initial rate of the accumulation of the colored reaction product [39]. Briefly, 20 µL of diluted enzyme (culture supernatant) was added into each microplate well (pre-heated at 70 °C for 10 min) containing 180 µL of 5 mM *p*NPG in 50 mM sodium citrate buffer, pH 5.0 as the substrate. The plate was incubated at 70 °C for 10 min before stopping the reaction by adding 100 µL of 1 M cold sodium carbonate. The release of *p*-nitrophenyl by enzymatic hydrolysis was indicated by the appearance of yellow color and monitored

at 405 nm by xMark Microplate Spectrophotometer (Bio-Rad, Canada). The absorbance of the samples was normalized by the enzyme blanks (20 µL enzyme and 180 µL of the assay buffer) and the substrate blank (20 µL of the assay buffer and 180 µL of the substrate).

The optimal pH of BGLI activity was measured at pH ranging from 3.0 to 10.0 under the standard assay conditions at 70 °C for 10 min. The buffers used in the experiment were 50 mM sodium citrate (pH 3.0-6.0), 50 mM sodium acetate (pH 4.0-6.0), 50 mM MOPS (pH 6.0-8.0), and 50 mM Tris (pH 8.0-10.0). The pH stability was analyzed by pre-incubating 10 µL of BGLI in 90 µL of buffers mentioned above at 25 °C for 4 h. Of this, 20 µL of the enzyme mixture was then used to determine remaining activity at 70 °C in sodium citrate buffer, pH 5.0, for 10 min [15].

The optimal temperature of BGLI activity was determined by incubating the enzyme (aliquots of supernatant) at different temperatures ranging from 20 to 90 °C in 50 mM sodium citrate pH 5.0 for 10 min. The thermostability of the enzyme was analyzed by measuring the residual activity at the optimal conditions (70 °C and 50 mM sodium citrate buffer, pH 5.0, for 10 min) after pre-incubating the enzyme at 30-90 °C for 30-120 min. Relative activity was calculated as enzymatic activity at the indicated temperature divided by the maximal activity at the optimal temperature [15].

For detection of in gel β-glucosidase activity, samples were analyzed by native PAGE using 8% and 5% polyacrylamide as separation and stacking gels, respectively. Electrophoresis was run at a constant current of 25 mA at 4 °C for 5 h using Hoefer SE 600 Ruby (Amersham Biosciences, USA). Gel was washed with distilled water and overlaid with 5 mM 4-methylumbelliferyl β-D-glucopyranoside (MUG, Sigma-Aldrich, Canada) in 50 mM sodium citrate buffer (pH 5.0) and incubated at 70 °C for 10 min. The presence of fluorescent reaction product was visualized under UV light using a gel documentation system (Syngene, Canada).

Total cellulase activity was measured using a microplate based filter paper assay according to a method described previously [37], which is a 25-fold scale-down of the International Union of Pure and Applied Chemistry (IUPAC) protocol for FPA assay [40-42]. In the assay, 6 mm diameter filter paper

disk (Whatman No. 1, with average weight of 3.0 mg each, ThermoFisher Scientific, Canada) in 75 mM citrate buffer (pH 4.8) was used as the substrate. The reducing sugars released were measured using 3,5-dinitrosalicylic acid (DNS reagent) with the absorbance measured at 540 nm. The substrate control (containing only the filter paper, and the buffer) and enzyme control (containing enzyme and the buffer with no filter paper) were also tested and subtracted from the absorbance. Total reducing sugars generated during the assay were estimated as glucose equivalents. Filter paper unit (FPU/mL) was first calculated using the equation previously described by Xiao et al. [42] and then converted to FPU/mg using the protein concentration accordingly. One FPU is defined as an average of one μmole of glucose equivalents released per min in the assay reaction.

Endoglucanase activity (EG) was measured using 2% (w/v) carboxymethylcellulose (CMC) in citrate buffer (50 mM, pH 4.8) as the substrate according to the method described by Zhang et al. [43]. The enzymes were added to the substrate solution (pre-equilibrated at 50 °C) and incubated at 50 °C for 30 min. Glucose released was measured by DNS method at 540 nm and using a glucose standard curve after deduction of the enzyme blank absorbance. Exoglucanase activity (Exo) was measured using 1.25% (w/v) Avicel (PH 105) in sodium acetate buffer (0.1 M, pH 4.8) as the substrate according to the method described by Zhang et al. [43]. The enzymes were added to the substrate solution (pre-equilibrated at 50 °C) and incubated at 50 °C for 2 h. The total soluble sugars released in the assay were determined using phenol (5%)-sulfuric acid (98%) assay at 490 nm. The enzyme activity was calculated on the basis of a linear relationship between the total soluble sugar released and enzyme dilution [43]. One unit of exoglucanase activity is defined as the amount of enzyme that releases one micromole of glucose equivalent per minute from Avicel.

3.3.8. Enzymatic hydrolysis of biomass by the BGLI-overexpressing T. reesei transformants

In order to evaluate hydrolysis activity of the engineered *T. reesei* with enhanced β-glucosidase activity, either 3% (w/v) of barley straw (untreated, Organosolv- or NaOH-pretreated) or 3% of

microcrystalline cellulose were used according to method described by Cheng et al [44]. Substrate hydrolysis was catalyzed by the culture supernatants collected as described above. Experiments were started with 3% substrate concentration in 750 μl buffer (50 mM sodium citrate buffer at pH 5.0 with 1 mM sodium azide to prevent microbial contamination) and 750 μl crude enzyme dosage at 50 °C for 72 h. For a control sample, the crude enzyme was replaced with the buffer. Samples were taken every 24 h and subjected to determination of the glucose and reducing sugar levels in the supernatant. The reducing sugars were detected by DNS method and the glucose concentration was measured using glucose oxidase assay kit (QuantiChrom™ Glucose Assay Kit, Medicorp, Canada).

3.3.9. Data processing and statistical analysis

All experimental points are the average values of three independent experiments. The data was collected in a Microsoft Excel spreadsheet where the average and standard error of the mean were determined. The graphs were created using the software PRISM 5.0. A one-way analysis of variance (one-way ANOVA) at a confidence level of 99% ($\alpha = 0.01$) was carried out with the software PRISM 5 to test the statistical significance of differences between the Exoglucanases as well as Endoglucanases activities of the four *T. reesei* transformant strains (T1-T4) compared to the parental *T. reesei* strain (Figure 3C).

3.4 Results and discussion

3.4.1. Overexpression of extracellular β-glucosidase from Periconia sp. in T. reesei QM9414

To increase the production of β-glucosidase, and ultimately the overall cellulolytic ability of *T. reesei*, the *bgl1* gene encoding an extracellular β-glucosidase (BGLI) in *Periconia sp*. was selected. This gene was selected as it has been previously shown to produce a thermotolerent β-glucosidase [15]. Ultimately, *bgl1* was isolated by Harnpicharnchai et al. (2009) from the strain and subsequently cloned

into *P. pastoris* [15]. The optimal temperature and pH for the enzyme was reported to be 70 °C and 5.0-6.0, respectively. The enzyme retained 60% of its activity at 70 °C after 1.5 h incubation. Moreover, the enzyme remained fully active when incubated for 2 h at pH ≥ 6.0 [15]. BGLI belongs to family 3 glycoside hydrolases (EC 3.2.1.21) and as the other members of the group showed high activity toward aryl β-*D*-glucosides, cellobiose, and cellooligosaccharides [15, 16]. Finally, the addition of BGLI to a commercial cellulase (Celluclast ® 1.5L) improved the hydrolysis rate of rice straw into simple sugars [15]. These attractive features were the basis for selecting BGLI as candidate for overexpression and thus improvement of β-glucosidase activity in *T. reesei*.

In our study, *Periconia sp.* total RNA was extracted and cDNA was constructed. The 2601 bp *bgl1* gene was amplified through PCR using Full-Beta primers (Table 2) designed according to cDNA sequence of *Periconia sp bgl1* reported by Harnpicharnchai et al (Accession No. EU304547). However, our cDNA sequencing results showed that our cloned *bgl1* sequence has 6 nucleotide differences compared to the reported cDNA sequence. To confirm the result, *Periconia sp. bgl1* genomic DNA was also cloned and sequenced. The sequencing results using the genomic DNA confirmed the 6 nucleotide difference. These nucleotides included A660G, C678T, C851T, A903T, A914C and G921T (numbers are based on the cDNA sequences). Four of the six variations made changes in the codon, but coded the same amino acids (aa 220; A660G, aa226; C678T, aa301; A903T and aa307; G921T). The other two however, coded different amino acids (C851T: T→I, and A914C: N→T, aa 284 and aa 305, respectively). The 2847 bp *bgl1* genomic DNA contained three exons (1-60, 252-310 and 366-2847) and two introns (61-191 and 180-250) was submitted to the GenBank [Accession No. JQ239427]. *Periconia sp. bgl1* genomic DNA and cDNA were aligned using MultAlin software (multiple sequence alignment by Florence Corpet) and presented in Figure 1 [17].

The *T. reesei* cellobiohydrolases I terminator region was used as the terminator to generate the *bgl1-cbh1* expression cassette. The *bgl1-cbh1* expression cassette was cloned into pPtef1 expression vector under the promoter regions of highly expressed gene *tef1α* to generate pPtef1-*bgl1-cbh1* (Figure

2A) and then transformed into the *T. reesei* QM9414. The plasmid contains hygromycin B phosphotransferase (*hph*) expression cassette which was used as the selection marker. Four mitotic stable hygromycin-resistant transformants were obtained and were selected for the single spore isolation to insure for the pure culture. The four transformants (named T1-T4) were cultured for more than 6 generations and *bgl1* was confirmed to be in the genome of the transformants via PCR and DNA sequencing (Figure 2B). The copy number of the *bgl1* expression cassette integrated into the *T. reesei* genome of the transformants was analyzed by quantitative Real-Time PCR (qRT-PCR) (Figure 2C). The result showed that three of the four transformants (T1, T2 and T3) obtained only one copy of the *bgl1* cassette whereas T4 received two copies.

To test the β-glucosidase activity of the BGLI-overexpressing *T. reesei* transformants (T1-T4), they were cultivated in a microcrystalline cellulose containing medium as the cellulase inducer for 144 h. The β-glucosidase activity of the transformants was measured using the culture supernatant every 24 h and was compared to the parent strain (*T. reesei* QM9414) (Figure 3A). All the transformants showed very high β-glucosidase activity compared to the parent strain over the time course study. High β-glucosidase activity in the transformants was seen at the first time point (24 h) after the induction with about 23.9 IU/mg for T3 which is 10.5-fold higher than the parental β-glucosidase activity (Figure 3A). The β-glucosidase activity of the transformants and the parent strain slowly increased over the rest of the time points with a peak at 120 h post induction. The maximum activity was seen in two transformants (T2 and T3) at about 27 IU/mg at 120 h. The parent strain also showed the maximum activity at 120 h with about 8.4 IU/mg, which is 3.2-fold lower than the maximum activity obtained for the transformants (T2 and T3). Although the transformant T4 received two copies of the *bgl1* expression cassette, our results showed that its β-glucosidase activity resembled the same as the other transformants with the single integrated expression cassette (Figure 3A). Levels of transcription and recombinant protein production in fungal transformants are mainly correlated with two factors including the gene copy number and the loci of the integration [18]. However, it has been reported that in some cases, high copy number transformants

did not produce more recombinant protein in *T. reesei* [9, 18, 19]. This can be possibly due to different loci integration of the expression cassette in *T. reesei* genome. Improvement of β-glucosidase activity of *T. reesei* strains was previously reported by different groups where the activity was increased by 7.5 or 3.7-fold compared to the parent strain after 168 or 36 h incubation time, respectively [9, 10]. Our results indicated that the activity of our constructed transformants is higher than (10.5-fold) previous studies. In addition, the transformants showed high β-glucosidase activity within a shorter induction time (24 h). Our recombinant strain (T3) showed β-glucosidase activity of 23.9 IU/mg after the first 24 h of incubation which is 2.8-fold higher than the maximal β-glucosidase activity of the parent strain (8.4 IU/mg) after 120 h of incubation. Higher β-glucosidase production, as well as shorter incubation time, suggests that our transformants can be used for their potential industrial application for the production of β-glucosidase.

Despite *Aspergillus niger, T. reesei* produces very low amounts of BGL causing the accumulation of cellobiose as the end product and thus inhibits further cellulose hydrolysis [1, 20]. Thus, it has been suggested that by improving the BGL activity of *T. reesei* its cellulose hydrolysis activity would also improve. Different studies have shown that overexpression of different BGL in different *T. reesei* strains results in higher cellulolytic activity. For example, total cellulase activity of different recombinant *T. reesei* strains such as RUT-C30 or PC-3-7 were improved by 2.3 or 1.3-fold respectively, when compared to their parent strains [9, 10]. Our four transformants (T1-T4) were grown in a cellulase-inducing medium (MA-medium containing microcrystalline cellulose) for 144 h and their total cellulase activity (or filter paper assay, FPA) were compared to the parent strain every 24 h (Figure 3B). The maximum FPA activity was seen for T4 with about 51.9 FPU/mg activity after 120 h of incubation. The parent strain also reached its maximum FPA activity (about 32.8 FPU/mg) at 120 h, which is 1.58-fold less compared to the activity obtained for T4. FPA activity of the parent strain was very low over the first 48 h of the incubation whereas high FPA activity was obtained for all the four transformants after the first 24 h of incubation (Figure 3B). For example, T3 showed high FPA activity with about 39.0 FPU/mg at 24 h of incubation which is 1.18-fold higher than the maximal FPA activity of the parent strain (about 32.8 FPU/mg)

obtained after 120 h of incubation. Thus, all the four transformants showed higher FPA activity within a shorter incubation time compared to the parent strain (Figure 3B).

Cellobiohydrolase (exoglucanases, Exo) and endoglucanases (EG) activities of BGLI-overexpressing transformants and the parental strain were also measured at 120 h, as this is where the maximal BGL and FPA activities were seen (Figure 3C). All the four transformants showed similar level of Exo activity compared to the parent strain. Although T2 and T4 showed slight decreased in Exo activity compared to the parent strain but no significant difference was obtained (ANOVA, $P < 0.01$). This may also be a result of different loci integration of the BGLI-overexpressing transformants. Similar result was also reported by Zhang study (2010) in which the Exo activity of some of the overexpressing transformants were lower than that of the parent *T. reesei* strain [9]. In the case of EG activity, however, transformants T1-T3 showed lower level of EG activity whereas the transformant T4 showed almost the same activity compared to the parent strain (Figure 3C). However, no significant difference in EG activity was obtained between the four transformants and the parent strain (ANOVA, $P < 0.01$). The total cellulase activity of our transformants was significantly higher than the parent strain despite the fact that their Exo and EG activities in two of the four transformants were slightly decreased. This supports the hypothesis that the lower β-glucosidase production by *T. reesei* causes the inhibition of further hydrolysis of cellulosic residues to the end product [1].

3.4.2. Characterization of the BGLI-overexpressing transformants

Identification of β-glucosidase BGLI-overexpressing transformants (T1-T4) was done using 4-methylumbelliferyl β-D-glucopyranoside (MUG) in a MUG-zymogram assay (Figure 4). Proteins from the culture supernatant were first separated in an 8% native PAGE gel, with β-glucosidase activity ultimately detected using a MUG-zymogram assay (Figure 4). One enzymatically active protein was observed in the culture supernatant of both the BGLI-overexpressing strains as well as the parent strain *T. reesei* QM9414 (Figure 4, lanes T1-T4 and QM). This β-glucosidase activity has been reported as the native extracellular β-glucosidase in *T. reesei* strain (BGLI) with a molecular weight about 75 kDa [8].

However, a second active β-glucosidase with higher molecular weight was only obtained from the transformants, which is correlated to the recombinant BGLI (Figure 4, lanes T1-T4). A sample from the culture supernatant of *Periconia sp.* (*bgl1* donor) was also compared to confirm the presence of the recombinant BGLI in the BGLI-overexpressing transformants (Figure 4, lane P). Harnpicharnchai et al. (2009) also reported that the *Periconia sp.* BGLI showed a higher molecular weight band on the native gel (> 150 kDa) although its molecular weight predicted to be around 95 kDa [15]. This study also showed that overexpressed *Periconia* sp. BGLI (in *P. pastoris*) migrates slowly on a SDS-PAGE gel with a molecular weight around 130 kDa [15]. They proposed that the slower migration is due to the protein glycosylation (BGLI has 16 putative *N*-glycosylation sites predicted by PROSITE) which was also confirmed by the experimental approaches [15].

The effect of pH on β-glucosidase activity of BGLI-overexpressing transformants was determined using *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) as the substrate. The culture supernatant of the selected transformant T3 (based on its β-glucosidase activity) was incubated at 70 °C for 10 min at different pH (3.0-10.0). Although, the maximal enzyme activity was obtained at pH 5.0, the enzyme showed over 80% activity in a pH range from 4.0 to 6.0 (Figure 5A). Harnpicharnchai and et al. (2009) reported similar results using purified *Periconia sp.* BGLI overexpressed in *P. pastoris* expression system [15]. For the determination of enzyme stability at different pH, the culture supernatant was incubated in the various pH buffers (3.0-10.0) for 4 h at 25 °C before determining the remaining β-glucosidase activity (Figure 5B). Our results indicated that the β-glucosidase remain over 88% active in a pH range from 3.0 to 9.0. However, the remaining activity decreased to about 76% when the pH increased to 10.0 (Figure 5B). Similar results were obtained for the pH ranges from 6.0-10.0 by the Harnpicharnchai study using the purified BGLI enzyme overexpressed in *P. pastoris* [15]. Nazir et al. (2009), also reported an active β-glucosidase (BGI) from *Aspergillus terreus* after 4 h of incubation under a broad range of pH (5.0-10.0) [21]. In another study by Ng et al. (2010) pH stability of the crude β-glucosidase from *Penicillium citrinum* YS40-5 was tested over a wide pH range from 1.0-10.0 after 24 h incubation at 4 °C [22].

134

Surprisingly, the enzyme retained over 85% of its maximal activity over the entire pH range [22]. Since citrate buffer at pH 5.0 gave the highest level of enzyme activity, it was chosen to be used as the standard assay condition in the all following experiments when the BGLI activity was measured.

The temperature influence on β-glucosidase activity of BGLI-overexpressing transformants was determined by monitoring the hydrolysis of pNPG at 20-90 °C (Figure 5C). The maximal enzyme activity obtained at 70 °C but it exhibited more than 80% of the maximal activity between 65 and 75 °C. This is in accordance to the reported optimal enzyme temperature using pure *Periconia sp.* BGLI overexpressed in *P. pastoris* expression system [15] and also it is in the range of reported optimal temperature for different β-glucosidase (45-75 °C) [1]. For example, the optimal temperature of a thermotolerant β-glucosidase from moderately thermophilic fungus *Talaromyces emersonii* also overexpressed in *T. reesei* was determined to be at 71.5 °C [23]. The thermal stability of BGLI was tested by incubating the enzyme at different temperature (30-90 °C) for 30-120 min (Figure 5D). The residual activity was measured every 30 min at the optimal pH and temperature (pH 5.0 and 70 °C). The enzyme maintained over 85% of its maximal activity after 2 h incubation at different temperatures up to 60 °C. The enzyme activity, however, decreased to about 40% of its maximal activity after 30 min of incubation at 70 °C. Higher temperatures (≥80 °C) inactivated the enzyme and only about 5% of the maximal activity was maintained after 30 min of incubation. Thermotolerant β-glucosidase from *T. emersonii* was reported to maintain 50% of its maximal activity at 65 °C after 62 min of incubation [23].

Our overexpressed BGLI also maintained its maximal activity after four months incubation at 4 °C (data not shown here). Overall, our BGLI-overexpressing transformants showed very high β-glucosidase activity which can tolerate a wide range of pH (3.0-10.0), high temperature (up to 60 °C) and also remain fully active after long time storage at 4 °C in the absence of any stabilizer. These unique features suggest that our BGLI-overexpressing transformants are superior candidates for their potential biotechnological applications. Many recent bioconversion research studies focused on two main strategies including enhancing overall fungal hydrolytic activities as well as identifying stable enzymes able to

function under harsh conditions [1]. Acidic pretreatment of lignocellulosic residues seems a preferred option due to fungal cellulolytic enzymes activity at lower pH (usually 4.0-5.0). Enzymes able to remain active at higher temperatures and also retain their activity at lower pH values are more suitable for pretreatment methods where acid and high temperatures are applied [1]. The overall hydrolytic activity can be enhanced by thermostable enzymes through their higher specific activity and higher stability [1]. β-glucosidases able to tolerate harsh conditions (i.e. acidic and/or basic pH conditions as well as high temperatures) have great potential biotechnological applications in different industries such as food [12], wine [13] and textile production [14]. Thermotolerant β-glucosidases, for example, represent valuable characteristics such as reduction of the risks associated with microbial contamination in the process as well as substrate viscosity which leads to higher reaction velocities and thus improved hydrolysis efficiency.

3.4.3. Enzymatic hydrolysis of biomass by the BGLI-overexpressing T. reesei transformants

Efficient hydrolysis of cellulose and hemicelluloses of lignocellulosic residues to their soluble monomeric sugars is an important step in bioconversion process. For this reason, much effort has been done to obtain efficient microorganisms with robust lignocellulolytic activities [1]. To this end, the hydrolysis efficiency of the BGLI-overexpressing *T. reesei* transformants using barley straw was investigated (Figure 6A-H).

For the untreated barley straw (Figure 6A and B), all the transformants released higher amount of reducing sugars (RS) (maximum 4.21 mg/mL for T3) and glucose (G) (maximum 1.92 mg/mL for T3) which were 90 and 30% higher than the parent strain (2.21 mg/mL RS and 1.48 mg/mL G) over the first 48 h of hydrolysis incubation time, respectively. Longer incubation time (72 h) slightly increased the RS and G released by the transformants (maximum for T2 with 4.90 and 3.0 mg/mL RS and G, respectively). In the case of the parental strain, the longer incubation time (72 h) also improved the hydrolysis rate and the level of RS and G was 3.92 and 2.14 mg/mL, respectively. This was 25 and 40% lower than the maximal RS and G productions by the transformant T2. This suggest that in the absence of any

pretreatment, our BGLI-overexpressing transformants efficiently hydrolyze barley straw within a shorter time compared to the parent strain.

Hydrolysis of barley straw treated with NaOH was also tested using all four transformants and the parental strain (Figure 6C and D). The results indicated that after 72 h, all four transformants released higher amount of RS and G (maximum 24.96 and 11.0 mg/mL for T4, respectively), which were 2.33- and 1.77-fold higher than the enzymatic hydrolysis by the parent strain (10.71 and 6.18 mg/mL, respectively). Similar results were also observed when Organosolv-treated barley straw was used (Figure 6E and F). After 72 h of the incubation time, the maximal RS and G released was obtained for T2 (19.42 and 9.71 mg/mL, respectively), which were 3.67- and 2.55-fold higher than the parent strain (5.28 and 3.80 mg/mL, respectively). Hydrolysis of microcrystalline cellulose was also tested using all four transformants as well as the parental strain (Figure 6G and H). After 72 h of the incubation time, all four transformants released higher amount of RS and G (maximum 22.80 and 12.20 mg/mL for T1, respectively), which were 2.34- and 1.89-fold higher than the enzymatic hydrolysis by the parent strain (9.71 and 6.45 mg/mL, respectively). Our results demonstrated that the BGLI-overexpressing transformants produced more efficient enzymes which facilitate hydrolysis of the cellulosic substrates. This may be a direct result of removing β-glucosidase limitation on further cellulose hydrolysis [1].

3.5 Conclusion

In our study, a thermotolerant β-glucosidase (BGLI) from *Periconia sp.* was overexpressed in *T. reesei* QM9414. The β-glucosidase activity and total cellulase activity of the recombinant *T. reesei* strains overexpressing BGLI are significantly increased. The BGLI-overexpressing transformants showed higher biomass hydrolytic efficiency, suggesting that they can be used in the hydrolysis step in biomass conversion. High β-glucosidase activity, wide pH range tolerant and high temperature resistance makes

the transformants excellent candidates for their potential application for the production of β-glucosidase as well as improving the biomass conversion using cellulases.
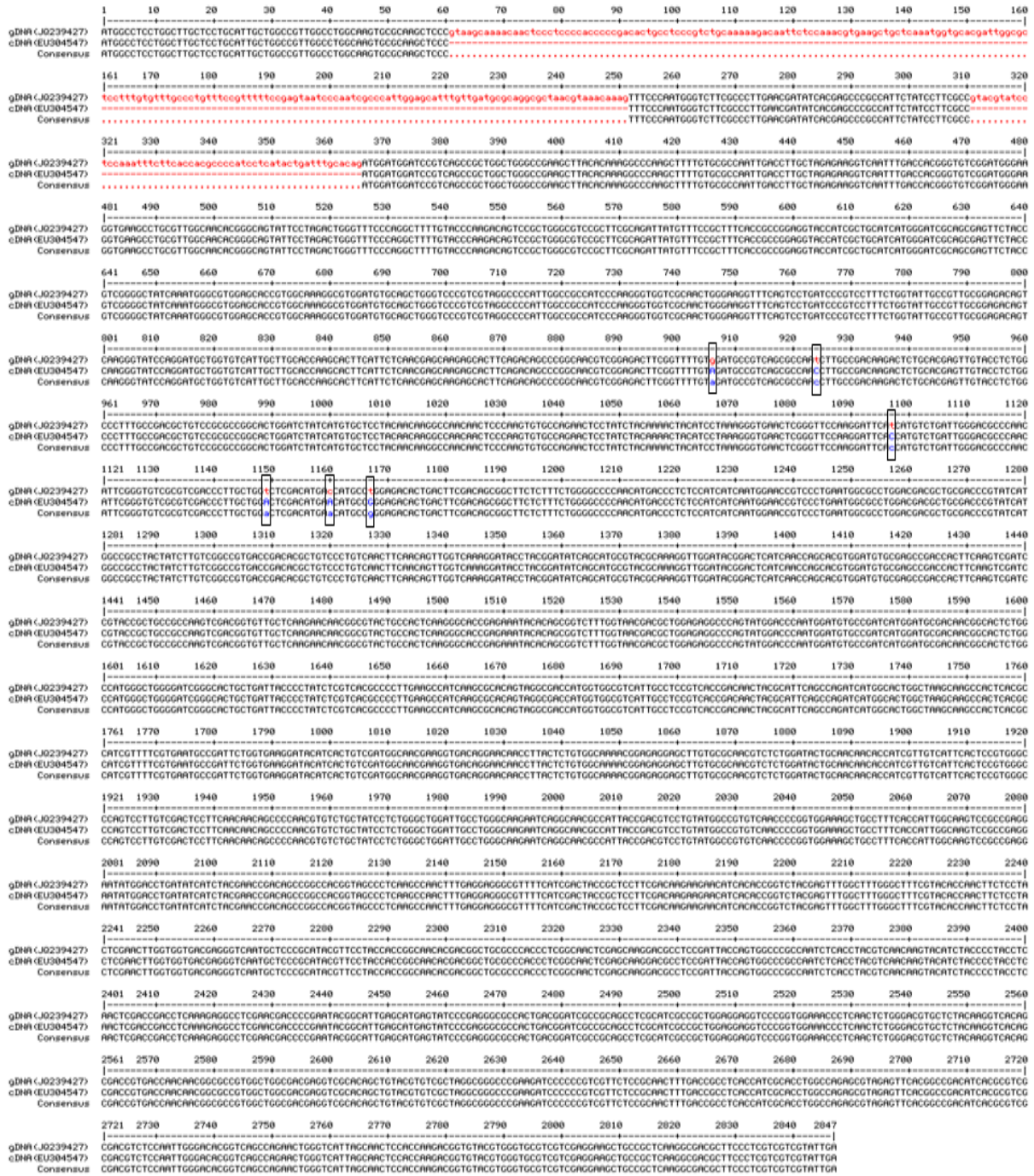

Acknowledgements

Table 1. Composition of barley straw used in the study. Data are represented as the mean of three independent experiments and error denote standard error of the mean.

	Soluble part	Hemicellulose	Cellulose	Acid-soluble lignin	Acid-insoluble lignin	Ash
Untreated barley straw (%)	13.13 ± 0.40	26.46 ± 3.67	32.60 ± 4.10	1.49 ± 0.53	24.01 ± 4.79	1.03 ± 0.06

Table 2. List of oligonucleotides used in the study for the genes amplification (restriction sites are underlined).

Primers set/gene	Primer name	Sequence (5'-3')	Expected fragment size (bp)
bgl1	Full-Beta-F Full-Beta-R	ATGGCCTCCTGGCTTGCT TCAATACGACGACGAGGGAA	2601
cbh1	*cbh1*-F *cbh1*-R	AGGTCACCTTCTCCAACATC AGAGCGGCGATTCTACGGGT	573
bgl1-cbh1 fusion	F1 Fuse-REV1 Fuse- F2 REV2	AG<u>ATCGAT</u>ATGGCCTCCTGGCTTGCTCCTG AGAAGGTGACCTTCAATACGACGACGAGGGAA GTCGTATTGAAGGTCACCTTCTCCAACATC GG<u>ATCGAT</u>AGAGCGGCGATTCTACGGGTTA	3187
In-Fusion	In-Fusion-F In-Fusion-R	CACAAACCGTC<u>ATCGAT</u>ATGGCCTCCTGGCTTGCT TGCAGGTCGAC<u>ATCGAT</u>AGAGCGGCGATTCTACGGGTTA	3205
bgl1	*bgl1*-Realtime-F *bgl1*-Realtime-R	GCCATTCTATCCTTCGCC TGCCAACGCAGGCTTCAC	154
tef1a	*tef1a*-Realtime-F *tef1a* -Realtime-R	ACCAAGGCTGGCAAGTTC GACACCAGTCTCGATACG	162

Figure 1 Alignment of *Periconia sp. bgl1* genomic DNA and cDNA using MultAlin software. Intron regions are shown in lower case in red while the coding regions are shown in upper case and black. The 6 single-nucleotide differences between the genomic DNA (Accession No. JQ239427) and previously reported cDNA (Accession No. EU304547) (including: A660G, C678T, C851T, A903T, A914C and G921T) are shown in lower case and highlighted in boxes.
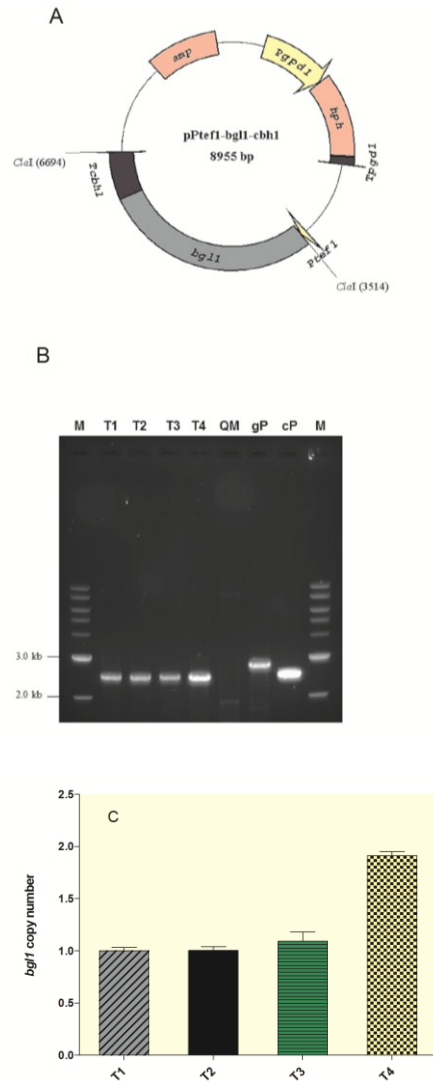
140

Figure 2 Construction of BGLI-overexpression *T. reesei* strain. (A) The structure of pPtef1-*bgl1-cbh1* expression vector using SimVector software. Ptef1 represents *tef1* promoter; *bgl1* represents β-glucosidase gene from *Periconia sp.*; T*cbh1* indicates the *cbh1* terminator; P*gpd1* represents *gpd1* promoter (glyceraldehyde-3-phosphate dehydrogenase gene); *hph* represents hygromycin B phosphotransferase gene; T*gpd1* indicates the *gpd1* terminator. (B) PCR assay of the *bgl1* gene in the selected overexpression transformants using full-bgl1 primers. All transformants (T1-T4) showed a 2601 bp band whereas the parent strain (QM) did not amplified any specific PCR products. *Periconia sp.* genomic DNA (gP) as well as cDNA (cP) were used as the positive controls which amplified 2847 and 2601 bp products, respectively. (C) qPCR analysis of the isolated genomic DNA from the transformants T1-T4 using Real-Time *bgl1* and *tef1a* primers. The transformants T1-T3 received one copy of the *bgl1* gene whereas T4 obtained two copies.
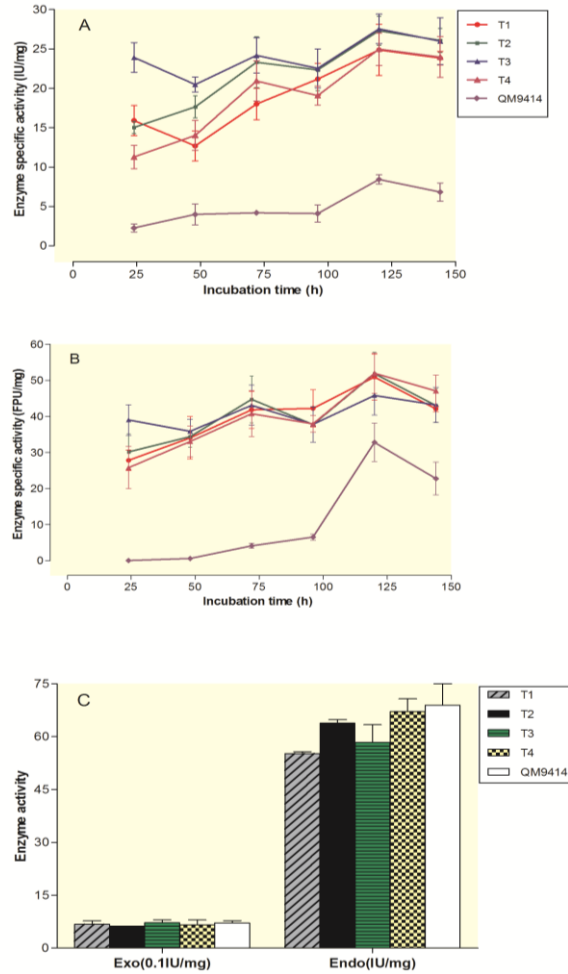
Figure 3 The enzyme activities and time course study of BGLI-overexpressing transformants (T1-T4) and the parental strain (*T. reesei* QM9414). The strains were pre-grown on MA-medium containing 1% glucose for 24 h and then mycelia were washed and grown for 144 h on MA-medium containing 1% microcrystalline for the induction. Enzyme activities were expressed as specific activities using international units per mg protein in the supernatant. (A) The β-glucosidase activity was measured every 24 h using the culture supernatant as the enzyme sources. The enzyme was incubated at 70 °C for 10 min, pH 5.0 using 50 mM sodium citrate buffer. (B) Filter paper activity (FPA) was measured every 24 h using the culture supernatant and incubated at 50 °C for 60 min, pH 4.8 using 75 mM citrate buffer. (C) Exoglucanases (Exo), and endoglucanases activities (EG) of the strains were measured after being induced with 1% microcrystalline cellulose for 120 h. No significant difference was obtained for Exo and EG activities of the four transformants compared to the parent strain using one-way ANOVA at a confidence level of 99% (α= 0.01). Data are represented as the mean of three independent experiments and error bars denote standard error of the mean.
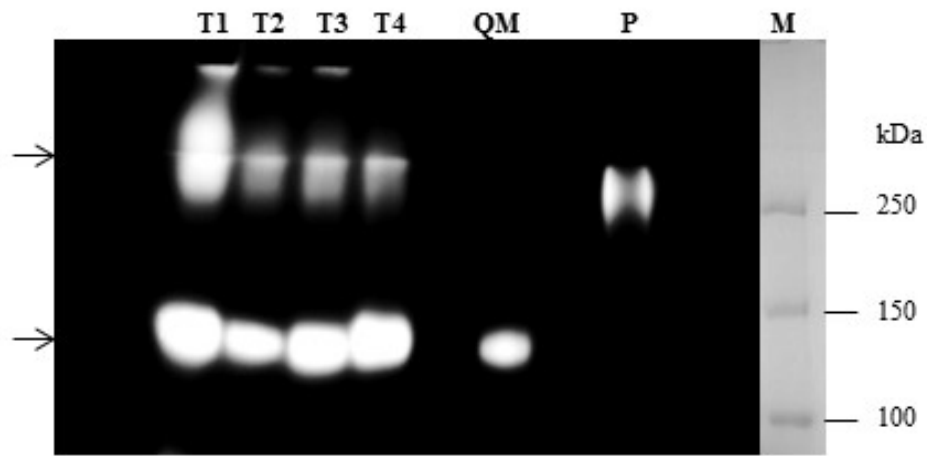
Figure 4 Identification of β-glucosidase in BGLI-overexpressing transformants (T1-T4) by MUG-zymogram assay. Proteins (culture supernatant) from BGLI-overexpressing transformants (T1-T4) and the parental strain (*T. reesei* QM9414, lane QM) were separated in 8% native PAGE. Culture supernatant of *Periconia sp.* (*bgl1* donor strain) (lane P) grown on MA-medium containing 1% microcrystalline was used as the positive control. β-glucosidase activity was detected by MUG-zymogram assay. Lower arrow indicates the native extracellular β-glucosidase found in *T. reesei* strain (lanes T1-T4 and QM) whereas the upper arrow represents active β-glucosidase correlated to *Periconia sp.* BGLI only found in the BGLI-overexpressing transformants as well as *Periconia sp.* (lanes T1-T4 and P). Lane M, molecular weight marker.
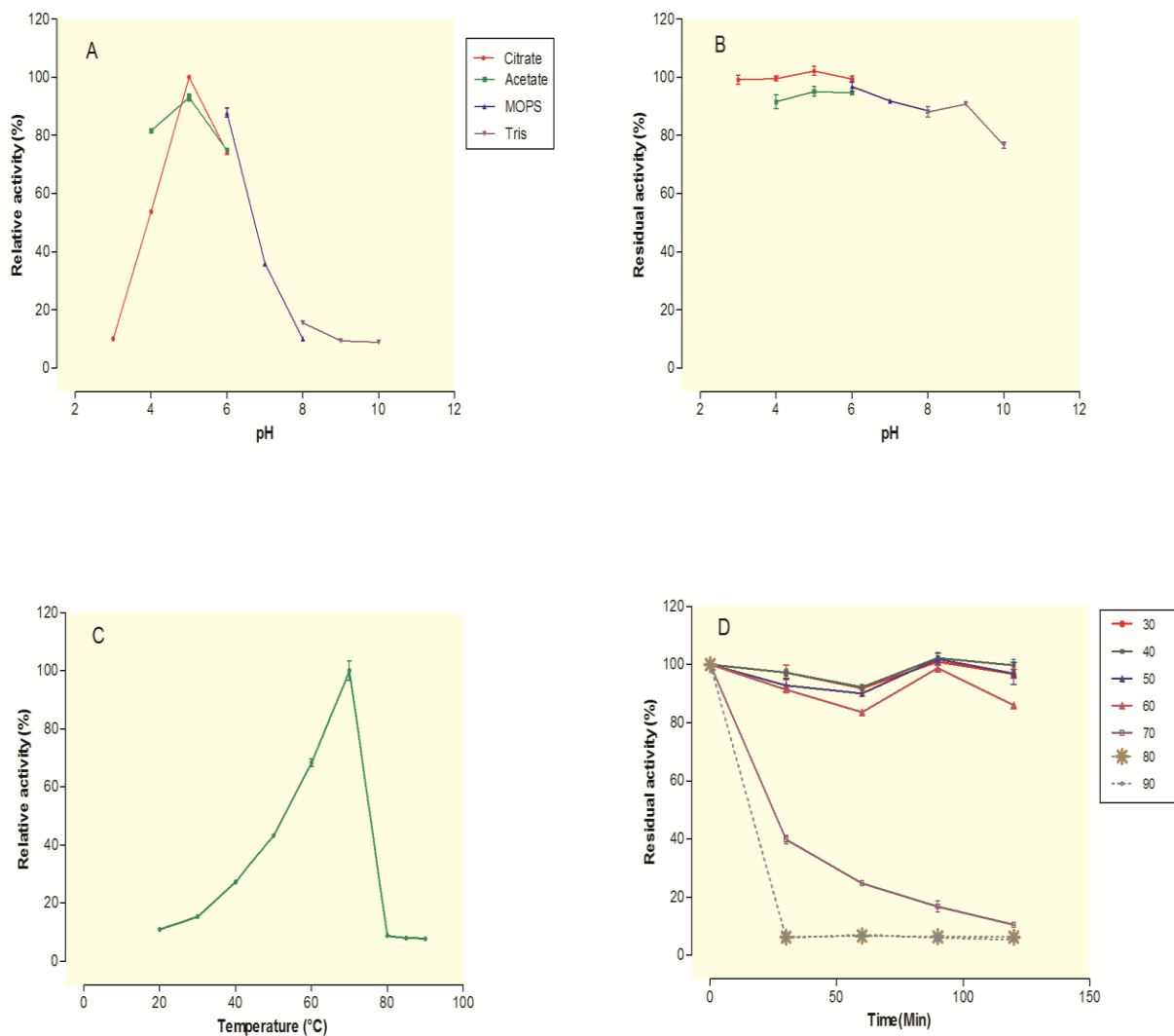
Figure 5 Effect of pH and temperature on β-glucosidase activity of the selected BGLI-overexpressing transformant (T3). (A) pH profile was determined by incubating the enzyme (culture supernatant) at 70 °C for 10 min at different pH, using 50 mM sodium citrate (pH 3.0-6.0), sodium acetate (pH 4.0-6.0), MOPs (pH 6.0-8.0) and Tris buffer (pH 8.0-10.0). (B) The remaining β-glucosidase activity was determined (at 70 °C in 50 mM sodium citrate buffer, pH 5.0 for 10 min) after incubating the enzyme at 25 °C for 4 h at the different pHs. (C) Temperature profile was determined by incubating the enzyme (culture supernatant) in 50 mM sodium citrate buffer (pH 5.0) for 10 min at different temperature (30-90 °C). (D) Thermal stability was carried out by incubating the enzyme in 50 mM sodium citrate buffer (pH 5.0) at different temperature (30-90 °C) for 30, 60, 90 and 120 min before the remaining activity was assayed (at 70 °C in 50 mM sodium citrate buffer, pH 5.0 for 10 min).
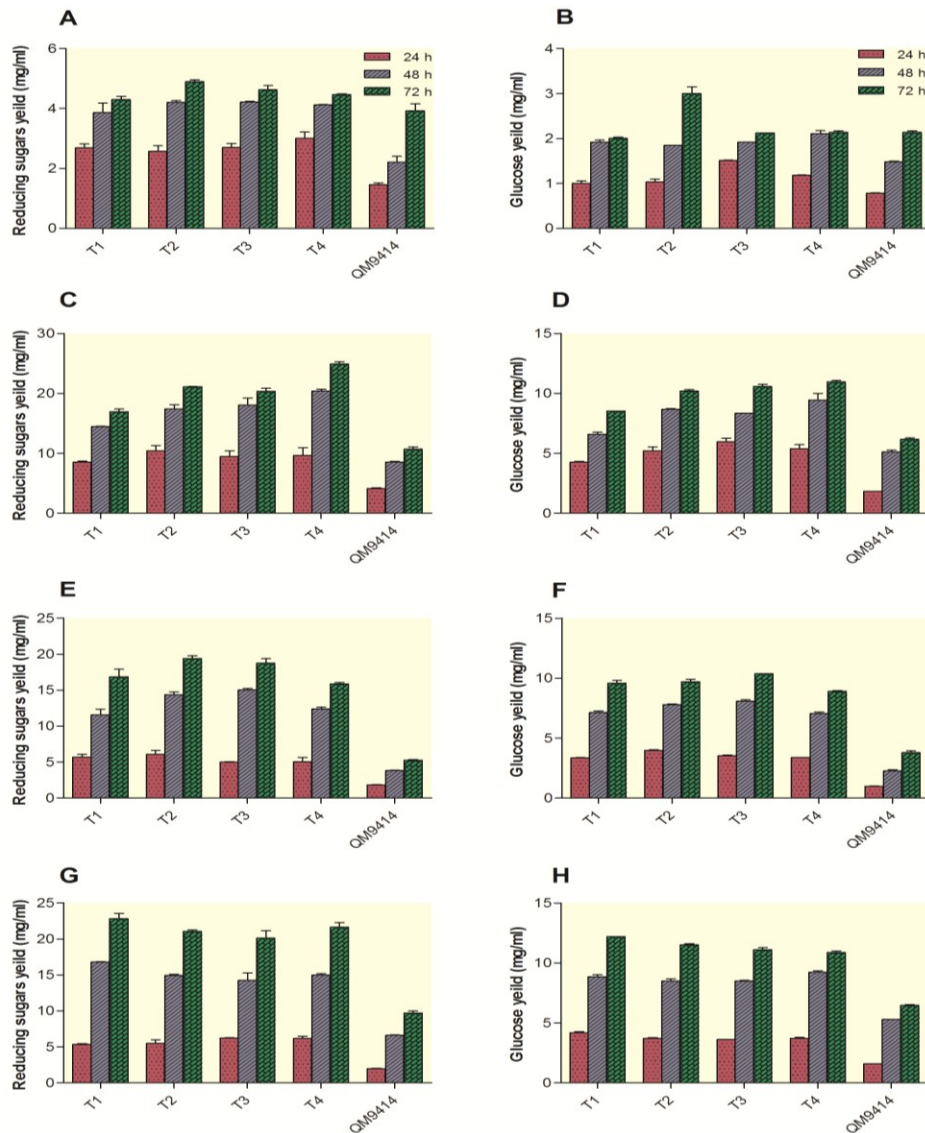
Figure 6 Enzymatic hydrolysis of barley straw and microcrystalline cellulose by BGLI-overexpressing transformants (T1-T4) and the parental strain (*T. reesei* QM9414). (A and B) Reducing sugars and glucose yield (mg/mL) released using untreated barley straw, respectively. (C and D) Reducing sugars and glucose yield (mg/mL) released using NaOH-pretreated barley straw, respectively. (E and F) Reducing sugars and glucose yield (mg/mL) released using Organosolv-pretreated barley straw, respectively. (G and H) Reducing sugars and glucose yield (mg/mL) released using microcrystalline cellulose, respectively. 750 μl of the culture supernatants (as the enzyme source) were added to tubes containing 750 μl of 50 mM sodium citrate buffer (pH 5.0) and 0.045 g (3%) either barley straw or microcrystalline cellulose. The tubes were incubated at 50 °C for 72 h and reducing sugars as well as glucose released were measured every 24 h. Data are represented as the mean of three independent experiments and error bars denote standard error of the mean. Total protein loading was as follow; T1: 0.22 mg; T2: 0.26 mg; T3: 0.24 mg; T4: 0.19 mg, and QM9414: 0.22 mg.

References

1.	Dashtban M, Schraft H, Qin W: Fungal bioconversion of lignocellulosic residues; opportunities & perspectives. *Int J Biol Sci* 2009, 5(6):578-595.

2.	Seidl V, Seiboth B: Trichoderma reesei: genetic approaches to improving strain efficiency. *Biofuels* 2010, 1(2):343-354.

3.	Kubicek CP, Mikus M, Schuster A, Schmoll M, Seiboth B: Metabolic engineering strategies for the improvement of cellulase production by Hypocrea jecorina. *Biotechnol Biofuels* 2009, 2:19.

4.	Fowler T, Brown RD, Jr.: The bgl1 gene encoding extracellular beta-glucosidase from Trichoderma reesei is required for rapid induction of the cellulase complex. *Mol Microbiol* 1992, 6(21):3225-3235.

5.	Messner R, Kubicek CP: Evidence for a single, specific β-glucosidase in cell walls from *Trichoderma reesei* QM 9414. *Enzyme Microb Technol* 1990(12):685-690.

6.	Umile C, Kubicek CP: A constitutive, plasma-membrane bound β-glucosidase in *Trichoderma reesei*. *FEMS Microbiol Lett* 1986(34):291-295.

7.	Saloheimo M, Kuja-Panula J, Ylosmaki E, Ward M, Penttila M: Enzymatic properties and intracellular localization of the novel Trichoderma reesei beta-glucosidase BGLII (cel1A). *Appl Environ Microbiol* 2002, 68(9):4546-4553.

8.	Mach RL, Seiboth B, Myasnikov A, Gonzalez R, Strauss J, Harkki AM, Kubicek CP: The bgl1 gene of Trichoderma reesei QM 9414 encodes an extracellular, cellulose-inducible beta-glucosidase involved in cellulase induction by sophorose. *Mol Microbiol* 1995, 16(4):687-697.

9.	Zhang J, Zhong Y, Zhao X, Wang T: Development of the cellulolytic fungus Trichoderma reesei strain with enhanced beta-glucosidase and filter paper activity using strong artificial cellobiohydrolase 1 promoter. *Bioresour Technol* 2010, 101(24):9815-9818.

10.	Rahman Z, Shida Y, Furukawa T, Suzuki Y, Okada H, Ogasawara W, Morikawa Y: Application of Trichoderma reesei cellulase and xylanase promoters through homologous recombination for enhanced production of extracellular beta-glucosidase I. *Biosci Biotechnol Biochem* 2009, 73(5):1083-1089.

11.	Nakazawa H, Kawai T, Ida N, Shida Y, Kobayashi Y, Okada H, Tani S, Sumitani JI, Kawaguchi T, Morikawa Y *et al*: Construction of a recombinant Trichoderma reesei strain expressing Aspergillus aculeatus beta-glucosidase 1 for efficient biomass conversion. *Biotechnol Bioeng* 2012, 109(1):92-99.

12.	Bhatia Y, Mishra S, Bisaria VS: Microbial beta-glucosidases: cloning, properties, and applications. *Crit Rev Biotechnol* 2002, 22(4):375-407.

13.	Palmeri R, Spagna G: β-Glucosidase in cellular and acellular form for winemaking application. *Enzyme and Microbial Technology* 2007, 40(3):382-389.

14.   Song J, Imanaka H, Imamura K, Kajitani K, Nakanishi K: Development of a highly efficient indigo dyeing method using indican with an immobilized beta-glucosidase from Aspergillus niger. *J Biosci Bioeng* 2010, 110(3):281-287.

15.   Harnpicharnchai P, Champreda V, Sornlake W, Eurwilaichitr L: A thermotolerant beta-glucosidase isolated from an endophytic fungi, Periconia sp., with a possible use for biomass conversion to sugars. *Protein Expr Purif* 2009, 67(2):61-69.

16.   Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B: The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. *Nucleic Acids Res* 2009, 37(Database issue):D233-238.

17.   Corpet F: Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res* 1988, 16(22):10881-10890.

18.   Graessle S, Haas H, Friedlin E, Kurnsteiner H, Stoffler G, Redl B: Regulated system for heterologous gene expression in Penicillium chrysogenum. *Appl Environ Microbiol* 1997, 63(2):753-756.

19.   Verdoes JC, van Diepeningen AD, Punt PJ, Debets AJ, Stouthamer AH, van den Hondel CA: Evaluation of molecular and genetic approaches to generate glucoamylase overproducing strains of Aspergillus niger. *J Biotechnol* 1994, 36(2):165-175.

20.   Kovacs K, Szakacs G, Zacchi G: Comparative enzymatic hydrolysis of pretreated spruce by supernatants, whole fermentation broths and washed mycelia of Trichoderma reesei and Trichoderma atroviride. *Bioresour Technol* 2009, 100(3):1350-1357.

21.   Nazir A, Soni R, Saini HS, Kaur A, Chadha BS: Profiling differential expression of cellulases and metabolite footprints in Aspergillus terreus. *Appl Biochem Biotechnol* 2009, 162(2):538-547.

22.   Ng IS, Li CW, Chan SP, Chir JL, Chen PT, Tong CG, Yu SM, Ho TH: High-level production of a thermoacidophilic beta-glucosidase from Penicillium citrinum YS40-5 by solid-state fermentation with rice bran. *Bioresour Technol* 2010, 101(4):1310-1317.

23.   Murray P, Aro N, Collins C, Grassick A, Penttila M, Saloheimo M, Tuohy M: Expression in Trichoderma reesei and characterisation of a thermostable family 3 beta-glucosidase from the moderately thermophilic fungus Talaromyces emersonii. *Protein Expr Purif* 2004, 38(2):248-257.

24.   Cheng S, I. Dc, Wang M, Leitch M, Xu M: Highly Efficient Liquefaction of Woody Biomass in Hot-Compressed Alcohol−Water Co-solvents. *Energy Fuels* 2010, 24 (9):4659-4667.

25.   Deshpande P, Nair S, Khedkar S: Water Hyacinth as Carbon Source for the Production of Cellulase by Trichoderma reesei. *Appl Biochem Biotechnol* 2009, 158:552–560.

26.   Arora DS, Sharma RK: Comparative ligninolytic potential of Phlebia species and their role in improvement of in vitro digestibility of wheat straw *Journal of Animal and Feed Sciences* 2009, 18:51-161.

27. Sharma RK, Arora DS: Production of lignocellulolytic enzymes and enhancement of in vitro digestibility during solid state fermentation of wheat straw by Phlebia floridensis. *Bioresour Technol*, 101(23):9248-9253.

28. TAPPI T 222 om-02: Acid-insoluble lignin in wood and pulp, in: 2002-2003 TAPPI Test Methods. 2002:Tappi Press, Atlanta, GA, USA.

29. TAPPI: TAPPI Useful Method UM 250: Acid-solube lignin in wood and pulp. 1985:TAPPI Useful Methods, TAPPI, Atlanta, GA, USA.

30. Benko Z, Drahos E, Szengyel Z, Puranen T, Vehmaanpera J, Reczey K: Thermoascus aurantiacus CBHI/Cel7A production in Trichoderma reesei on alternative carbon sources. *Applied biochemistry and biotechnology* 2007, 137-140(1-12):195-204.

31. Mandels MM, Andreotti RE: The cellulose to cellulase fermentation. *Proc Biochem* 1978, 13:6-13.

32. Akel E, Metz B, Seiboth B, Kubicek CP: Molecular regulation of arabinan and L-arabinose metabolism in Hypocrea jecorina (Trichoderma reesei). *Eukaryot Cell* 2009, 8(12):1837-1844.

33. Szewczyk E, Nayak T, Oakley CE, Edgerton H, Xiong Y, Taheri-Talesh N, Osmani SA, Oakley BR: Fusion PCR and gene targeting in Aspergillus nidulans. *Nat Protoc* 2006, 1(6):3111-3120.

34. Solomon PS, Ipcho S.V.S., Hane JK, Tan K-C, Oliver R.P.: A quantitative PCR approach to determine gene copy number. *Fungal Genetics Reports* 2008, 55:5-8.

35. Nakari T, Alatalo E, Penttila ME: Isolation of Trichoderma reesei genes highly expressed on glucose-containing media: characterization of the tef1 gene encoding translation elongation factor 1 alpha. *Gene* 1993, 136(1-2):313-318.

36. Martinez D, Berka RM, Henrissat B, Saloheimo M, Arvas M, Baker SE, Chapman J, Chertkov O, Coutinho PM, Cullen D *et al*: Genome sequencing and analysis of the biomass-degrading fungus Trichoderma reesei (syn. Hypocrea jecorina). *Nat Biotechnol* 2008, 26(5):553-560.

37. Dashtban M, Buchkowski R, Qin W: Effect of different carbon sources on cellulase production by *Hypocrea jecorina* (*Trichoderma reesei*) strains. *Int J Biochem Mol Biol* 2011, 2(3).

38. Cianchetta S, Galletti S, Burzi PL, Cerato C: A novel microplate-based screening strategy to assess the cellulolytic potential of Trichoderma strains. *Biotechnology and bioengineering* 2010, 107(3):461-468.

39. Korotkova OG, Semenova MV, Morozova VV, Zorov IN, Sokolova LM, Bubnova TM, Okunev ON, Sinitsyn AP: Isolation and properties of fungal beta-glucosidases. *Biochemistry (Mosc)* 2009, 74(5):569-577.

40. Ghose TK: Measurement of cellulase activities. *Pure Appl Chem* 1987, 59(2):257-268.

41. Dashtban M, Maki M, Leung KT, Mao C, Qin W: Cellulase activities in biomass conversion: measurement methods and comparison. *Critical reviews in biotechnology* 2010, 30(4):302-309.

42.   Xiao Z, Storms R, Tsang A: Microplate-based filter paper assay to measure total cellulase activity. *Biotechnology and bioengineering* 2004, 88(7):832-837.

43.   Zhang Y-HP, Hong J, Ye X: Cellulase assays. *Methods in molecular biology (Clifton, NJ* 2009, 581:213-231.

44.   Cheng Y, Song X, Qin Y, Qu Y: Genome shuffling improves production of cellulase by Penicillium decumbens JU-A10. *J Appl Microbiol* 2009, 107(6):1837-1846.

Chapter 4: Xylitol production by genetically engineered *Trichoderma reesei* strains using barley straw as feedstock

Adapted from: Mehdi Dashtban [1, 2], Greg Kepka [1, 3], Bernhard Seiboth[4] and Wensheng Qin[1, 2*]

[1]Biorefining Research Institute (BRI)
Lakehead University
955 Oliver Road
Thunder Bay, Ontario, P7E 5E1 Canada

[2]Department of Biology
Lakehead University
955 Oliver Road
Thunder Bay, Ontario, P7E 5E1 Canada

[3]Instrumentation Lab
Lakehead University
955 Oliver Road
Thunder Bay, Ontario, P7E 5E1 Canada

[4] Research Division Biotechnology and Microbiology
 Institute of Chemical Engineering
 Vienna University of Technology, Austria

*Corresponding author: Wensheng Qin

4.1 Abstract

Xylitol, a naturally occurring five-carbon sugar alcohol derived from *D*-xylose, is currently in high demand by industries. *Trichoderma reesei*, a prolific industrial cellulase and hemicellulase producing fungus, is able to selectively use *D*-xylose from hemicelluloses for xylitol production. The xylitol production by *T. reesei* can be enhanced by genetic engineering of blocking further xylitol metabolism in the *D*-xylose pathway. We have used two different *T. reesei* strains which are impaired in the further metabolism of xylitol including a single mutant in which the xylitol dehydrogenase gene was deleted (Δ*xdh1*) and a double mutant where additionally *L*-arabinitol-4-dehydrogenase, an enzyme which can partially compensate for xylitol dehydrogenase function, was deleted (Δ*lad1*Δ*xdh1*). Barely straw was first pretreated using NaOH- and Organosolv-pretreatment methods. The highest xylitol production of 6.1 and 13.22 g/L was obtained using medium supplemented with 2% Organosolv-pretreated barley straw and 2% *D*-xylose by the Δ*xdh1* and Δ*lad1*Δ*xdh1* strains, respectively.

Keywords: Agricultural residues, *Trichoderma reesei*, xylitol production

4.2 Introduction

Large amount of lignocellulosic wastes are produced by different industries such as forestry, agriculture and food during pre-harvest, post-harvest and the processing steps (Dashtban et al., 2009). These lignocellulosic wastes are mainly composed of cellulose, hemicellulose and lignin. Recently, there is high interest in using hemicellulose-rich wastes generated as by-products from agricultural and food industries to produce high value bio-product such as xylitol, *D*-mannitol and *D*-xylonic acid (Nygard et al., 2011; Yu et al., 2011). Additionally, these wastes can be further processed to extract their monomeric sugars, such as *D*-xylose and *L*-arabinose which can be fermented to ethanol by microorganisms able to utilize pentose sugars (Dashtban et al., 2009; Seiboth and Metz, 2011). Pretreatment of lignocellulosic

151

waste however is a prerequisite step since lignin is the main barrier for accessing cellulose and

hemicellulose by the catalyzing enzymes (Dashtban et al., 2010b; Taherzadeh and Karimi, 2008).

D-xylose as the monomeric 5-carbon sugar creates the backbone of the β-1,4-xylan which is the

major constituent of plant hemicelluloses (Dashtban et al., 2009). Different microorganisms including

bacteria and fungi can use xylan as a carbon source for their growth. These microorganisms hydrolyze β-

1,4 linkages in xylan to produce oligomers using xylanases. Ultimately, the oligomers can be further

hydrolyzed into D-xylose monomers by β-xylosidase (Dashtban et al., 2009). In fungi such as $T. reesei$,

D-xylose enters into a three-step pathway before entering into the Pentose Phosphate Pathway (PPP)

(Fig.1) (Seiboth et al., 2003). In the first step, D-xylose will be converted by D-xylose-reductase (XYL1;

EC 1.1.1.21) into the 5-carbon sugar alcohol xylitol (Seiboth et al., 2007). Xylitol will be then used to

produce D-xylulose by xylitol dehydrogenase (EC 1.1.1.9). Finally, D-xylulose will be converted into D-

xylulose-5-phosphate by D-xylose kinase (EC 2.7.1.17) and enters into the PPP (Seiboth et al., 2003).

Deletion of the xylitol dehydrogenase gene $xdh1$ in $T. reesei$ (Δ$xdh1$) only partially impairs the growth of

the Δ$xdh1$ strain on D-xylose and reduces the growth rates to about 50% (Seiboth et al., 2003). Thus, it

was suggested that a second enzyme with xylitol dehydrogenase activity in $T. reesei$ would exist. The

responsible enzyme was identified as the L-arabinitol-4-dehydrogenase (LAD1) which could partially

compensate for the loss of XDH1 function in Δ$xdh1$ strains and consequently a strain with deletions in

both dehydrogenases (Δ$lad1$Δ$xdh1$) failed to grow on D-xylose and xylitol (Seiboth et al., 2003). Xylitol

is also the first common intermediate of the interconnected D-xylose and L-arabinose pathways (Seiboth

et al., 2007; Seiboth and Metz, 2011).

Xylitol as a naturally occurring polyalcohol has been used as a natural sweetener due to its high

sweetening power for years. Besides this, it has been used as an anticariogenic for oral health and caries

prevention by preventing the growth of microorganisms involved in tooth decay (Yu et al., 2011).

Additionally, oral administration of xylitol in animal models showed to be important for bone tissue and

affect bone metabolism and ultimately increase bone density (Sato et al., 2011). According to Prakasham

et al., (2009), the annual global market for xylitol is $340 million and is estimated to grow. Xylitol can be produced either chemically using pure *D*-xylose or by applying biotechnological processes including fermentation by microorganisms and/or enzymatic approaches. Although, chemical production of xylitol has some disadvantages such as high cost and energy investment, there is still a case for its industrial production. Biotechnological methods for large-scale xylitol production which can replace the chemical method however are not yet available (Santos et al., 2008). Significant efforts, many of which have been successful, have been made to improve xylitol production by different microorganisms. These include genetic engineered bacteria, yeast and fungi (Ahmad et al., 2012; Akinterinwa et al., 2008; Khankal et al., 2008; Seiboth et al., 2003). Besides, many recent studies focused on using pretreated agricultural residues as the feedstock for xylitol production by xylitol hyper-producing microorganisms. To this end, different lignocellulosic biomass such as corn cobs (El-Batal and Khalaf, 2004), sugarcane bagasse (Santos et al., 2008), and corn stover (Rodrigues et al., 2011) were pretreated applying different pretreatment methods and ultimately the hydrolyzates were used for the xylitol production.

In our study, we investigated the effects of two different pretreatment methods including Organosolv- and NaOH-pretreatment of barley straw. The hydrolyzates were used as the feedstock for xylitol production by two metabolically engineered *T. reesei* strains including single ($\Delta xdh1$) and double ($\Delta lad1\Delta xdh1$) deletion strains. To maximize xylitol production by these strains, different media compositions were also tested. Additionally, hydrolysis efficiency of the enzymes produced by the mutant strains was investigated using untreated and pretreated barley straw.

4.3 Materials and Methods

4.3.1. Chemicals

Chemicals and reagents used in the study were of analytical grade. Barley straw (obtained from Gammondale Farm, Thunder Bay, Canada) was first ground in a Wiley mill, then sieved to less than 20 mesh and dried in an oven to a constant weight at 70 °C before use.

153

4.3.2. Pretreatment of barley straw

Barley straw (50 g of previously ground) was subjected to Organosolv pretreatment (in Dr. Charles Xu's lab, Lakehead University, Canada) using a 1 L autoclave reactor (Autoclave Engineers, USA) and according to the method described by Cheng et al., (2010). Alkali treatment of ground barley straw (2% w/v) was done according to the method described by Deshpande et al., (2009).

4.3.3. Particle size and particle size distribution of untreated and pretreated barley straw

Particle size measurement was done in the instrumentation lab at Lakehead University (Ontario, Canada) using a Malvern Mastersizer 2000 particle size analyzer with Hydro 2000MU (Malvern Instruments Ltd, UK). An ultrasonic probe disperser was applied to all the sample measurements due to the high electro-static charge of the samples. The experiment was done using approximately 5 g of barley straw (untreated, Organosolv- or NaOH-pretreated) according to the manufacture's instruction. The size of the particles were recorded and analyzed three times by the provided manufacture's software using the same SOP.

4.3.4. FTIR analyses of untreated and pretreated barley straw

To investigate the modification of lignin functional groups of pretreated barley straw, Fourier transform infrared spectroscopic (FTIR) spectra (4000 to 800 cm^{-1}) were obtained and compared to untreated barley straw (instrumentation lab, Lakehead University, Ontario, Canada) using a Tensor 37 FTIR (Bruker Optics, Germany) with a resolution of 4 cm^{-1} and 32 scans per sample. The experiment was done using approximately 2 mg of each sample and applying a MIRacle ATR accessory with high-pressure clamp (PIKE Technologies, Madison, WI, USA).

4.3.5. Strains and culture conditions

T. reesei knockout mutant *xdh1* ($\Delta xdh1$) strain and double knockout mutant *lad1 xdh1* ($\Delta lad1\Delta xdh1$) strain were used in xylitol production experiments (Seiboth et al., 2003). The strains were grown and maintained on potato dextrose agar (PDA). Strains were grown in 50 mL of medium described

by Mandels and Andreotti, (1978) (MA-medium) in 250 mL flasks using a rotary shaker (200 rpm) at 30 °C supplemented with different carbon sources at a final concentration of 2% (w/v). The media was autoclaved at 121 °C (15 lb psi) for 15 min.

4.3.6. Inoculum preparation and xylitol production by two mutant T. reesei strains

The greenish conidia from the mutant *T. reesei* strains incubated for 14 days at 30 °C on PDA plates was suspended in 5 mL of sterile saline solution (0.9% w/v, NaCl). Ultimately, gentle filtration was applied in order to separate the spores from the mycelia using 12 layers of lens paper (Fisher Scientific, Canada), followed by spore counting using a Petroff-Hausser cell counter (American Optical, USA). The isolated spores were used to inoculate (1.0×10^7, final concentration) 250 mL flasks containing 50 mL medium (MA-medium) with 2% glycerol (w/v) as the carbon source and incubated at 30 °C for a total of 48 hours. These pre-grown mycelia were then washed three times by MA-medium without any carbon source to remove any residual glycerol. Equal amounts of mycelia (1 g) were then transferred into 250 mL flasks containing 50 mL xylitol production media (MA-medium) in which 2% glycerol (w/v) was substituted with 2% (w/v) barley straw (untreated, Organosolv- or NaOH-pretreated) with or without 2% (w/v) monomeric carbon sources (either *D*-glucose or *D*-xylose). Two biological replicates were done for each experiment. To examine xylitol production, a time course trial of 168 h was conducted at 30 °C. Samples of 500 µL each were taken from each of the flasks every 24 h and were centrifuged at 16060 rcf for 5 min. The supernatant was used as the source for the xylitol measurement as well as xylanases enzyme assays (Gamerith et al., 1992).

4.3.7. Analytical analyses

During the 168 h incubation period, samples of 500 µL were extracted at 24 h intervals and centrifuged (16060 rcf for 5 min). The resultant supernatants were kept at 4 °C for further substrate and product analyses. These supernatants were used to determine extracellular concentration of xylitol produced by the two mutant *T. reesei* strains. The supernatant was appropriately diluted, followed by

filtration using 0.2 μm syringe filters (Ultident, Canada). Ultimately, they were analyzed by High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD) using a Dionex ICS3000 system (Dionex, Sunnyvale, USA) equipped with a 3x150 mm CarboPac PA20 Carbohydrate Column and Guard (Dionex, Sunnyvale, USA). 0.52 M NaOH (isocratic) eluent was used at a flow rate of 0.5 mL min^{-1} and a full loop injection volume of 25 μL. The column was maintained at 30 °C, and a gold (Au) electrode with quadruple potential was used for separation. Applying the same method, either *D*-Xylose or *D*-glucose concentrations in the supernatant were measured only whenever either of them presented in the supernatant (xylitol production experiment, section 3.3). The method was modified in order to determine *D*-glucose, *D*-Xylose and *L*-arabinose concentrations in the supernatant in the case of enzymatic hydrolysis of barley straw (section 3.4), where these sugars were released and mixed together. To this end, 0.36 M NaOH was used as eluent.

4.3.8. Enzyme assays

Xylanases (EC 3.2.1.8) activity was determined by measuring the release of reducing sugars using a dinitrosalicylic acid method (DNS) (Miller, 1959). The reaction mixture containing 25μL of 1% (w/v) birch wood xylan was prepared in 50 mM sodium citrate buffer (pH 4.8), and 25 μL of suitably diluted enzyme solution (culture supernatant) in the same buffer and incubated at 50°C for 10 min. The reaction was stopped by adding 150 μL DNS solution and kept in a boiling water bath for 5 min. Absorbance was read at 570 nm for xylanases activity (Gamerith et al., 1992). The xylanases activity was defined as IU/mL and one international unit (IU) was the amount of enzyme that released 1 μmole of reducing sugar (xylose) equivalents per minute calculated using a xylose standard curve. The background reducing sugars found in the enzyme supernatant were excluded from the results by adding negative controls (enzyme control) in the assay. The absorbance of the enzyme control sets and substrate control were subtracted from the absorbance of the activity assay (Gamerith et al., 1992).

Total cellulase activity was also measured using the DNS method and applying a microplate based filter paper assays according to a method described previously (Dashtban et al., 2011). Total reducing sugars released during the assay were estimated as glucose equivalents and represented as filter paper units (FPU/mL) where one FPU is defined as the amount enzyme that released one μmole of glucose equivalents per min in the assay reaction (Dashtban et al., 2010a). The protein concentration in the supernatant was measured using the Fermentas Bradford Reagent according to the manufacturer's protocol and also a Fermentas bovine serum albumin (BSA) standard set as the standard (Fermentas, Canada).

4.3.9. Enzymatic hydrolysis of barley straw by two T. reesei mutant strains

Hydrolysis activity of the mutant *T. reesei* strains was investigated using 3% (w/v) of barley straw (untreated, Organosolv- or NaOH-pretreated) according to the method described before (Dashtban and Qin, 2012). Substrate hydrolysis was catalyzed using the culture supernatants of the mutant strains grown on MA-medium supplemented with 1% microcrystalline cellulose (MCC) for 168 h. Enzymatic hydrolysis of barley straw was started with 3% substrate concentration in 50 mM sodium citrate buffer at pH 5.0 (supplemented with 1 mM sodium azide to prevent microbial contamination) and equal amount of crude enzyme dosage for both strains at 50 °C for 120 h. A negative control was added in which the crude enzyme was replaced with the buffer. Samples were taken every 24 h and subjected to determination of released reducing sugars (RS) as well as the *D*-glucose, *D*-xylose and *L*-arabinose levels in the supernatant. The released reducing sugars were detected by the DNS method while the *D*-glucose, *D*-xylose and *L*-arabinose concentrations were measured using HPAE-PAD.

4.3.10. Data processing and statistical analysis

All experimental points presented in the study are the average values of two independent repeats. Microsoft Excel spreadsheet was used to collect the data and for determination of average and standard error of the mean. The graphs were created using the software PRISM 5.0.

4.4 Results and discussion

4.4.1. Effect of pretreatment on Particle size and particle size distribution of barley straw

The effect of two different pretreatment methods on barley straw particle size distribution is shown in Fig. 2, A. The particle size profile of untreated barley straw presented a main peak with the volume percentage about 8.5% at 700 μm. Additionally, smaller particle size with very low percentage of particles was observed for untreated barley straw in the region ranging from 2-110 μm. However, different profiles were obtained for NaOH- and Organosolv-pretreated barley straw compared to untreated barely straw and the main peak was shifted to150 and 70 μm for the treatments, respectively (Fig. 2, A). In the bioconversion process, pretreatment of lignocellulosic biomass becomes an essential step before hydrolysis takes place. This is due to the fact that the hydrolysis of untreated lignocellulosic biomass is very slow which leads to lower product yield (Dashtban et al., 2009). It has been shown that particle size affects bioconversion of lignocellulosic biomass in which smaller particle size positively correlated with higher conversion rates (Vidal et al., 2011). For example, a study by Pedersen and Meyer (2009) showed that reduced particle size in pretreated wheat straw with about 53-149 μm particle sizes (oxidized) released more *D*-glucose and *D*-xylose compared to the untreated sample. Our results (section 3.5, enzymatic hydrolysis of biomass) also showed higher amounts of reducing sugars released from both pretreated barley straw compared to the untreated sample. However, it has been suggested that other important factors such as cellulose crystallinity also plays a major role in the hydrolysis process beside the particle size in pretreated biomass (da Silva et al., 2010; Dashtban et al., 2009).

4.4.2. FTIR analyses of untreated and pretreated barley straw

The FTIR was used to qualitatively determine the chemical changes in pretreated barley straw. The FTIR spectra for NaOH- and Organosolv-pretreated barley straw samples were compared to untreated barley straw and shown in Fig. 2 (B and C) while the main assignment of functional groups in FTIR bands are listed in Table 1. The general trends of chemical changes in barley straw after the

pretreatments (4000 to 800 cm^{-1}) are compared to untreated barley straw (Fig. 2, B). The peaks around

3,350 cm^{-1} are associated with O-H stretching of hydrogen bonds in cellulose (Chundawat et al., 2007;

Hsu et al., 2010). Untreated and NaOH-pretreated barley straw showed similar absorption peaks however,

at decreased levels in the Organosolv-pretreated barley straw. This indicated that similar to untreated

barley straw most of the crystalline cellulose in NaOH-pretreated barley was not disrupted by the

treatment. Organosolv-pretreatment however showed to be a more effective method and decreased the

crystallinity of barley straw. The band at 2,900 cm^{-1} is attributed to C-H stretching of methylene cellulose

which was less intense in both pretreatment methods used in this study (Hsu et al., 2010; Kumar et al.,

2009). The bands at 1200-1000 cm^{-1} representing structural features of carbohydrates and polysaccharides

were also less intense in the pretreated barley straw compared to the untreated sample (Hsu et al., 2010;

Wang et al., 2010) (Fig. 2, C). The peaks at 1,610 and 1,516 cm^{-1} are attributed to aromatic skeletal

stretching in lignin and the bands at 2,860, 1,460 and 1,425 cm^{-1} are attributed to C-H deformation within

the methyl groups of lignin (Guo et al., 2008; Hsu et al., 2010). Both pretreated barley straw samples

showed decreased adsorption in bands compared to the untreated sample suggesting that lignin was

removed in the pretreated samples. The band at 1,720 cm^{-1} is attributed to ester linkage C=O which is

categorized as hemicellulose-lignin linkage and/or the acetyl group existing in hemicellulose structure

(Chundawat et al., 2007; Hsu et al., 2010). Our results showed that the band adsorption in the

Organosolv-pretreated barley straw decreased suggesting that the linkage between lignin and

hemicellulose was removed. However, NaOH-pretreated barley straw showed almost the same band

adsorption as untreated barley straw. The decrease in the 1,720 cm^{-1} band is suggested to be related to

delignification of hemicellulose in the pretreated sample (Chundawat et al., 2007). The band at 1,640 cm^{-1}

attributed to the aldehyde group in hemicellulose and the decrease in the band represents hemicellulose

hydrolysis which was the case for the both pretreatment methods used in the study (Chundawat et al.,

2007) (Fig. 2, C).

4.4.3. Xylitol production by the mutant T. reesei strains and the effect of different pretreatment methods on the production

Two *T. reesei* strains which are blocked in the step from xylitol to D-xylulose were used for the xylitol production. Strain Δ*xdh1* is partially impaired in xylitol oxidation while strain Δ*xdh1Δlad1* is completely blocked in the further conversion of xylitol. Barley straw was used as the complex medium and also as an example for the application of agricultural waste. This would be essential to investigate since usually complex lignocellulosic wastes (agriculture or forestry-based) are targeted for bio-product productions by different microorganisms. In this study, MA-medium was supplemented with barley straw containing relatively high hemicellulose content (26.46 ± 3.67%). Barley straw was either pretreated with Organosolv or 2% NaOH. Alkali or Organosolv pretreatment methods have been shown to be able to remove lignin and possibly also part of the hemicellulose fraction and enhance saccharification rate by increasing cellulose accessibility to cellulases (Taherzadeh and Karimi, 2008). The xylitol production by the two *T. reesei* strains using untreated, Organosolv- and NaOH-pretreated barley straw is shown in Fig. 3 (A-F). The media was also supplemented by either 2% *D*-glucose or *D*-xylose in order to enhance the production and increase the cell growth on the complex media. As shown in Fig. 3 (A and B), very low xylitol production was observed using untreated, Organosolv- and NaOH-pretreated barley straw in the absence of the supplementary sugars (*D*-glucose or *D*-xylose). The maximum production of 1.25 and 1.13 g/L was obtained for single and double deletion strains using untreated and NaOH-pretreated barley straw after 96 and 168 h of incubation, respectively (Fig. 3, A and B). Our results showed that adding *D*-glucose to the complex media did not improve xylitol production by either strain (Fig. 3, C and D). This confirmed that xylitol production is through a separate pathway and does not directly correlate with the cell growth on *D*-glucose. A study by Prathumpai et al., (2004) also showed that wild-type or mutant *Aspergillus nidulans* strains produced very low amount of xylitol (58.7 and 12.7 mg/L, respectively) either in the presence or absence of *D*-glucose in the fermentation medium. The highest xylitol production however was obtained when the media were additionally supplemented with *D*-xylose (Fig. 3, E and F).

For the Δ*xdh1* strain, the maximum xylitol production of 6.1 g/L was obtained after 96 h of incubation using MA-medium supplemented with 2% Organosolv-pretreated barley straw and 2% *D*-xylose (Fig. 3, E). The Δ*xdh1*Δ*lad1* strain however, produced 2.17-fold more xylitol (13.22 g/L) compared to the single mutant strain after 168 h of incubation using the same medium condition (Fig. 3, F). This is in a good accordance with the previous studies where a partial compensation for the loss of *xdh1* function in the Δ*xdh1* strain was observed (Seiboth et al., 2003). Lower xylitol was produced using other media compositions including untreated and NaOH-pretreated barley straw supplemented with *D*-xylose. This suggests that the Organosolv pretreatment method favors xylitol production by both strains from barley straw. A study by Prakash et al., (2011) also showed that higher xylitol production by *Debaryomyces hansenii* was observed when the hemicellulose fraction from sugarcane bagasse was supplemented with 2% *D*-xylose.

D-Glucose and *D*-xylose consumption by the two strains were monitored over the time course study (Fig. 3, C-F). As expected, both strains showed high *D*-glucose consumption within a short time and *D*-glucose concentration in the medium decreased from 20 g/L to about 5 g/L after the first 24 h of incubation (Fig. 3, C and D). Our results showed that *D*-glucose concentration in the medium remained at almost the same level over the rest of the incubation times. However, the two strains showed different trends for *D*-xylose consumption (Fig. 3, E-F). *D*-xylose was slowly used by the Δ*xdh1* strain over the time point study and the concentration gradually decreased from 20 g/L to about 0.32 g/L after 168 h of incubation (Fig. 3, E). However, for the Δ*xdh1*Δ*lad1* strain no major changes in *D*-xylose concentrations were observed over the whole period and about the same amount of *D*-xylose was found at the end of the cultivation (Fig. 3, F).

4.4.4. Xylanases activity of the mutant T. reesei strains and the effect of different pretreatments on the activity

Xylanases activity of the two *T. reesei* strains grown on MA-media supplemented with barley straw (untreated or pretreated) and monomeric sugars (section 3.3) was also investigated here (Fig. 4, A-

F). The single Δ*xdh1* strain showed high xylanases activity using MA-media supplemented with only 2% barley straw (Organosolv- or NaOH-pretreated) with the highest activity at about 336 (IU/mL) after 168 h of incubation using MA-medium supplemented with NaOH-pretreated barley straw (Fig. 4, A). The Δ*xdh1*Δ*lad1* strain however showed high xylanases activity only when the MA-medium was supplemented with Organosolv-pretreated barley straw at about 292 IU/mL after 120 h of incubation (Fig. 4, B). Both strains showed the same trend for xylanases activity when the medium was additionally supplemented with 2% *D*-glucose (Fig. 4, C and D). Our results indicated that addition of *D*-glucose lowers the xylanases activity in most cases except when NaOH-pretreated barley straw was used. In this case xylanases activities of 253 and 267 IU/mL were obtained for Δ*xdh1* and Δ*xdh1*Δ*lad1* strains grown on MA-medium supplemented with 2% NaOH-pretreated barley straw and 2% *D*-glucose after 144 and 168 h of incubation, respectively (Fig. 4, C and D). Lower xylanases activity was also obtained when the medium was supplemented by *D*-xylose (Fig. 4, E and F). However, the Δ*xdh1*Δ*lad1* strain showed higher activity compared to the single Δ*xdh1* strain. Specifically, the Δ*xdh1*Δ*lad1* strain grown on MA-medium supplemented with 2% Organosolv-pretreated barely straw and 2% *D*-xylose showed high xylanases activity at about 338 IU/mL after 144 h of incubation (Fig. 4, F). This is in accordance with the xylitol production results (Fig. 3, F, section 3.3) where the highest xylitol production by the Δ*xdh1*Δ*lad1* strain was obtained.

4.4.5. Enzymatic hydrolysis of biomass by the mutant T. reesei strains

Efficient hydrolysis is an important step in the bioconversion process where initially lignocellulosic polymers including cellulose and hemicelluloses break down to their monomeric soluble sugars (Dashtban et al., 2009). Thus, many studies have been focused on improving lignocellulolytic activity of different microorganisms in addition to their ability to produce value added bio-products (Dashtban et al., 2009). To this end, the hydrolysis efficiency of the two mutant *T. reesei* strains using barley straw was investigated here. In order to choose the right medium condition for the efficient hydrolysis activity, both strains were grown in different medium conditions and their total cellulases

activity as well as xylanases activity was monitored over a 168 h time course study (Fig. 5, A-D). In this experiment, MA-medium was supplemented with 1% microcrystalline cellulose (MCC) based on our previous study where the highest cellulase activity was obtained in three *T. reesei* strains (Dashtban et al., 2011).

Our results showed that high total cellulase activity was seen for both mutant strains only when grown on MA-medium supplemented with 1% MCC or a combination of MCC and Organosolv-pretreated barley straw (Fig. 5, A and B). Total cellulase activity reached 20.83 and 14.46 FPU/mL using MA-medium supplemented with either 1% MCC or 1% MCC plus 1% Organosolv-pretreated barley straw for single or double mutant strains after 168 h of incubation, respectively (Fig. 5, A and B). However, very low cellulase activity was obtained for both strains using MA-medium supplemented with 1% MCC in combination with 1% NaOH-pretreated barley straw. In the case of xylanases activity however, overall very high activity was obtained for both strains under the same culture condition over the entire time point study (Fig. 5, C and D). For the single mutant strain, the highest xylanases activity of about 281 IU/mL was obtained when MA-medium was supplemented with 1% Organosolv-pretreated barley straw in addition to 1% MCC (Fig. 5, C). The double mutant strain showed higher xylanases activity at about 342 IU/mL using MA-medium only supplemented with 1% MCC after 168 h of incubation (Fig. 5, D). Since high cellulases activity as well as high xylanases activity were obtained for both mutant strains using MA-medium supplemented with 1% MCC, the culture supernatants were used as the enzyme source for the following enzymatic hydrolysis of biomass experiment.

Hydrolysis efficiency of both mutant strains was tested using 3% barley straw as the lignocellulosic biomass over a 120 h incubation time (Fig. 6, A-F). For the untreated barley straw (Fig. 6, A and B), both single and double mutant strains released the highest reducing sugars (RS) at the last time point (120 h) over the studied time points with about 200 and 230 mg/g, respectively. *D*-Glucose, *D*-xylose and *L*-arabinose released by both strains using untreated barley straw were also monitored over the time points. The single mutant strain produced about 110, 41 and 5 mg/g, *D*-glucose, *D*-xylose and *L*-

arabinose after 120 h of incubation while the double mutant strain was able to release about 115, 48 and 7 mg/g, *D*-glucose, *D*-xylose and *L*-arabinose after 120 h of incubation, respectively (Fig. 6, A and B).

Hydrolysis efficiency of both mutant strains was tested using Organosolv-pretreated barley straw (Fig. 6, C and D). The results indicated that both strains released higher amounts of RS compared to untreated barley straw. The amount of released RS gradually increased over the time point study with the highest production of 592 and 600 mg/g after 120 h of incubation for single and double mutant strains, respectively (Fig. 6, C and D). These were 2.96- and 2.59-fold higher than the maximum released RS from untreated barley straw by both single and double mutant strains, respectively. In the case of *D*-glucose, the maximum production of 537 and 479 mg/g was achieved for single and double mutant strains after 120 h of incubation. This suggests that Organosolv-pretreatment significantly increased the accessibility of cellulose in barley straw leading to higher levels of release of *D*-glucose compared to untreated barley straw (Fig. 6, C and D). In the case of *D*-xylose and *L*-arabinose however, no detectable sugars were released by either strains indicating that most of the hemicellulose part of barley straw was removed following the Organosolv-pretreatment. This is in good accordance with the results obtained by Pan et al studies where most of the monomeric and oligomeric hemicellulosic sugars of Poplar became water-soluble after Organosolv-pretreatment and separated from solid residue (mostly cellulose) (Pan et al., 2006).

Similar results with high released RS were also obtained when NaOH-pretreated barley straw was used as the substrate for the enzymatic hydrolysis experiment (Fig. 6, E and F). After 120 h of incubation time, the maximum RS released was obtained for both single and double mutant strains (692 and 685 mg/g, respectively), which were 3.46- and 2.96-fold higher than the released RS from untreated barley straw by single and double mutant strains, respectively (Fig. 6, E and F). The single mutant strain produced about 417, 177 and 17 mg/g, *D*-glucose, *D*-xylose and *L*-arabinose after 120 h of incubation which was 3.78-, 4.33- and 3.2-fold higher than the released sugars using untreated barley straw, respectively. The double mutant strain however produced about 404, 155 and 17 mg/g, *D*-glucose, *D*-

xylose and *L*-arabinose after 120 h of incubation which was 3.49-, 3.21- and 2.42-fold higher than the released sugars using untreated barley straw, respectively (Fig. 6, E and F).

4.4. Conclusion

In our study, we have used two *T. reesei* mutant strains as the cell factories for xylitol production. Barley straw was used as an example of agricultural residue. Barley straw was subjected to two pretreatment methods including NaOH- and Organosolv-pretreatment. High xylitol production was achieved when barley straw (untreated or pretreated) was supplemented with 2% *D*-xylose whereas *D*-glucose supplement did not lead to any significant difference. The production reached to 6.1 and 13.22 g/L using medium supplemented with 2% Organosolv-pretreated barley straw and 2% *D*-xylose by single and double mutant strains, respectively. Saccharification of pretreated barley straw showed that NaOH-pretreatment significantly increases the accessibility of cellulose/hemicellulose leading to a greater release of reducing sugars, *D*-glucose, *D*-xylose and *L*-arabinose compared to untreated barley straw. Hydrolysis of Organosolv-pretreatment barley straw led to higher amounts of released *D*-glucose compared to untreated barley straw however, no detectable *D*-xylose or *L*-arabinose were found in the hydrolyzates using both strains. This indicated that Organosolv-pretreatment removed most available hemicellulose in barley straw. Organosolv-pretreatment was more effective than NaOH-pretreatment since it significantly increased xylitol production by both *T. reesei* strains. However, this was only the case when the medium was also supplemented with 2% *D*-xylose. This suggests that beside the accessibility of xylan, *D*-xylose concentration is also important for the xylitol production. The results suggest that agricultural residues, such as barley straw, could be a suitable resource for bioconversion to produce value-added products such as xylitol.

Acknowledgement

Table 1. Main assignments of lignin and polysaccharides FTIR bands of barley straw.

|	Wavenumber (cm⁻¹)	Assignments	References
Lignin			
	2860	C-H stretching of methyl, methylene or methane groups of lignin	(Guo et al., 2008; Hsu et al., 2010)
	1610	Aromatic skeletal vibrations	(Guo et al., 2008; Hsu et al., 2010)
	1516	Aromatic C=Cstretching from aromatic ring of lignin plus C=O stretch	(Guo et al., 2008; Hsu et al., 2010)
	1460	Aromatic C-H vibrations	(Guo et al., 2008; Hsu et al., 2010)
	1425	Aromatic C=C stretching from aromatic ring	(Guo et al., 2008; Hsu et al., 2010)
Polysaccharides	3350	O-H stretching (hydrogen bonds in cellulose)	(Chundawat et al., 2007; Kumar et al., 2009)
	2900	C-H stretching (methyl/methylene group of cellulose)	(Hsu et al., 2010; Kumar et al., 2009)
	1720	Ester linkage C=O in hemicellulose-lignin linkage	(Chundawat et al., 2007; Hsu et al., 2010)
	1640	Aldehyde group in hemicellulose	(Chundawat et al., 2007)
	1030-1170	O-H stretching in alcohols	(Chundawat et al., 2007)
	1098	C-O, C-C stretching or C-OH bending in hemicellulose and cellulose	(Wang et al., 2010)
	900	C-O-C vibrations at β-glucosidic linkages in hemicellulose and cellulose)	(Wang et al., 2010)
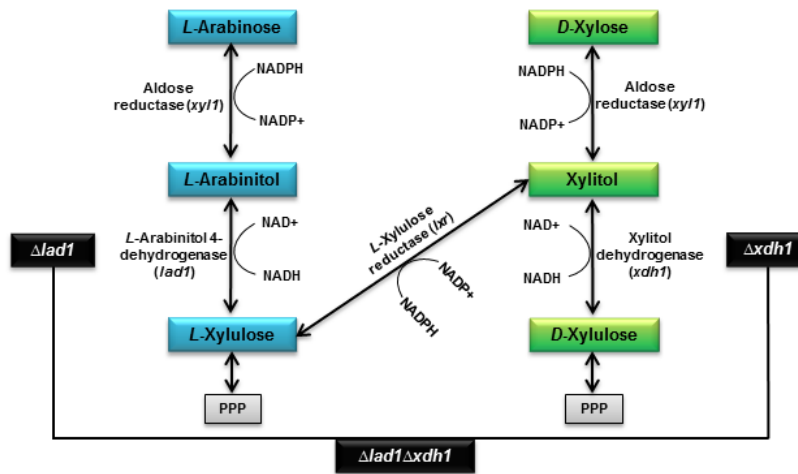
Fig. 1. *T. reesei D*-xylose and *L*-arabinose catabolism pathway and created *T. reesei* single (Δ*xdh1*) and double mutant (Δ*lad1*Δ*xdh1*) strains (modified from Seiboth et al., 2007).
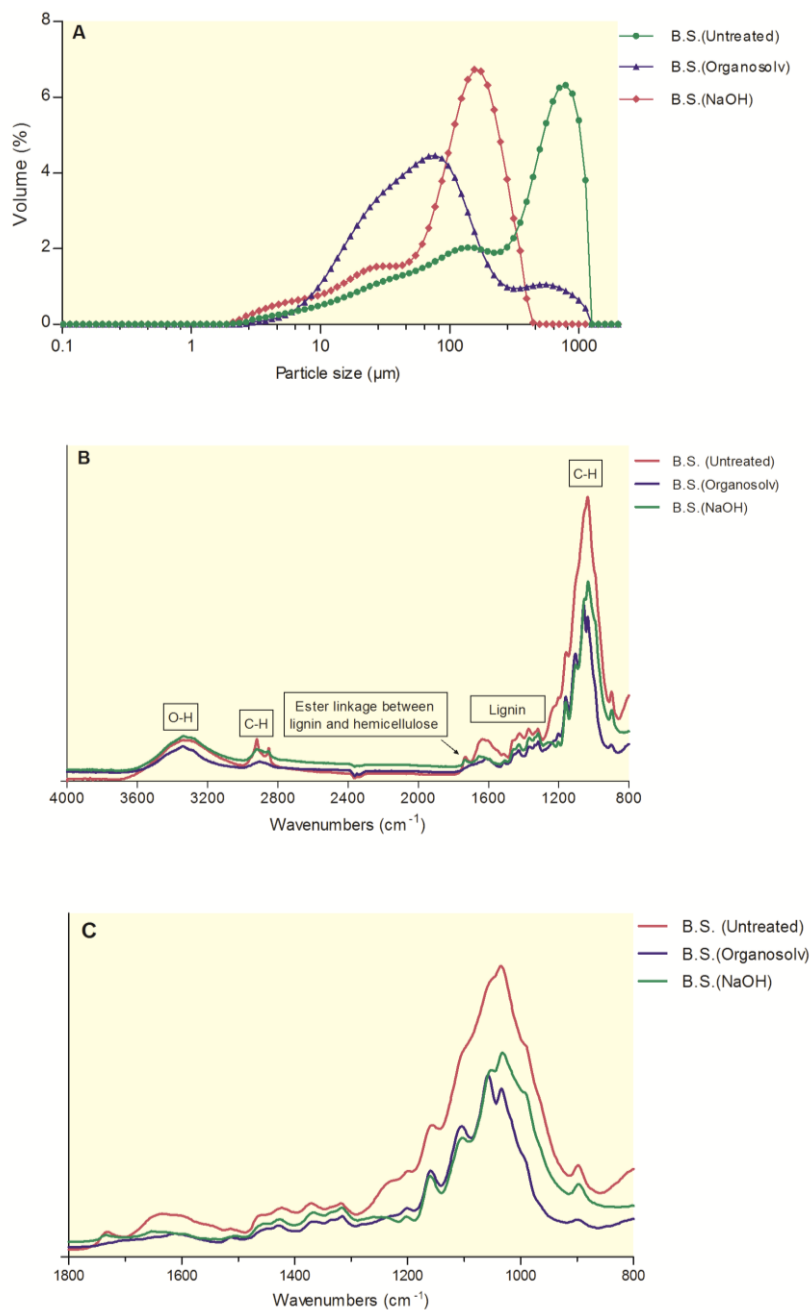
Fig. 2. (A) Particle size distribution of untreated barley straw as well as NaOH- and Organosolv-pretreated. (B and C) FTIR spectra for untreated and NaOH- and Organosolv-pretreated barley straw. (B) General trends of chemical changes in barley straw after the treatments and (C) selected FTIR spectra (1800 to 800 cm⁻¹) region for functional changes after the treatments.
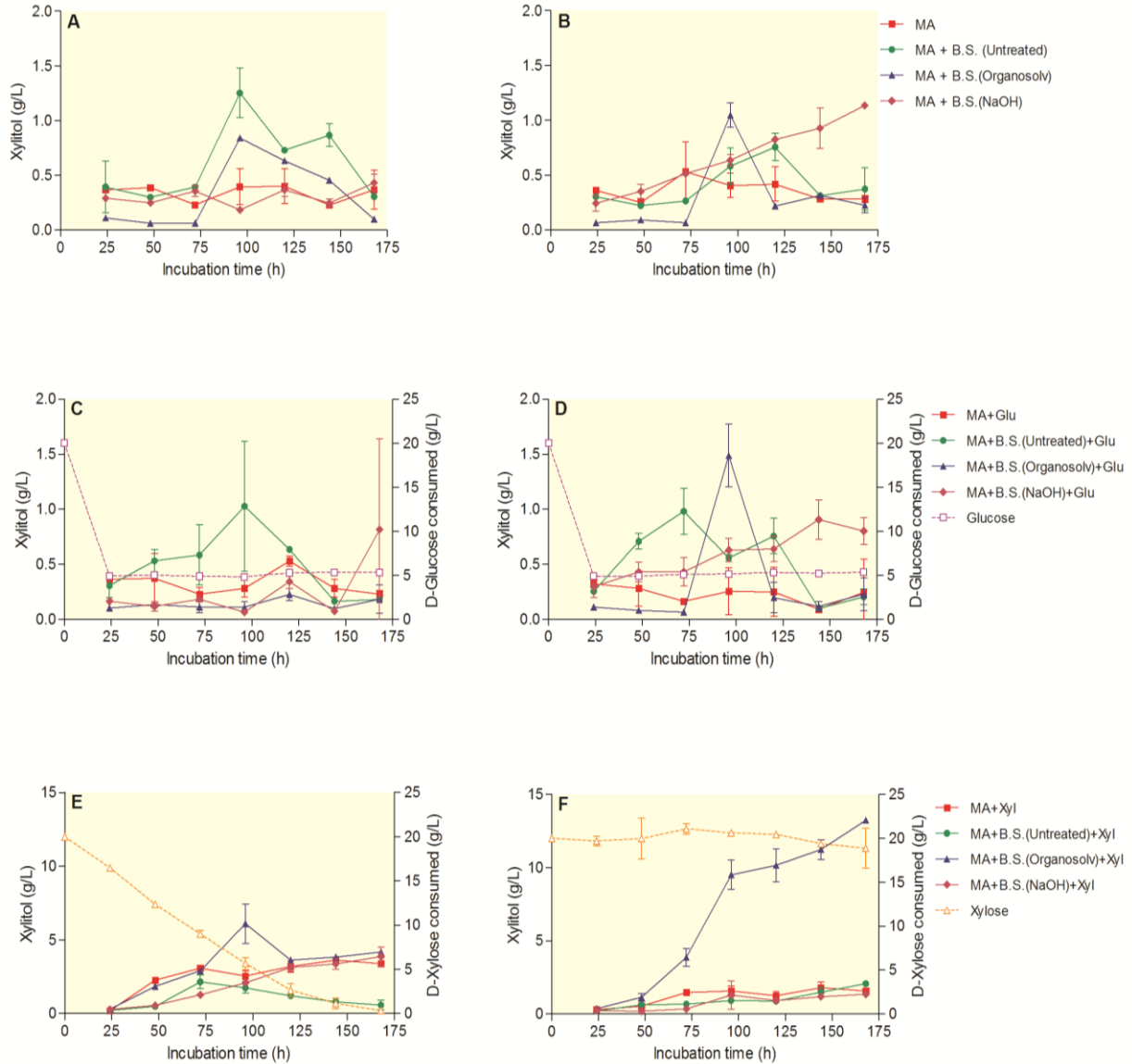
Fig. 3. Xylitol production by two mutant *T. reesei* strains using barley straw. (A and B) xylitol production by single or double mutant strains on MA-medium supplemented with 2% barley straw, respectively. (C and D) Xylitol production by single or double mutant strains on MA-medium supplemented with 2% barley straw in addition to 2% *D*-glucose, respectively. (E and F) Xylitol production by single or double mutant strains on MA-medium supplemented with 2% barley straw in addition to 2% *D*-xylose, respectively. As the controls, xylitol production by both strains was measured using MA-medium without any carbon source (MA, in A and B), or MA-medium supplemented either with 2% *D*-glucose (MA + Glu, in C and D) or *D*-xylose (MA + Xyl, E and F). *D*-Glucose (C and D) or *D*-xylose consumption by both strains (E and F) are also measured over the time point study. Data is represented as the mean of two independent experiments and error bars denote standard error of the mean.
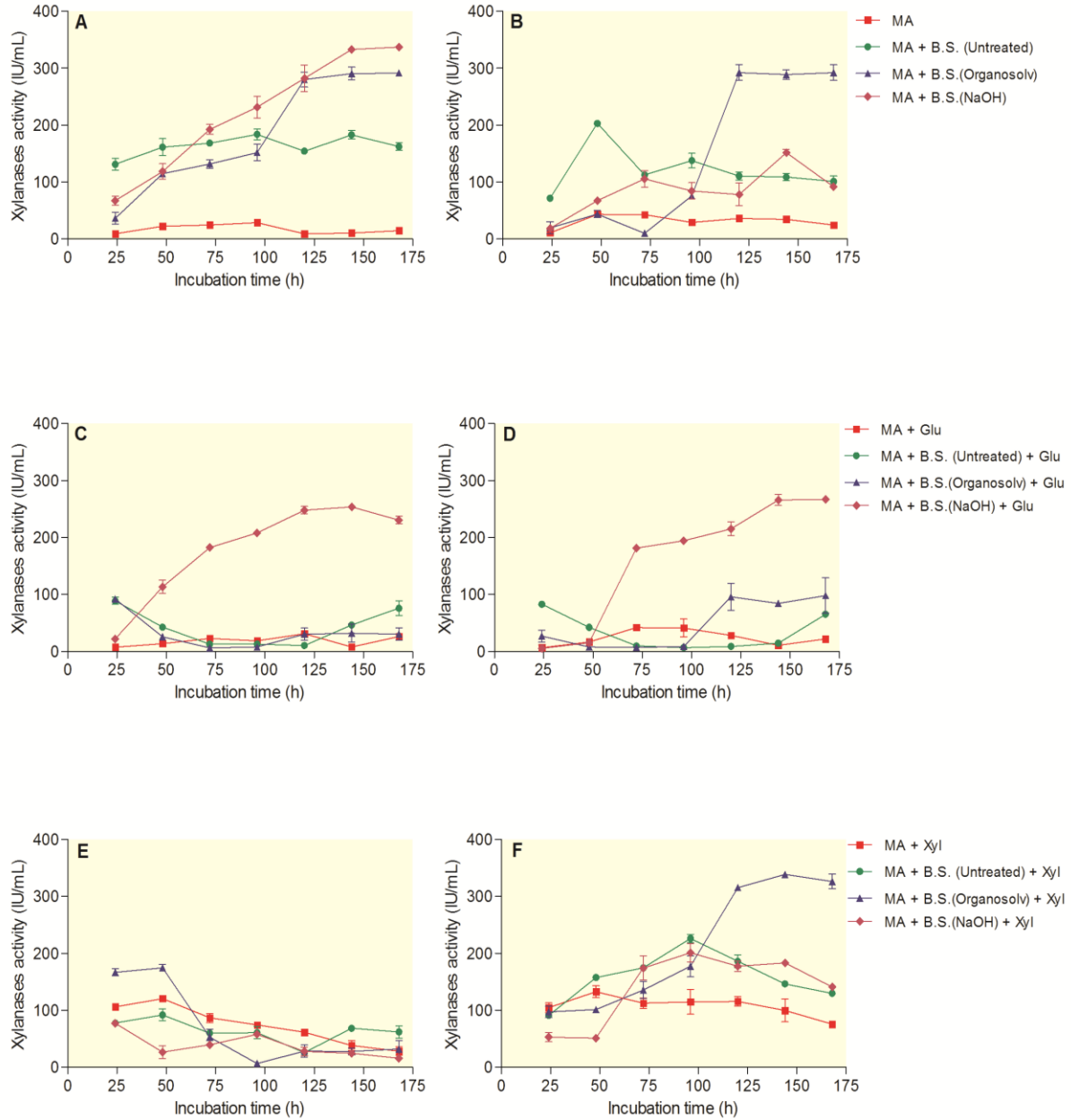
170

Fig. 4. Xylanases activity of two mutant *T. reesei* strains grown on MA-media used in xylitol production experiment (Fig. 3). (A and B) Xylanases activity of single or double mutant strains on MA-medium supplemented with 2% barley straw, respectively. (C and D) Xylanases activity of single or double mutant strains on MA-medium supplemented with 2% barley straw in addition to 2% *D*-glucose, respectively. (E and F) Xylanases activity of single or double mutant strains on MA-medium supplemented with 2% barley straw in addition to 2%*D*-xylose, respectively. Data is represented as the mean of two independent experiments and error bars denote standard error of the mean.
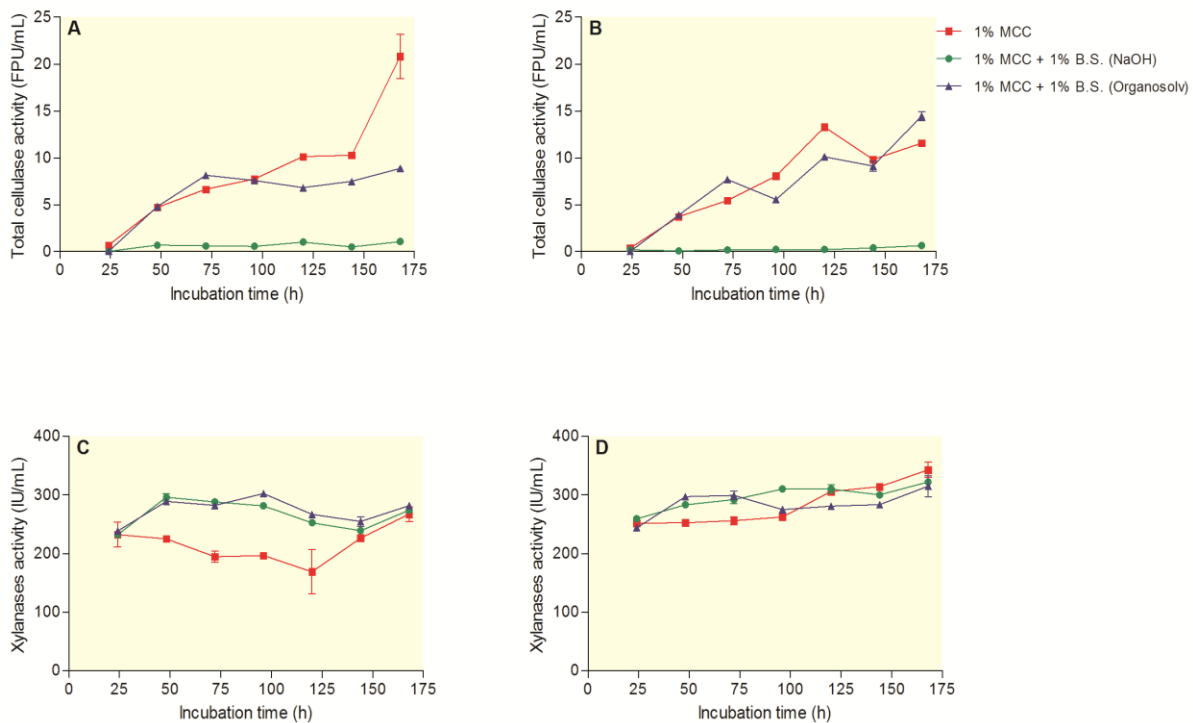
171

Fig. 5. Cellulases and xylanases activities of two mutant *T. reesei* strains grown on MA-media.(A and B) Cellulases activity of single or double mutant strains grown on MA-medium supplemented with either 1% microcrystalline cellulose (MCC) or a combination of 1% MCC and 1% barley straw (untreated or pretreated), respectively. (C and D) Xylanases activity of single or double mutant strains grown on MA-medium supplemented with either1% microcrystalline cellulose (MCC) or a combination of 1% MCC and 1% barley straw (untreated or pretreated), respectively. Data is represented as the mean of two independent experiments and error bars denote standard error of the mean.
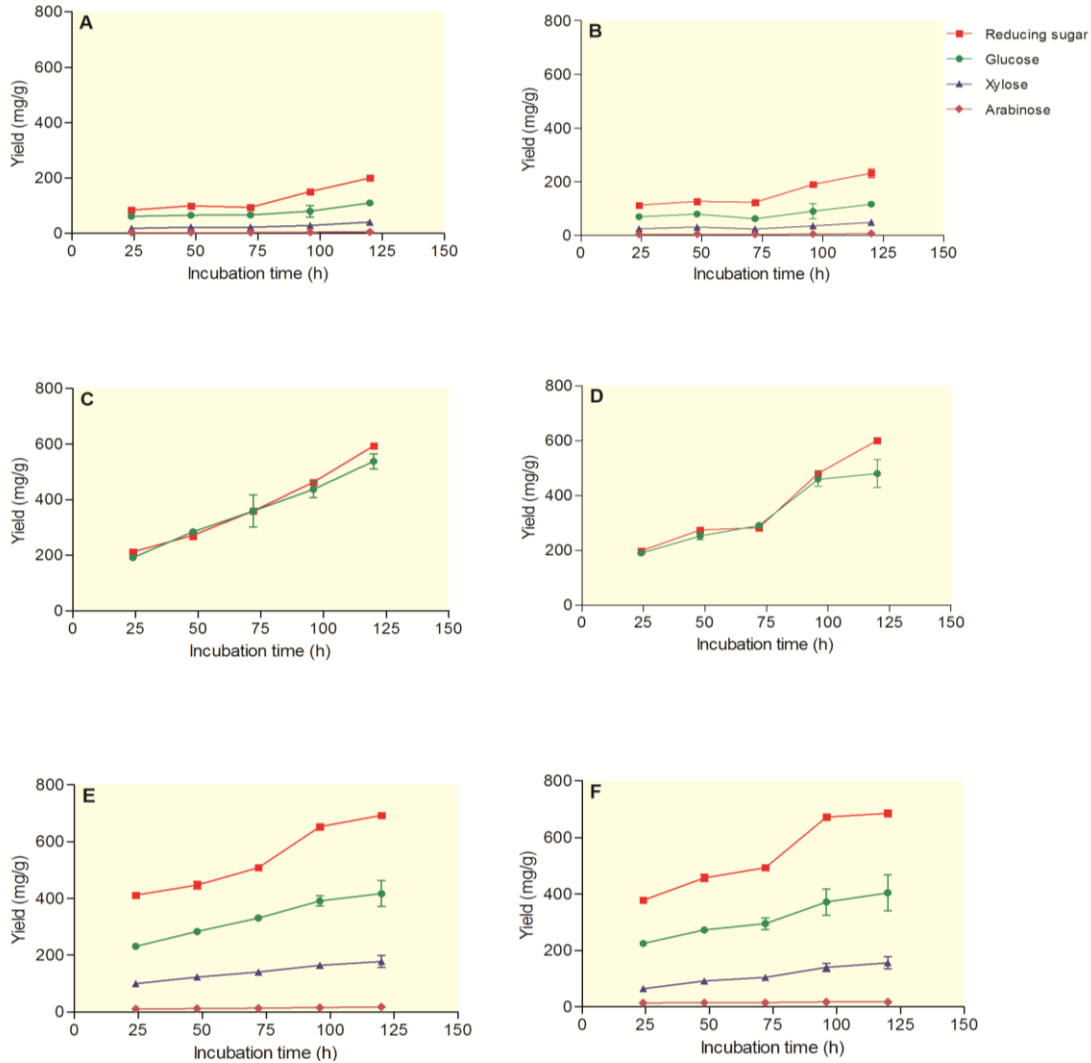
Fig. 6. Enzymatic hydrolysis of barley straw by two mutant *T. reesei* strains. (A and B) Reducing sugars (RS), *D*-glucose, *D*-xylose and *L*-arabinose yield (mg/g) released by single or double mutant strains using untreated barley straw, respectively. (C and D) Reducing sugars, *D*-glucose, *D*-xylose and *L*-arabinose yield (mg/g) released by single or double mutant strains using NaOH-pretreated barley straw, respectively. (E and F) Reducing sugars, *D*-glucose, *D*-xylose and *L*-arabinose yield (mg/g) released by single or double mutant strains using Organosolv-pretreated barley straw, respectively. 1000μl of the culture supernatants previously grown on MA-medium supplemented with 1% MCC (as the enzyme source) were added to tubes containing 1000 μl of 50 mM sodium citrate buffer (pH 5.0) and 0.06 g (3%) barley straw. The tubes were incubated at 50 °C for 120 h and reducing sugars as well as *D*-glucose and *D*-xylose released were measured every 24 h. Data is represented as the mean of two independent experiments and error bars denote standard error of the mean. Total protein loading of 0.5 mg (single or double mutant culture supernatant) was used and the volume was adjusted by the buffer.

References

Ahmad, I., Shim, W. Y., Jeon, W. Y., Yoon, B. H., Kim, J. H., 2012. Enhancement of xylitol production in Candida tropicalis by co-expression of two genes involved in pentose phosphate pathway. Bioprocess Biosyst Eng. 35**,** 199-204.

Akinterinwa, O., Khankal, R., Cirino, P. C., 2008. Metabolic engineering for bioproduction of sugar alcohols. Curr Opin Biotechnol. 19**,** 461-7.

Cheng, S., I., D. c., Wang, M., Leitch, M., Xu, M., 2010. Highly Efficient Liquefaction of Woody Biomass in Hot-Compressed Alcohol−Water Co-solvents. Energy Fuels. 24 4659-4667.

Chundawat, S. P., Venkatesh, B., Dale, B. E., 2007. Effect of particle size based separation of milled corn stover on AFEX pretreatment and enzymatic digestibility. Biotechnol Bioeng. 96**,** 219-31.

da Silva, A. S., Inoue, H., Endo, T., Yano, S., Bon, E. P., 2010. Milling pretreatment of sugarcane bagasse and straw for enzymatic hydrolysis and ethanol fermentation. Bioresour Technol. 101**,** 7402-9.

Dashtban, M., Buchkowski, R., Qin, W., 2011. Effect of different carbon sources on cellulase production by Hypocrea jecorina (Trichoderma reesei) strains. Int J Biochem Mol Biol. 2**,** 274-286.

Dashtban, M., Maki, M., Leung, K. T., Mao, C., Qin, W., 2010a. Cellulase activities in biomass conversion: measurement methods and comparison. Crit Rev Biotechnol. 30**,** 302-9.

Dashtban, M., Qin, W., 2012. Overexpression of an exotic thermotolerant β-glucosidase in *Trichoderma reesei* and its significant increase in cellulolytic activity and saccharification of barley straw Microbial Cell Factories. 11:63.

Dashtban, M., Schraft, H., Qin, W., 2009. Fungal bioconversion of lignocellulosic residues; opportunities & perspectives. Int J Biol Sci. 5**,** 578-95.

Dashtban, M., Schraft, H., Syed, T. A., Qin, W., 2010b. Fungal biodegradation and enzymatic modification of lignin. Int J Biochem Mol Biol. 1**,** 36-50.

Deshpande, P., Nair, S., Khedkar, S., 2009. Water Hyacinth as Carbon Source for the Production of Cellulase by Trichoderma reesei. Appl Biochem Biotechnol 158**,** 552–560.

El-Batal, I. A., Khalaf, S. A., 2004. Xylitol Production from Corn Cobs Hemicellulosic Hydrolysate by *Candida tropicalis* Immobilized Cells in Hydrogel Copolymer Carrier. International Journal of Agriculture & Biology. 6**,** 1066-1073.

Gamerith, G., Groicher, R., Zeilinger, S., Herzog, P., Kubicek, C. P., 1992. Cellulase-poor xylanases produced by *Trichoderma reesei* RUT C-30 on hemicellulose substrates. Appl Microbiol Biotechnol 38**,** 315-322.

Guo, G. L., Chen, W. H., Men, L. C., Hwang, W. S., 2008. Characterization of dilute acid pretreatment of silvergrass for ethanol production. Bioresour Technol. 99**,** 6046-53.

Hsu, T. C., Guo, G. L., Chen, W. H., Hwang, W. S., 2010. Effect of dilute acid pretreatment of rice straw on structural properties and enzymatic hydrolysis. Bioresour Technol. 101**,** 4907-13.

Khankal, R., Chin, J. W., Cirino, P. C., 2008. Role of xylose transporters in xylitol production from engineered Escherichia coli. J Biotechnol. 134**,** 246-52.

Kumar, R., Mago, G., Balan, V., Wyman, C. E., 2009. Physical and chemical characterizations of corn stover and poplar solids resulting from leading pretreatment technologies. Bioresour Technol. 100**,** 3948-3962.

Mandels, M. M., Andreotti, R. E., 1978. The cellulose to cellulase fermentation. Proc. Biochem. 13**,** 6-13.
Miller, G. L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem. 31**,** 426-428.

Nygard, Y., Toivari, M. H., Penttila, M., Ruohonen, L., Wiebe, M. G., 2011. Bioconversion of D-xylose to D-xylonate with Kluyveromyces lactis. Metab Eng. 13**,** 383-91.

Pan, X., Gilkes, N., Kadla, J., Pye, K., Saka, S., Gregg, D., Ehara, K., Xie, D., Lam, D., Saddler, J., 2006. Bioconversion of hybrid poplar to ethanol and co-products using an organosolv fractionation process: optimization of process yields. Biotechnol Bioeng. 94**,** 851-61.

Pedersen, M., Meyer, A. S., 2009. Influence of substrate particle size and wet oxidation on physical surface structures and enzymatic hydrolysis of wheat straw. Biotechnol Prog. 25**,** 399-408.

Prakash, G., Varma, A. J., Prabhune, A., Shouche, Y., Rao, M., 2011. Microbial production of xylitol from D-xylose and sugarcane bagasse hemicellulose using newly isolated thermotolerant yeast Debaryomyces hansenii. Bioresour Technol. 102**,** 3304-8.

Prakasham, R., Rao, R., Hobbs, P., 2009. Current trends in biotechnological production of xylitol and future prospects. Curr Trends Biotechnol Pharm. 3**,** 8-36.

Prathumpai, W., McIntyre, M., Nielsen, J., 2004. The effect of CreA in glucose and xylose catabolism in Aspergillus nidulans. Appl Microbiol Biotechnol. 63**,** 748-53.

Rodrigues, R. C., Kenealy, W. R., Jeffries, T. W., 2011. Xylitol production from DEO hydrolysate of corn stover by Pichia stipitis YS-30. J Ind Microbiol Biotechnol. 38**,** 1649-55.

Santos, D. T., Sarrouh, B. F., Rivaldi, J. D., A., C., Silva, S. S., 2008. Use of sugarcane bagasse as biomaterial for cell immobilization for xylitol production. Journal of Food Engineering. 86**,** 542-548.

Sato, H., Ide, Y., Nasu, M., Numabe, Y., 2011. The effects of oral xylitol administration on bone density in rat femur. Odontology. 99**,** 28-33.

Seiboth, B., Gamauf, C., Pail, M., Hartl, L., Kubicek, C. P., 2007. The D-xylose reductase of Hypocrea jecorina is the major aldose reductase in pentose and D-galactose catabolism and necessary for beta-galactosidase and cellulase induction by lactose. Mol Microbiol. 66**,** 890-900.

Seiboth, B., Hartl, L., Pail, M., Kubicek, C. P., 2003. D-xylose metabolism in Hypocrea jecorina: loss of the xylitol dehydrogenase step can be partially compensated for by lad1-encoded L-arabinitol-4-dehydrogenase. Eukaryot Cell. 2**,** 867-75.

Seiboth, B., Metz, B., 2011. Fungal arabinan and L-arabinose metabolism. Appl Microbiol Biotechnol. 89**,** 1665-73.

Taherzadeh, M. J., Karimi, K., 2008. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review. Int J Mol Sci. 9**,** 1621-51.

Vidal, B. C., Jr., Dien, B. S., Ting, K. C., Singh, V., 2011. Influence of feedstock particle size on lignocellulose conversion--a review. Appl Biochem Biotechnol. 164**,** 1405-21.

Wang, B., Wang, X., Feng, H., 2010. Deconstructing recalcitrant Miscanthus with alkaline peroxide and electrolyzed water. Bioresour Technol. 101**,** 752-60.

Yu, C., Cao, Y., Zou, H., Xian, M., 2011. Metabolic engineering of Escherichia coli for biotechnological production of high-value organic acids and alcohols. Appl Microbiol Biotechnol. 89**,** 573-83.

Chapter 5: General discussion and future directions

Many reasons such as rising energy consumption, depletion of fossil fuels and increased environmental concerns have shifted the focus of energy generation towards biofuel use. Lignocellulose is a renewable organic material and is the major structural component of all plants. Lignocellulosic wastes are produced in large amounts by many industries including those of forestry, pulp and paper, agriculture, and food [1-4]. Lignocellulose mainly consists of three major components: cellulose, hemicellulose and lignin. Significant efforts, many of which have been successful, have been made to convert these lignocellulosic residues to valuable products such as biofuels, chemicals and animal feed [1,5]. Using lignocellulosic wastes as the main source for ethanol production will eliminate the concern originally caused by bioethanol industries where they have used sugars or corn as the feedstock [1].

In nature, many microorganisms including fungi and bacteria had been found to degrade cellulose and other plant cell wall fibres. Degradation of cellulosic biomass is performed by mixtures of hydrolytic enzymes collectively known as cellulases which work in a synergistic manner in biomass-degrading microbes [1,6]. The breakdown of lignocellulosic biomass involves the formation of long-chain polysaccharides, mainly cellulose and hemicellulose, and the subsequent hydrolysis of these polysaccharides into their component 5- and 6-carbon chain sugars. In biofuel production, these sugars can be converted to bioethanol through fermentation processes [6].

The primary challenge in biomass conversion to bioethanol is achieving yields that make it cost-competitive with current fossil fuel sources. Cellulose in the plant cell wall is not readily available to enzymatic hydrolysis. Pretreatment of the lignocellulosic residues is necessary because hydrolysis of non-pretreated materials is slow, and results in low product yield [7]. Many pretreatment methods rely on applying high temperature, and addition of acids or bases at industrial scales [1,7]. Thus, additional steps are included prior to the hydrolysis step by enzymes which make the overall process expensive, slow and inefficient. Additionally, pretreatment releases inhibitors which lower overall yield of the fermentation process.

The main goal of this thesis was to approach the above mentioned challenges in biofuel production mainly using fungi. Obviously, fungi contribute significantly to the decay of lignocellulosic residues in nature by producing many different lignocellulolytic enzymes. Most fungal strains produce various enzymes in large amounts which are released in the environment and act in a synergistic manner [1]. More recently, fungi became industrially important and act as cell factories due to their high potential for protein production (native or heterologous) and thus can be a successful replacement for bacteria. *T. reesei* is one of the most studied and industrially important cellulolytic fungus capable of efficiently degrading plant cell wall polysaccharides such as cellulose and hemicelluloses [1,8]. During this Ph.D. study, we have used different *T. reesei* strains for three main projects as they are presented in chapters 2 to 4.

In our first study, we tried to understand the biology underlying cellulase hyperproduction of *T. reesei*, and the conditions for the enzyme induction. We have used three different strains of *T. reesei*, including QM6a (wild-type), and mutants QM9414 and RUT-C30. To this end, two parallel experiments were carried out. These included the effect of different carbon sources as well as a time point study to investigate the cellulase production by the three *T. reesei* strains. We have tested 14 different carbon sources including soluble and insoluble carbon sources (pure cellulose and lignocellulosic). We were also interested to investigate how the medium composition affects the fungal growth and ultimately the relationship between the cell growth and their cellulolytic activity. The carbon source utilization profiles of these strains closely resembled each other although the cellulase activity of the *T. reesei* mutant strains QM9414 and RUT-C30 were appreciably higher than the wild-type QM6a. For all three strains, maximum cellulase production was observed using two different types of microcrystalline celluloses. The other findings of the study included determining optimum culture conditions such as incubation time and the concentrations of carbon sources for the highest cellulase production by all three strains. These findings were applied in the other studies carried out later on during the Ph.D. study and they can also serve as a reference for other research studies using the same strains. Additionally, our study using the *T.*

reesei strains indicated that the enzyme production is not directly correlated with the growth of the mycelium, and may be a result of complex inducing factors. Thus, more in-depth studies need to be done to investigate different parameters involved in controlling the cell growth and cellulase production. Future studies on *T. reesei* strains can focus on the genes mainly involved in regulation of cellulase production. For example, transcription factor studies will provide valuable information for the conditions in which the cellulase genes are induced or repressed. Perhaps, discovery of the regulatory processes altered in cellulases hyperproducing *T. reesei* mutant strains can be one of the main tasks.

Despite the fact that some fungal strains have the advantages of being thermostable and producing cellulases, most of these fungal strains do not produce sufficient amounts of one or more lignocellulolytic enzymes [1]. These enzymes are required for efficient bioconversion of lignocellulosic residues to fermentable sugars. *T. reesei*, for example, produces small amounts of β-glucosidase which inhibit further cellulose hydrolysis due to accumulation of the end product inhibitor (cellobiose) [1,8]. This became the key bottleneck in the process of biofuel production and thus new biotechnological solutions are needed to improve the efficiency. Many recent studies focused on improving fungal hydrolytic activity as well as finding stable enzymes capable of tolerating extreme conditions.

The main focus of my Ph.D. study was to improve cellulase activity of the *T. reesei* strain, through increasing its β-glucosidase activity using genetic engineering and molecular biology techniques. For decades, *T. reesei* was targeted by many research studies and traditionally random mutagenesis was applied to improve its cellulolytic activity, leading to the creation of different mutant strains with several-fold increases in the amount of secreted cellulolytic enzymes compared to the wild-type strain [9-11]. However, the hypercellulase producing *T. reesei* mutant strains still showed lower β-glucosidase activity. In our study we tried to improve β-glucosidase activity and ultimately its overall cellulase activity by the relief of end-product inhibition.

A β-glucosidase gene (*bgl1*) from an endophytic fungus *Periconia sp.* was selected since the fungus showed to have very high β-glucosidase activity at elevated temperatures [12]. Thermostable β-

glucosidases with high enzyme activity are especially useful for the bioconversion of lignocellulosic residues at elevated temperature [1]. This will be beneficial to biofuel industries by eliminating the extra step required to cool down pretreated lignocellulosic residues. In our study, the gene was successfully overexpressed in *T. reesei* QM9414 under the control of a promoter region of *T. reesei tef1α* (encoding translation elongation factor 1-alpha). Four mitotic stable BGLI-overexpressing *T. reesei* transformants were obtained in this study. Our results showed that the β-glucosidase activity and total cellulase activity of the recombinant *T. reesei* strains overexpressing BGLI are significantly increased. The BGLI-overexpressing transformants showed higher biomass hydrolytic efficiency, suggesting that they can be used in the hydrolysis step in biomass conversion. High β-glucosidase activity, wide pH range tolerance and high temperature resistance makes the transformants excellent candidates for their potential application for the production of β-glucosidase, as well as improving the biomass conversion using cellulases. As future direction for the project, the activity of the recombinant *T. reesei* can be tested towards other biomass such as corn stover, wheat straw and the results can be compared to that of barley straw. Additionally, the β-glucosidase production by recombinant *T. reesei* can be studied using different conditions such as submerged and solid state fermentation. The results of these studies will be very useful for the potential application of the recombinant *T. reesei* by different industries such as biofuel, textile and food, and help for its transition from lab scale to actual industrial scale.

During the cloning of *bgl1* from *Periconia sp.*, we have also identified three smaller genes with high sequence similarity to the full size *bgl1*. These genes were cloned and fully sequenced. Sequencing results showed that the smaller genes lost mainly part of their third exons. Our current and continuing future work related but not presented here has included the cloning and expression of these genes in a *E. coli* system. Our preliminary results showed very low expression level in the bacterial system. However, the proteins did not show any β-glucosidase activity toward the substrate suggesting that the expression system did not favor the protein folding. We are now working on the cloning and expression of these

genes in a fungal system (e.g. *T. reesei*) and ultimately, their activity will be compared toward the full size gene.

In our third study, we were interested to apply our previously established fungal techniques and used *T. reesei* strains to produce bio-product. Our targeted product was xylitol which is a naturally occurring five-carbon sugar alcohol derived from *D*-xylose. Xylitol is currently in high demand by industries and biotechnological methods became more attractive for large-scale xylitol production. Agricultural residue was selected for xylitol production because of the large amount of availability and high hemicellulose content. *T. reesei* is capable of selectively using *D*-xylose for xylitol production as an intermediate metabolite and its production can be enhanced by genetic engineering of the metabolic pathway. In our study, we used two xylitol hyperproducing *T. reesei* mutant strains including a single mutant (Δ*xdh1*) and a double mutant (Δ*lad1*Δ*xdh1*). Barley straw was subjected to two pretreatment methods including NaOH- and Organosolv-pretreatment. High xylitol production by both strains was achieved when barley straw (untreated or pretreated) was supplemented with 2% *D*-xylose whereas *D*-glucose supplement did not lead to any significant difference. High xylitol production of 6.1 and 13.22 g/L was obtained after 96 and 168 h of incubation using medium supplemented with 2% Organosolv-pretreated barley straw and 2% *D*-xylose by single and double mutant strains, respectively.

The effect of pretreatments on the cellulose and hemicellulose accessibility, as well as lignin modification of barley straw was tested using different techniques such as particle size distribution, FTIR analysis and saccharification. Our saccharification results showed that NaOH-pretreatment significantly increased the accessibility of cellulose/hemicellulose leading to increase the release of reducing sugars, *D*-glucose, *D*-xylose and *L*-arabinose compared to untreated barley straw. Organosolv-pretreatment also led to higher amounts of released *D*-glucose compared to untreated barley straw however, no detectable *D*-xylose or *L*-arabinose were found in the hydrolyzates. Although this indicated that Organosolv-pretreatment removed most of available hemicellulose in barley straw, higher xylitol production by the *T. reesei* strains was achieved using medium containing Organosolv-pretreated barley straw supplemented

with *D*-xylose. This suggests that the pretreatment in combination with the added sugar favored the xylitol production. Thus, we suggest that beside the accessibility, *D*-xylose concentration is also important for xylitol production. Additional work can be done to determine the right *D*-xylose concentration which can optimize xylitol production. Moreover, it would be interesting to study the effect of other available pretreatment methods on barley straw and ultimately the xylitol production by mutant *T. reesei* strains. The results suggest that agricultural residues, such as barley straw, could be a suitable resource for bioconversion to produce value-added products such as xylitol. However, other lignocellulosic biomass such as corn stover, or wheat straw can be also pretreated and used as the feedstock for xylitol production by *T. reesei* mutant strains as the cell factories.

Overall, my Ph.D. study opened a new toolbox for *T. reesei* strain improvement strategies as well as their industrial applications. *T. reesei* QM6a (wild-type) genomic DNA is now fully sequenced and the information can be used for further improvement strategies [9,10]. Future work on the strain improvement can focus on three main areas including; (1) further increasing enzyme expression levels by applying genetic engineering approaches, (2) increasing the efficiency of the enzyme mixture, and (3) metabolic engineering of the strain. These studies will accelerate the development of improved industrial strains that are highly efficient for production of biofuels and biochemical and ultimately will help to overcome the bottlenecks currently available in those industries.

References

[1] Dashtban M, Schraft H, Qin W. Fungal bioconversion of lignocellulosic residues; opportunities & perspectives. *Int J Biol Sci* 2009;5**:**578-95.

[2] United Nations Department of Economic and Social Affairs PD: World Population Prospects, The 2006 Revision, Highlights. 2007:Working Paper No. ESA/P/WP.202.

[3] Schiffer H-W: WEC energy policy scenarios to 2050. Energy Policy 2008;36:2464-2470.

[4] Howard RL, Abotsi E, Jansen van Rensburg EL, Howard S. Lignocellulose biotechnology: issues of bioconversion and enzyme production. *African Journal of Biotechnology* 2003;2:602-619.

[5] Sanchez C. Lignocellulosic residues: biodegradation and bioconversion by fungi. *Biotechnol Adv* 2009;27:185-194.

[6] Percival Zhang YH, Himmel ME, Mielenz JR. Outlook for cellulase improvement: screening and selection strategies. *Biotechnol Adv* 2006;24:452-481.

[7] Zhang YH, Ding SY, Mielenz JR, Cui JB, Elander RT, Laser M, Himmel ME, McMillan JR, Lynd LR. Fractionating recalcitrant lignocellulose at modest reaction conditions. *Biotechnol Bioeng* 2007;97:214-223.

[8] Dashtban M, Qin W,. Overexpression of an exotic thermotolerant β-glucosidase in *Trichoderma reesei* and its significant increase in cellulolytic activity and saccharification of barley straw *Microbial Cell Factories* 2012;11:63.

[9] Kubicek CP, Mikus M, Schuster A, Schmoll M and Seiboth B. Metabolic engineering strategies for the improvement of cellulase production by Hypocrea jecorina. *Biotechnol Biofuels* 2009; 2: 19.

[10] Seidl V and Seiboth B. Trichoderma reesei: genetic approaches to improving strain efficiency. *Biofuels* 2010; 1(2):343-354.

[11] Martinez D, Berka RM, Henrissat B, Saloheimo M, Arvas M, Baker SE, Chapman J, Chertkov O, Coutinho PM, Cullen D *et al*: Genome sequencing and analysis of the biomass-degrading fungus Trichoderma reesei (syn. Hypocrea jecorina). *Nat Biotechnol* 2008, 26(5):553-560.

[12] Harnpicharnchai P, Champreda V, Sornlake W, Eurwilaichitr L: A thermotolerant beta-glucosidase isolated from an endophytic fungi, Periconia sp., with a possible use for biomass conversion to sugars. *Protein Expr Purif* 2009, 67(2):61-69.

Publication List

1) **Dashtban M.**, Schraft H. and Qin W. Fungal Bioconversion of Lignocellulosic Residues; Opportunities & Perspectives. *International Journal of Biological Sciences* 2009; 5:578-595 (received H.O.P.E. Award in 2009) (Chapter 1, 1.2).

2) **Dashtban M.**, Schraft H., Syed T. A. and Qin W. Fungal Biodegradation and Enzymatic Modification of Lignin. *International Journal of Biochemistry and Molecular Biology*. 2010; 1(1):36-50 (Chapter 1, 1.3).

3) **Dashtban M.***, Maki M.*, Leung K. T., Mao C. and Qin W. Cellulase Activities in Biomass Conversion: Measurement Methods and Comparison. *Critical Reviews in Biotechnology* 2010; 30(4): 302-9 (*authors with equal contributions) (Chapter 1, 1.4).

4) **Dashtban M.**, Buchkowski R. and Qin W. Effect of Different Carbon Sources on Cellulase Production by *Hypocrea jecorina* (*Trichoderma reesei*) Strains. *International Journal of Biochemistry and Molecular Biology*. 2011, 2(3):274-286 (Chapter 2).

5) **Dashtban M.**, and Qin W. Overexpression of an exotic thermotolerant β-glucosidase in *Trichoderma reesei* and its significant increase in cellulolytic activity and saccharification of barley straw. *Microbial Cell Factories*. 2012, 11: 63 (Chapter 3).

6) **Dashtban M.**, Kepka G., Seiboth B. and Qin W. Xylitol production using barley straw as feedstock by genetically engineered *Trichoderma reesei* strains. *Metabolic Engineering* (submitted in May 2012, submission number MBE-S-12-00108) (Chapter 4).

Gene bank:

1) **Dashtban M.**, and Qin W. *Periconia sp.* BCC 2871 β-glucosidase genomic DNA (2847 bp), GenBank; Accession No. JQ239427. April 14, 2012 (Chapter 3).