Budapest University of Technology and Economics

Production and characterization of novel cellulase and hemicellulase enzymes by selected filamentous fungi

DOCTORAL THESIS

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List of publications

1. Van Gool, M.P., Vancso, I., Schols, H.A., Szakacs, G., **<u>Toth, K.,</u>** Gruppen, H.: Screening for distinct xylan degrading enzymes in complex shake flask fermentation supernatants Bioresource Technology 102: 6039-6047 (2011) IF: 4.253

2. Van Gool, M.P., **<u>Toth, K.,</u>** Schols, H.A., Szakacs, G., Gruppen, H.: Performance of hemicellulolytic enzymes in a wide range of fermentation supernatants depends on substrate choice Bioresource Technology 114: 523-528 (2012) IF: 4.253

3. **<u>Toth K.,</u>** Van Gool M.P., Schols H.A., Samuels G.J., Gruppen H., Szakacs G.: Diversity in production of xylan degrading enzymes among species belonging to the *Trichoderma* section *Longibrachiatum*. Bioenergy Research 6(2): 631-643 (2013) IF: 3.562

4. Marjamaa K., **<u>Toth K.,</u>** Bromann P.A., Szakacs G., Kruus K.: Novel *Penicillium* cellulases for total hydrolysis of lignocellulosics. Enzyme and Microbial Technology. 52:358-369 (2013) IF: 2.367

Other publication

1. Szabo O.E., Csiszar E., **<u>Toth K.,</u>** Szakacs G., Koczka B.: Ultrasound-assisted extraction and characterization of hydrolytic and oxidative enzymes produced by solid state fermentation and their use in biopreparation of linen. Bioenergy Research (submitted, 2013) IF: 4.250

Presentations

1. **<u>Toth K.,</u>** Van Gool M.P., Schols H.A., Samuels G.J., Gruppen H., Szakacs G.: Diversity in production of xylan degrading enzymes among species belonging to the *Trichoderma* section *Longibrachiatum*. 15th European Congress on Biotechnology, 23-26 September 2012, Istanbul, Turkey (poster presentation)

2. **<u>Toth K.,</u>** Van Gool M.P., Schols H.A., Szakacs G.: Hemicellulóz lebontó enzimek termelése és vizsgálata *Trichoderma* fonalasgomba fajokkal. XXXV. Kémiai Előadói Napok (KEN), 29-31 October 2012, Szeged, Hungary (oral presentation)

3. **<u>Toth K.,</u>** Van Gool M.P., Schols H.A., Szakacs G.: Xilanáz enzimek termelése és vizsgálata *Trichoderma* fonalasgomba fajokkal. MTA Természetes Polimerek Munkabizottsági Ülés, 28 November 2012, Budapest, Hungary (oral presentation)

4. **<u>Toth K.,</u>** Van Gool M.P., Schols H.A., Samuels G.J., Gruppen H., Szakacs G.: Xilán lebontó enzimek termelése és vizsgálata *Trichoderma* fonalasgomba fajokkal. X. Jubileumi Oláh György Doktori Konferencia, 07 February 2013, Budapest, Hungary (poster presentation)

Other presentation

1. **<u>Toth K.,</u>** Szakacs G.: 'TUB' mikrobiológiai törzsgyűjtemény gyógyszeripari hasznosíthatósága. BME-Richter Tudományos Nap, 12 December 2012, Budapest, Hungary (oral presentation)

Novel scientific findings

1. Novel screening method has been developed for investigation of various specific enzyme activities in crude fermentation supernatants. This method combines classical screening method together with different instrumental analytical tools such as High Performance Liquid Chromatography and Mass Spectrometry. (Paper #1)
2. This screening method is only effective when using the correct substrate. The substrate characteristics determine enzyme performance and screening results. Screening of xylan degrading enzymes was performed on soluble xylan rich substrates (wheat arabino-xylan, eucalyptus xylan hydrolysate) and insoluble xylan rich fractions (wheat straw water unextractable solids and corn fiber alcohol insoluble solids). (Paper #1 and #2)
3. Screening of 78 shake flask fermentation supernatants on different xylan rich substrates resulted in *Aspergillus*, *Penicillium*, *Trichoderma* and other non-identified species have high hemicellulolytic activity. These results could indicate further screening experiments of more strains. (Paper #2)
4. Screening of 15 species belonging to the *Trichoderma* section *Longibrachiatum* resulted in equally good or better isolates than control species *Trichoderma reesei* RUT C30 and QM6a. They are genetically close to *T. reesei* such as *T. reesei* CPK 155, *T. parareesei* TUB F-2535, and *T. gracile* TUB F-2543 isolates. They may represent a potential for industrial applications. (Paper #3)
5. Novel *Penicillium* cellulases were efficient in specific cellulase activity similarly to *Trichoderma reesei* RUT C30 cellulases. Also, higher β-xylosidase activity was investigated in *Penicillium* cellulase complex. The lignin inhibition of hydrolytic activity of the *Penicillium* cellulases was almost absent in case of herbaceous lignin being at a lower temperature (35°C). *P. pulvillorum* TUB F-2220 and *P. cf. simplicissimum* TUB F-2378 cellulases are competent in simultaneous saccharification and fermentation (SSF) at a lower temperature. (Paper #4)

Abstract

Filamentous fungi can be valuable sources of cellulosic and hemicellulsic enzymes for bioconversion of lignocellulosic biomass to biofuels. The present research work describes a new screening method using up-to-date methodology and instruments, by which specific xylan degrading enzymes can be detected. A great number of fungi enzyme mixtures have been produced in fermentation of different lignocellulosic carbon sources. Randomly selected enzyme preparations were tested in the hydrolysis of soluble xylan rich substrates and insoluble xylan rich fractions. Hydrolysis products were analyzed by high performance liquid chromatography and mass spectrometry. This screening method was also applied in study of supernatans of *Trichoderma Longibrachiatum* clade which are genetically close to *Trichoderma reesei*, one of the most promising cellulase and hemicellulase producers and the results might even have possess scientific value. These methods help to prove that sufficiency of degradation is depends not only on the selected fungi enzyme preparation but also the characteristics of xylan rich substrates.

Furthermore, cellulase enzyme systems of two novel *Penicillium* strains have been studied. The secreted enzyme mixtures of the recently found species were tested in the hydrolysis of different lignocellulose substrates and microcrystalline cellulose (Avicel PH-101). The sensitivity of these cellulase complexes was studied on softwood (spruce) and grass (wheat straw) lignins on different temperature and pH level. These *Penicillium* cellulases could be valuable in the simultaneous saccharification and fermentation (SSF) process performed at a lower temperature (e.g., 35°C) for pretreated lignocelluloses. Similar studies have been performed in subject of cellulase of different *Trichoderma* isolates. However, at this moment they are under in-depth analysis for a possible patent. This means that I am unable to publish the results until the end of the investigation.

List of Abbreviations

BSA	Bovine Serum Albumin
CBH	Cellobiohydrolase
CF AIS	Corn Fiber Alcohol Insoluble Solids
DM	Dry Matter
DP	Degree of Polimerization
DNS	3,5-Dinitrosalicylic acid
EXH	Eucalyptus Xylan Hydrolysate
FPA	Filter Paper Activity
FPU	Filter Paper Unit
GH	Glycoside Hydrolase
HPAEC	High Performance Anion-exchange Liquid Chromatography
HPLC	High Performance Liquid Chromatography
HPSEC	High Performance Size Exclusion Liquid Chromatography
IU	International Unit
MALDI-TOF MS	Matrix Assisted Laser Desorption/ionization Time-of-flight Mass Spectrometry
m-HDP	m-Hydroxydiphenyl
PDA	Potato Dextrose Agar
RACE PCR	Rapid Amplification of cDNA Ends by Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis
SP EnzHR	Enzyme Hydrolyzed Spruce Lignin
SSF	Simultaneous Saccharification and Fermentation
TUB	Technical University of Budapest
WAX	Wheat Arabino-xylan
WS EnzHR	Enzyme Hydrolyzed Wheat Straw Lignin
WS WUS	Wheat Straw Water Unextractable Solids

Table of content

1. General Introduction

Diminishing resources of fossil-fuel energy and the detrimental effects of their usage on the environment has generated the growth of the application of plant biomass for fuel and chemical production (Ragauskas et al., 2006; Himmel et al, 2007). First generation bioethanol plants (sugar cane and starch-based plant grains) are intensively studied, although their usage may increase the price of food and feed of animals. Therefore, the developing second generation biofuels produced from non-food biomass are also significant for future biorefineries (Sims et al., 2010; Xu et al, 2009). Source of second generation biofuel is raw materials such as energy plants and agricultural by-products e.g. wheat straw, corn stover and wood from agro- and forestry residues. Major components of lignocelluloses are cellulose, hemicelluloses and lignin. Natural carbon recycling of the polymers is based on lignocellulose degrading micro-organisms. These creatures produce a wide range of glycosidic hydrolases, which are also used in the industrial hydrolysis of lignocellulosic materials (Zhang et al., 2006; Jovanovic et al., 2009).

The present PhD research is part of the 7th EU Framework project, with the logo 'DISCO' ('Targeted DISCOvery of novel cellulases and hemicellulases and their reaction mechanisms for hydrolysis of lignocellulosic biomass', www.disco-project.eu). This project focuses on the development of efficient and cost-effective enzyme tools to degrade the lignocellulosic biomass to fermentable sugars, convert it to bioethanol and to investigate the performance of these enzymes. Our laboratory focused on the screening of novel cellulases and hemicellulases produced by selected lignocellulolytic filamentous fungi, enriched soil samples and enzyme collections to find enzymes being able to degrade selected materials. In the cellulase subject we collaborated with Technical Research Centre of Finland (VTT), while we also cooperated with Wageningen University (WU) in studying the degradation of hemicelluloses.

The present screening work was carried out approximately with 950 strains. Selected enzyme supernatants have been tested in hydrolysis experiments, in degradation of cellulose and hemicellulose rich carbon sources. Furthermore, performances of enyme mixtures were tested on different carbon sources in presence of different lignins. Sensitivity of enzymes was analyzed on different temperature and pH levels. Purification of enzyme preparation was performed and purified proteins were investigated.

1.1. Lignocellulosic Biomass

Lignocellulosic biomass represents a potential source of renewable raw materials for bioethanol production. Agricultural and forestry residues are a remarkable part of common solid waste (e.g. waste paper), as lignocellulosic materials are used as potential resources of biofuel production. The complex structure of biomass is made up of three important fractions: 35%–50% cellulose, 20%–35% hemicellulose and 12%–20% lignin. Furthermore, a minor amount of other polymeric components: starch, pectin, proteins, minerals, ash, etc (Gomez et al., 2008; Lynd et al., 2008). Celluloses and hemicelluloses connected with lignins to the form an insoluble, tridimensional network, is a constant behavior.

1.1.1. Cellulose

The most abundant component in the plant cell wall is cellulose. It is composed of *D*-glucose units that are linked with *β*-1,4-glycosidic bonds to each other. Cellulose has two general forms in biomass: crystalline and amorphous. Crystalline cellulose creates the main segment of cellulose, while a low amount of cellulose is in organized amorphous form. Cellulose is more competent to enzymatic degradation in its amorphous form (Lynd et al., 2002; Laureano-Perez et al., 2005). Cellobiose is the repeat unit created through this linkage and it composes cellulose chains. The long cellulose chains are stabilized by strong hydrogen and van der Walls bonds to create microfibrils in plant cell walls. **Figure 1** represents the secondary structure of cellulose.

Cellulose microfibrils have a high part of crystalline regions and a lower amount of amorphous regions . Strong hydrogen bonds hold the long cellulose chains tightly together in a crystalline structure, resisting to the degradation of plant (Himmel et al., 2010; Guo et al., 2008).

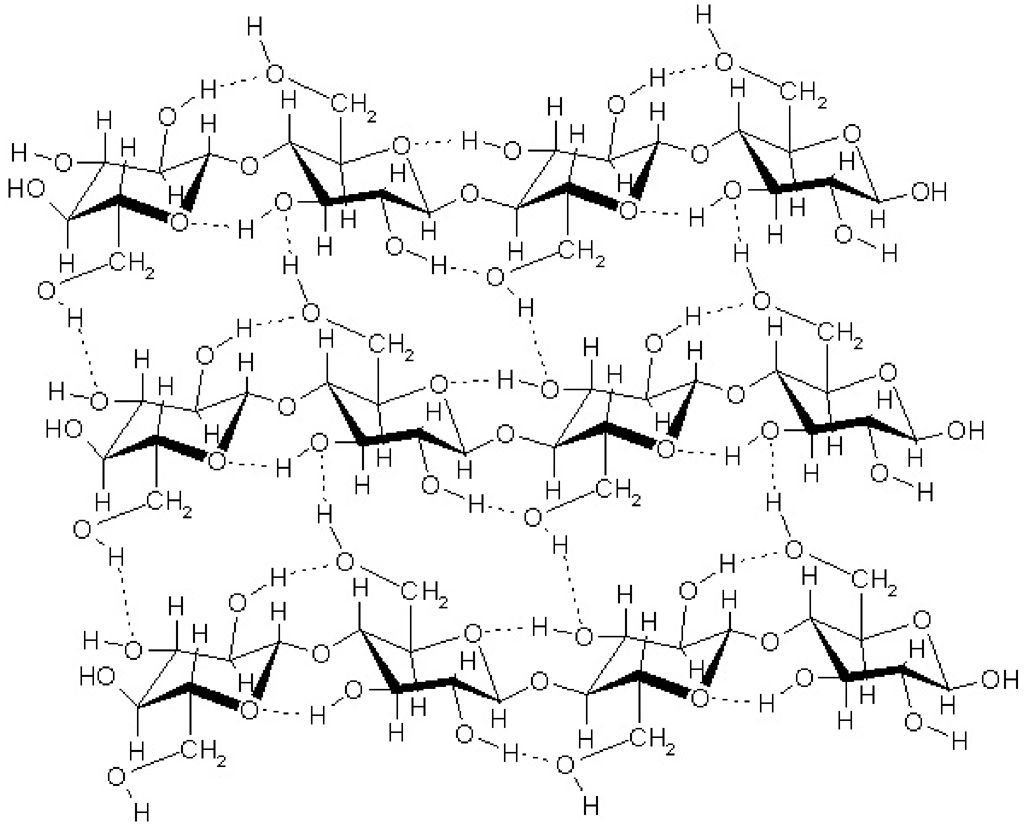


*Figure 1. The secondary structure of cellulose**

*http://www.doitpoms.ac.uk/tlplib/wood/structure_wood_pt1.php

Numerous organisms are able to digest cellulose and utilize as a source of food such as fungi, bacteria, plants and protists along with a wide variety of invertebrate animals, for example insects, crustaceans, annelids, molluscs and nematodes (Watanabe et al., 2001; Davison et al., 2005).

1.1.2. Hemicellulose

Hemicellulose is the second abundant material in the plant cell walls of both species (dicots and monocot). The structure of hemicellulose is made up of an amorphous chain that contains a mass of saccharides.The most frequently presented sugars in hemicellulose: *α*-D-galactose, β–D-glucose, β–D-mannose, β–D-xylose and α-L-arabinose. Besides, a smaller amount of other compounds are present in the structure of hemicellulose, such as acetic acid and uronic acids (α-D-glucuronic acid and α-4-*O*-methyl-D-glucuronic acid). And further lower amount of saccharides is attended in the structure of hemicellulose as well, for example α-L-rhamnose and α-L-fucose (Scheller et al., 2010).

Hemicellulose can be classified into four groups: xylans (β-1,4-linked D-xylose units), mannans (β-1,4-linked D-mannose units), arabinans (α-1,5-linked L-arabinose units), and galactans (β-1,3-linked D-galactose units) (da Silva et al., 2012). **Figure 2** represents the complex structure of xylan.

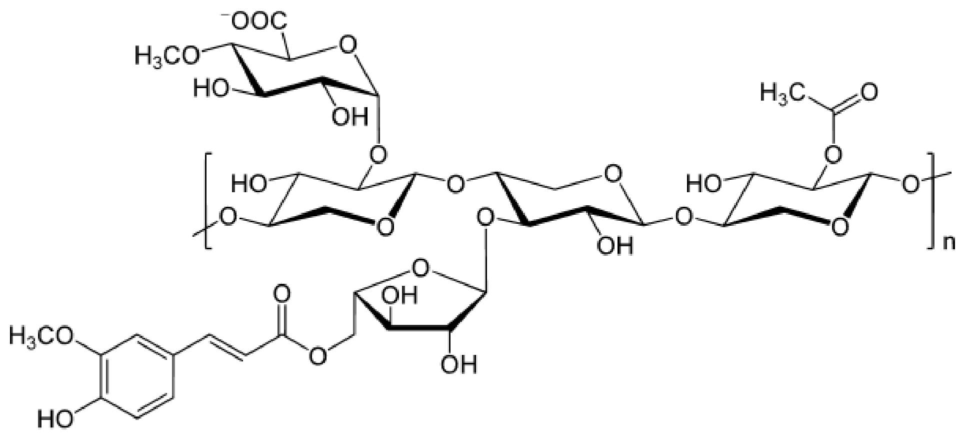


*Figure 2. The main components of the hemicellulose**

* http://upload.wikimedia.org/wikipedia/commons/4/46/Xylan.svg

The hemicellulose backbone has either the structure of homopolymer or heteropolymer with short junctions linked by β-(1,4)-glycosidic bonds and in some cases by β-(1,3)-glycosidic bonds**.** In contrast to cellulose, the polymers in hemicellulose are easily digestable. These polymers do not aggregate, even when they are connected to cellulose chains (Kumar et al., 2009).

1.1.2.1. Xylan as a Complex Hemicellulose

Xylan is the most common polymer from the hemicellulose group range, its polysaccharides include linear chains of β-(1,4)-D-xylanopyranosyl residues. It–can also be substituted by acetyl groups at the position of *O*-2 or *O*-3. Xylans of higher plants are also substituted by 4-*O*-methylglucuronosyl residues. Constitution of 4-*O*-methylglucuronic acid and glucuronoxylan is included in the secondary cell wall in dicots-but does not usually appear in the primary walls of dicots and the walls of monocots (Caffall et al., 2009). Numerous agricultural plants consist of xylans such as straw, sorghum, sugar cane, corn stalks and cobs, hulls and husks, as well as forest and pulping waste products from hardwoods and softwoods (Ebringerova and Hromadkova, 1999).

Recently, the following xylan groups have been distinguished according to their structure (da Silva et al., 2012):

Arabinoxylans: Xylan backbone substituted with β-(1,4)-D-xylopyranose–in position 2 or 3 with α-(1,3)-L-arabinofuranosyl can be esterified partly with phenolic acids. Arabinoxylans are presented in the starchy endosperm and the outer layers of cereal grains.

Glucuronoxylans: These can be partly acetylated and can have units substituted with α-(1,2)-4-*O*-methyl-D-glucopyranosyl uronic acids. They are presented in hardwood, depending on the treatment.

Glucuronoarabinoxylans: These have a substitution with α-(1,3)-L-arabinofuranosyl next to α-(1,2)-4-*O*-methyl-D-glucopyranosyl uronic acids. They are presented in softwoods.

Unsubstituted homoxylans: They are linear polysaccharides, presented in some kind of seaweed.

Heteroxylans: They are substituted with various mono- and/or oligosaccharides. They can be found in cereal bran, seed, and gum exudates.

Structure of xylans from different plants are extensively studied and characterized. More xylan extraction method have been developed, which provide a wide range of applying xylan extracts in further researches (da Silva et al., 2012).

1.1.3. Lignin

Lignin is hydrophobic in nature and bonds covalently to hemicellulose and cellulose to make the structure of lignocellulose more complex. Lignin is the roughest component of the plant cell wall as it has an important function in the physical characteristics of wood, and besides, it protects lignocellulosic plants against biological degradation (e.g. cellulose degrading microorganisms). Lignin has not a sugar-based construction however, it is made up of heterogeneous independent aromatic polymers of hydroxycinnamyl alcohols. Its major monocomponents are namely *p*-coumaryl, coniferyl and sinapyl alcohol (**Figure 3**) (Caffall et al., 2009).

Lignocellulosic biomass contains 5-25% of lignin. The quantity and conformation of lignin are different in plant species, diverse cell types and cell wall layers (Lin et al., 1992). Lignin enhances the rigidness of the cell walls and diminishes water permeability. Lignin concentration is more considerable in the softwood of coniferous trees (25-33%) than in the hardwood of broad leaved trees (20-25%). Lignin in softwood is mostly constructed of guaiacyl units originated from coniferyl alcohol, while lignins in hardwood are polymers of coniferyl and sinapyl alcohols.

During the industrial utilization of lignocelluloses, lignin is affected negatively by the limitation of access to glycosyl hydrolases (GHs) to the cellulose and hemicellulose and also by irreversible adsorption to GHs enzymes (Sewalt et al., 1997; Berlin et al., 2006; Nakagame et al, 2010; Rahikainen et al, 2011). This adsorption of proteins to lignin surfaces is typically operated by hydrogen bonding, electrostatic forces and hydrophobic interactions. The impact of lignin inhibiton depends on the composition of lignin as well as on the lignin origin (Pan et al., 2008; Tejirian et al., 2011).

Biological degradation of lignin is based on performance of soft- white- and brown- rot fungi. Lignin treatment of these fungi may be an efficient method of delignification in order to provide easier access to components of cellulose and hemicellulose (Gupta et al., 2011).

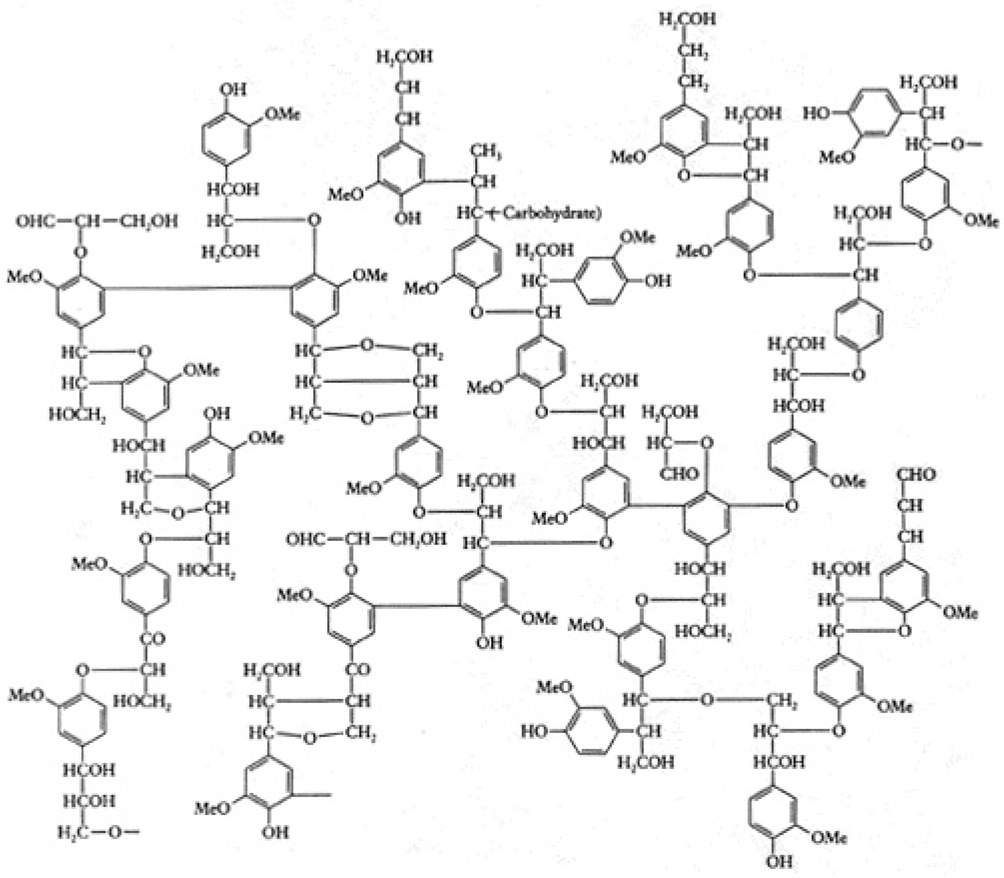


Figure 3. The complex structure of lignin[*]

*http://hu.wikipedia.org/w/index.php?title=F%C3%A1jl:Lignin_structure.svg&page=1&filetimestamp=2007042 4122513

1.1.3.1. Lignin Inhibition of Lignocellulose Hydrolysis

During the enzymatic hydrolysis of lignocellulose cellulose and hemicellulose degrading enzymes performance can be reduced by lignin interactions. Two possible inhibition ways are described: steric hindrance of the cellulose by lignin and the reversible or irreversible adsorptions of cellulases on lignin. This adsorption between lignin and cellulases can happen via hydrophobic interactions, ionic interactions or hydrogen-bonding interactions (Eriksson et al., 2002; Berlin et al., 2006; Pan et al., 2008).

In order to improve lignocellulose hydrolysis rate and recovery of degrading enzymes after the process the affinity of lignin binding to enzymes needs to be reduced in order to make the hydrolysis process more economical (Berlin et al., 2006). Further options have been developed to enhance the hydrolysis yield. For example the application of weak lignin-binding enzymes, which are mainly produced by fungi (Berlin et al., 2005). Another way is intensively increasing the temperature that reduces the cellulase-lignin interactions and decreases the release of cellulases from the lignin surface (Rahikainen et al., 2011). Delignification processes can also significantly improve the digestibility of lignocellulose by removing the lignin. This highlights the importance of pretreatment steps before hydrolysis (Pan et al., 2008).

1.2. Pretreatment of Lignocellulosic Materials

The impact of pretreatment of lignocellulosic biomass has been identified and written for a long time (McMillan et al., 1994). The intention of the pretreatment is to digest the plant cell wall and to remove lignin from cellulose and hemicellulose and besides to decrease cellulose crystallinity, as well as to increase the porosity of the lignocellulosic materials (**Figure 4**) (Merino et al., 2007).

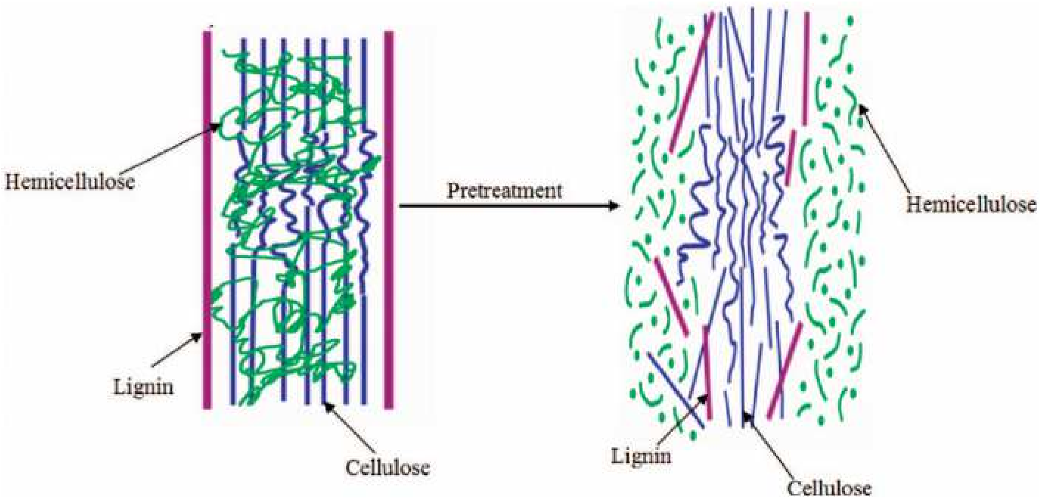


Figure 4. Scematical illustration about the effect of pretreatment of lignocellulose (adapted from Kumar et al., 2009)

The pretreatment has to suit some important requirements. First of all, it has to improve the formation of sugars or the ability to subsequently form sugars by enzymatic hydrolysis. It should prevent the degradation or loss of carbohydrate and prevent the formation of byproducts inhibitory to the subsequent hydrolysis and fermentation processes. Last but not least, it has to be economical (Galbe et al., 2007).

Numerous pretreatment technologies have been distinguished from extremely acidic to quite alkaline (e.g. acid-catalyzed steam explosion, ammonia fiber explosion and solvent extraction), breaking the composition of the biomass and developing it to be more accessible and digestable for degrading enzymes. For example, acidic pretreatment hydrolyzes the most amount of the hemicellulose but leaves a great amount of cellulose and lignin (Bura et al., 2002; Pan et al., 2005; Wyman et al., 2005; Agbor et al., 2011).

The hydrothermal- and steam pretreatment methods are commonly used technologies nowadays. These are supplemented with chemical digestion to improve the accessibility of carbohydrates by degradation of the tissue structure and modifing the spatial distribution of polymers (Merino et al., 2007).

Pretreatments may extremely improve enzymatic demolishibility of plant biomass-however, the pretreated lignocellulosic materials can still contain a considerable amount of lignin, which restricts enzymatic hydrolysis by irreversible conjuction of lignin to GHs. This negative factor enhances the cost of the process by increasing the dosage of enzymes and inhibiting the recycling of these enzymes. It has been investigated that lignins originated from softwood are more disadvantageous to the performance of enzyme than lignins from hardwood or grasses (Nakagame et al., 2010).

1.3. Microbial Degradation of Lignocelluloses

Efficient degradation of lignocellulosic biomass to oligo- and monosaccharides requires the cooperation of different microbial enzymes in synergism. Structurally two different enzymes are involved in the degradation of the plant cell wall, namely~~,~~ endo-enzymes and exo-enymes. Endo-enzymes cut the bonds inside the chain of polymers. The action of endo-enzymes results in the reduction of substrate molecula mass. Exo-enzymes digest the endings of the polymer chain and hydrolyse oligomers to monomers. Endo-enzymes may provide additional substrates for the exo-enzymes (Henrissat et al., 1997).

Numerous protist and polycellular creature can produce lignocellulolytic enzymes although the most utilized lignocellulose digesting enzymes are originated from bacterias and fungi. The most deeply investigated enzyme systems include cellulases, hemicellulases and some connecting glycoside hydrolases (Himmel et al., 2010).

Up until now there has been not enough efficient enzyem cocktails for total degradation of the plant cell walls and the quantity of these enzymes are not known exactly. However, the usage of cellulase and hemicellulase enzyme mixtures may drive for a better and better degradation of the lignocellulosic biomass (Himmel et al., 2007; Kristensen et al., 2008).

1.3.1. Cellulase

Recently used cellulase enzyme preparations are efficient in the hyrolization of cellulose**.** However, they are comparatively expensive, therefore it would be important to reduce the cost of their commercial usage in biorefineries. The cellulase enzyme complex contains three different kinds of enzyme that perform well in collaboration in degradation of cellulose (**Figure 5**). According to the region of substrate and hydrolysis products they are exoglucanases, endoglucanases and β-glucosidases (Wilson et al., 1999; Kubicek et al., 2009). They perform successantly in a good cooperation to breakdown break down the cellulose complex and produce a fermantable energy source (glucose) for bioethanol production (Beguin et al., 1994).

Cellulase enzyme complexes are distinguished as hydrolases which can break down *O*-glycosidic bonds between glucose units. They have been classified by the Enzyme Commission with the number 3.2.1.x, where x signs the different cellulolytic enzyme type. (Lynd et al., 2002).

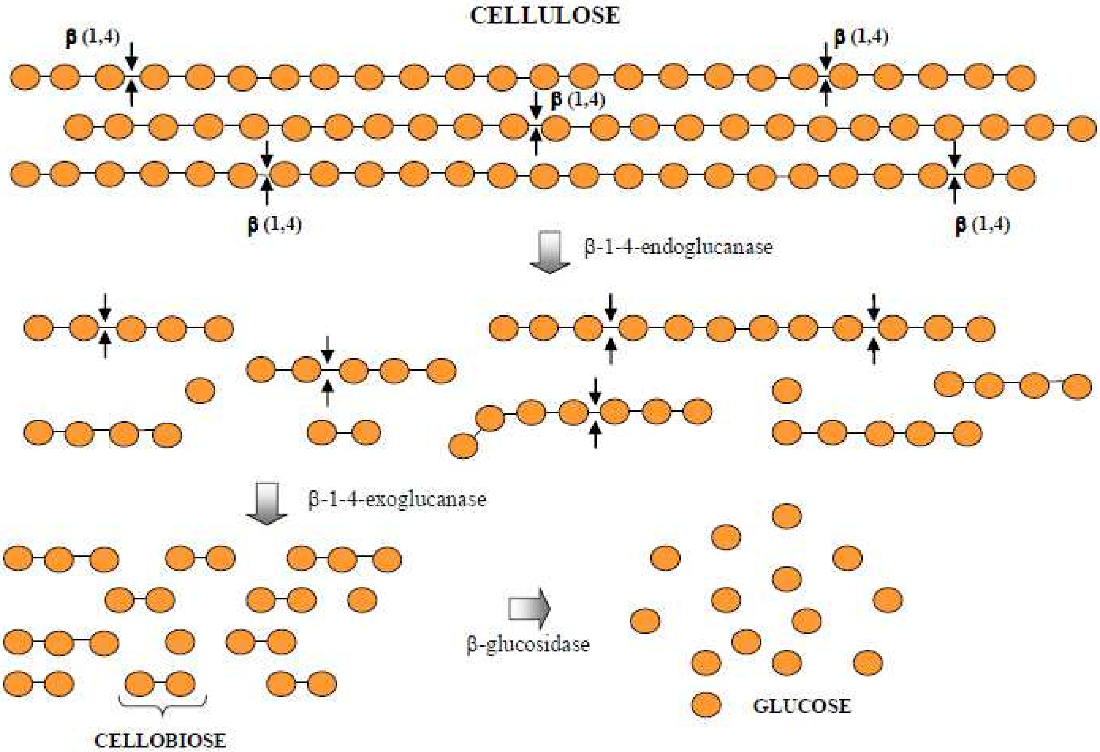


Figure 5. Efficiency in cooperation of members of cellulase enzyme system (adapted from Mussatto and Teixeria, 2010)

The following three classes of cellulase enzymes act in synergism during cellulose hydrolysis:

Endoglucanases: This cluster is represented by the β-1,4-endoglucanase (E.C. 3.2.1.4). It randomly digests the inner glycosid-linkages of the amorphous cellulose part, liberating polysaccharides with lower polymerization degree (DP) and soluble oligosaccharides (DP<7).

Exoglucanases: The most important enzymes of the group are cellobiohydrolases (CBH, E.C. 3.2.1.91) and glucanohydrolases (E.C. 3.2.1.74). Cellobiohydrolase indigests the cellobiose from the reducing (CBH I) and non-reducing (CBH II) terminals of cellulose chain. Glucanohydrolase liberates glucose units from the ends of the cellulose chain. It has been investigated in the past that both cellobiohydrolases (CBH, E.C. 3.2.1.91) and glucanohydrolases can be inhibited by their hydrolysis products, the glucose (de Castro et al., 2010; Lynd et al., 2002).

β-glucosidases: The third cluster from cellulase complex includes the β-1,4-glucosidase (E.C. 3.2.1.21), which induces the degradation of cellobiose and digests oligosaccharides to glucose, hydrolyzes the β-1,4 bonds in the cellulose chain randomly. This enzyme also can be inhibited by glucose (de Castro et al., 2010; Lynd et al., 2002).

The most prevalent commercial enzyme products for degradation of lignocellulosic biomass are produced by saprophytic mesophilic fungus *Trichoderma reesei* in submerged fermentation (Nydetzky et al., 1994). This microorganism was isolated for the first time over 60 years ago from decaying cotton tents during World War II (et al., 1976)**.** This *Trichoderma reesei* strain was a plentous producer of cellulase enzymes. Ever since the first isolation, several mutants have been investigated that increase the productivity of the strain by over 20-fold (Mandels et al., 1971; Montenecourt et al., 1979; Mirsha et al., 1984).

1.3.2. Hemicellulases

The enzymatic hydrolysis of hemicellulose requires the cooperation of a great number of enzymes. These hemicellulose degrading enzymes can be classified according to their actions in three main groups: endo-acting enzymes, exo-acting enzymes and further accessory enzymes. The endo-acting group has low efficiency on short oligomers, while exo-acting hemicellulases are induced by both short and long chain substrates as well. The accessory enzymes are responsible for the digestion of lignin-linked glycoside bonds liberating different substitutions from hemicellulose backbone (Himmel et al., 2010).

One of the most commonly known hemicelluloses is the xylan. The total degradation of a complex xylan is required first to release the substituents from the xylan backbone and afterwards the depolymerization of the xylan chain. The adequate hydrolysis of hemicellulose needs a simultaneous action of all these enzymes (Decker et al., 2009). Debranching of the side chains requires the performance of numerous accessory enzyme activities, including α-L-arabinofuranosidases, α-glucuronidases (E.C. 3.2.1.139), ferulic acid esterase (EC 3.1.1.73)~~,~~ and acetyl xylan esterases (EC 3.1.1.72) (Saha et al., 2003; Biely et al., 1997). Depolymerization of the backbone requires endo-xylanases (E.C. 3.2.1.8) that produces unbranched xylooligosaccharides, such as xylotriose and xylobiose, and β-xylosidases (E.C.

3.2.1.37) that split xylobiose and digests the non-reducing terminals of short chain xylooligosaccharides to release xylose (Saha et al., 2003).

Figure 6 presents a schematical view about complete degradation of xylan by co-operation of many enzymes:

- *Endo-xylanases* hydrolyse the glycosidic bonds of the β-1,4- or β-1,3- linked, unsubstituted xylan backbone to produce β-D-xylopyranosyl oligomers.

- *β-D-xylosidases* cleave xylooligosaccharides such as xylobiose from the non-reducing terminus liberating β-D-xylopyranosyl residues.

- *Acetyl xylan esterase* liberates the *O*-acetyl groups from β-D-xylopyranosyl remains of acetylated xylan. This enzyme is important in the degradation of xylan, because the acetyl groups can inhibit with the approach of endo-xylanases that digest the backbone.

- *α-L-arabinofuranosidase* or arabinoxylan hydrolases release α-arabinofuranosyl residues from the β-D-xylopyranose part.

- *α-Glucuronidase* enzyme degrade the α-1,2-bonds between β-D-xylopyranosyl units and the glucuronic acid (and also their *O*-methyl ethers).

- *Ferulic acid esterase* and *p-coumaric acid esterase* liberate ferulic acid and *p*-coumaric acid linked to arabinose units (Biely et al., 2003; .Polizeli et al., 2005).

Degradation of xyloglucan and xylomannan requires xyloglucanases (E.C. 3.2.1.151), β-glucosidases, endo-mannases (E.C. 3.2.1.78) and β–mannosidases (E.C. 3.2.1.25) as polymerizing enzymes, but also α–xylosidases (E.C. 3.2.1.177), α-galactosidases (E.C. 3.2.1.22), α-arabinofuranosidases (E.C. 3.2.1.55) and acetyl esterases (E.C. 3.2.1.6) as debranching enzymes.

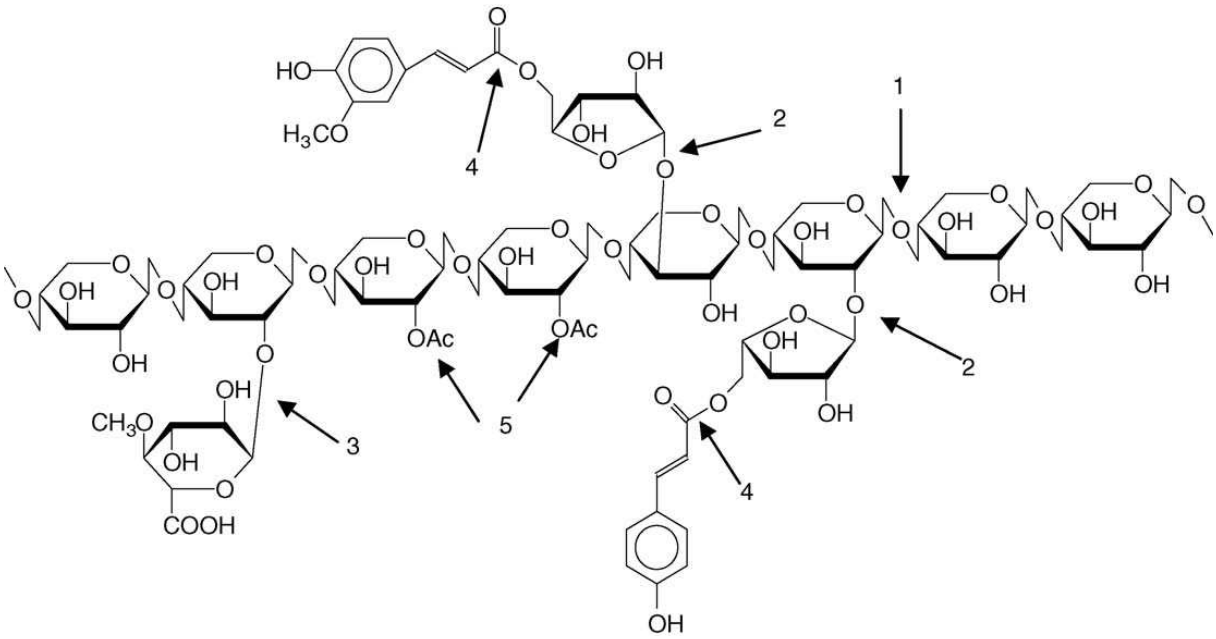


Figure 6. The structure of xylan and performances of the xylan degrading enzymes. 1: endo-xylanases; 2: α-L-arabinofuranosidases; 3: α-glucuronidases; 4: feruloyl and coumaroyl esterases; 5: acetyl xylan esterases (Chavez et al., 2006)

Hemicellulase enzymes are produced by many microorganisms, especially fungi and bacteria as well as by some plant–however, the commonly used xylanolytic fungi are mostly ascomycetes (Polizeli et al., 2005; Sizova et al., 2011; de Vries et al., 2011). Filamentous fungi have a crucial role in the industrial production of xylanase. They excrete hemicellulolytic enzymes in a much higher amount than yeasts or bacteria–and their enzymatic complexes are more complete. Commercial xylanase formulations have been produced, at an industrial scale, mainly from fungi of the genera *Trichoderma*, *Aspergillus*, and also *Penicillia* (Michelin et al., 2010; Lafond et al., 2011).

1.3.3. Industrial Application of Cellulase and Hemicellulase

A great number of the industrial sector applies cellulase and hemicellulase enzyme mixtures, such as the food- and feed industry, pulp- and paper industry, textile- and laundry industry, baking industry, waste treatment or alcohol production from biomass. Against the chemical treatments mild temperature is required in these processes in order to avoid the inactivation of enzymes (de Castro et al., 2010; Saha et al., 2003). These enzymes have been commercialized for more than 30 years (Kudah et al., 2011).

Pulp and Paper Industry: biotreatment pulping applying cellulases resulted in considerable energy savings (around 20–40%) during refining and improvements in handsheet strength properties. Besides, cellulases are used for dye removal from waste paper (Bhat et al., 2000). β-xylanases related from filamentous fungi are being used in the biobleaching of cellulose pulp. They have investigated that the usage of hemicellulase enzymes can materially influence the increase the brightness of kraft pulp. This process can replace the application of active chlorine, which makes it more environmentally friendly. Furthermore, these enzymes can be used in the biobleaching of sulfite pulps (Beg et al., 2001; Bhat et al., 2000; Viikari et al., 1994).

Textile Industry: Cellulases have been applied for the biostoning of jeans and biopolishing of cotton and other cellulosic textures. During the biostoning process, cellulases digest the small fiber ends from cellulose fabric on the yarn surface, dye release from textile therefore, it is easily removed by mechanical washing. Cellulase application benefits are the followings: increased productivity, less work-intensive and ecofriendly. Mainly monocomponent cellulases (selected endoglucanases) are applied in the textile industry (Kudah et al., 2011). Also the application of xylanases is outstanding in the textile industry in the process of plant fibres, such as linen. For this purpose, cellulase-free xylanases are more favourable in the textile industry (Csiszar et al., 2001).

Wine and Beer Industry: Glucanases from microorganisms are successfully used in fermentation industry to produce alcoholic beverages (beers and wines). These enzymes may increase both quality and yields of the fermented products. Glucanases are dosaged to hydrolyze glucan, to decrease the viscosity of wort, and to increase filterability (Bamforth et al., 2009).

Food Industry: Cellulase enzymes also play an important role in the application in food processing. The production of fruit and vegetable juices need increased treatments for extraction, clarification, stabilization and they can enhance the yield of juices as well. The benefits of digesting enzymes usage are improving both yield and process performance without further substantial investment (de Carvalho et al., 2008). Furthermore, usage of xylanases together with amylases, glucose oxidase and proteases is prevalent in baking industry. Xylanases degrade hemicellulose in wheat-flour, promoting the rearrangement of water and leaving the dough softer and much easier to knead. With the dosing of xylanases the volume of bread will increase, absorption of water will enhance and resistance to fermentation will improve. Furthermore, the high level of arabinoxylooligosaccharides in bread has positive effect on health. The production of fruit and vegetable juices needs treatments of extraction, clearing and stabilization where xylanases play an important role in these processes (Harbak et al., 2002; Camacho et al., 2003).

Animal Feed Industry: Pretreatment of agricultural silage and grain feed by cellulases or hemicellulases can increase the quantity of nutritions, hereby decreasing the price of the flesh. Application of cellulase and xylanase enzymes in the feed industry (especially for monogastric animals and broiler chicken) is also significant. Supplement of animal feed by xylanases and other enzymes (e.g. glucanases, pectinases, proteases, amylases, phytase, galactosidases and lipases) are more essential in feeding monogastric animals (e.g., pigs, broiler chickens) than in feeding ruminants (Shrivastava et al., 2011). In addition, xylanases degrade arabinoxylans in the hemicellulose part of the feed, decreasing the viscosity of the raw material (Twomey et al., 2003).

Agricultural Industry: Different enzyme preparations (various combinations of cellulases, hemicellulases, and pectinases) have extensive applications in agriculture in order to enhance the yield of crops and to decrease plant diseases. Saprophitic fungi cellulases are able to degrade the cell wall of plant pathogens hereby controlling the plant diseases. Numerous cellulolytic fungi such as *Trichoderma* species, *Gliocladium* species, *Chaetomium* species~~,~~ and *Penicillium* species are known as an outstanding performer in agriculture by promoting seed germination, plant growth and flowering, enhancing the root system and improving crop yields (Bailey et al., 1998; Harman et al., 1998).

Detergent Industry: Application of cellulase enzymes together with protease and lipase in the detergents is an improvement in this field of industry. Cellulase enzyme coctails play an important role in the modification of cellulose fibers increasing the color brightness, feel, de-pilling and dirt removal from the cotton. Mainly bacterial cellulases are used in detergents with slightly alkaline pH optima (Kudah et al., 2011).

Recycling of Waste: Waste produced from forests, agricultural sections~~,~~ and agroindustries comprise a large number of unappropriated cellulose sources, generating environmental pollution. Currently-these unutilized wastes are considerately used to make valuable materials such as enzymes, saccharides, biofuels (e.g. bioethanol), biochemicals, carbon sources for fermentation, enriched animal feeds and nutrients (Kudah et al., 2011; Zhang et al., 2006; Sukumaran et al., 2005; Milala et al., 2005).

Production of biofuels: Another potential usage of the cellulases and hemicellulases is in the saccharification of lignocelluloses in the process of bioconversion of lignocellulose to biofuels and biochemicals (Saha et al., 2003). **Figure 7** represents the main steps of biofuel production from lignocellulosic materials. Cellulase and hemicellulase enzymes play an important role in the hydrolysis of biomass to fermentable mono- and oligosaccharides (Kumar et al., 2009).

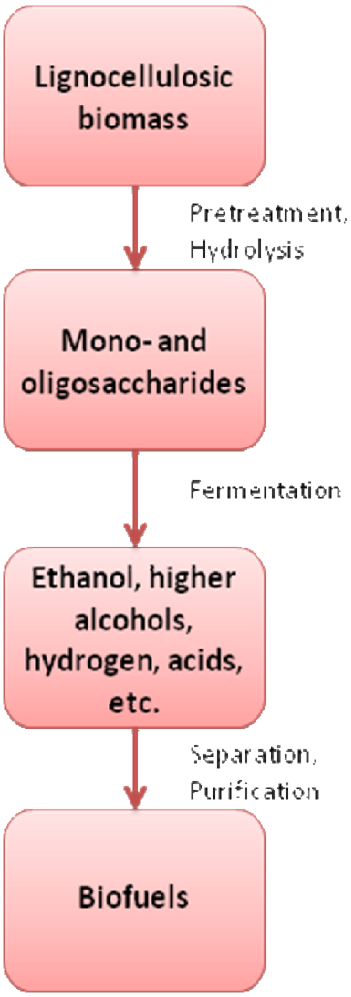


Figure 7. Schematically view of production biofuels from lignocellulose

1.4. Plant Cell Wall Degrading Fungi

Fungi play a crucial role in the degradation of the plant cell wall. Degrading enzymes are produced by many other creatures as well (bacteria, yeast, marine algae, snails, insects, etc.). They excrete extracellular enzymes, which together affect the plant cell wall materials releasing mono-, oligosaccharides and other cell wall components (Kulkarni et al., 1999; Polizeli et al., 2005). Three classes of decaying fungi are distinguished according to which part of the lignocelluloses decay: white-rot, brown-rot and soft-rot fungi. White-rot fungi can degrade the lignin part of the plant matter, while brown-rot fungi are able to release the cellulose and hemicellulose part though not the lignin. Soft-rot fungi produce both polysaccharide- and lignin-degrading enzymes (Kirk et al., 1983).

Classes of Basidiomycetes and Ascomycetes are known as efficient producers of wood decaying enzymes. *Trichoderma*, *Aspergilli* and *Penicillia* species are the most crucial microorganisms for cellulase/hemicellulase production and create the source of these enzymes for industrial applications, including the production of biofuels from plant biomass (Esposito et al., 2008; de Vries et al., 2001; Aro et al., 2005; Jorgensen et al., 2005; Chavez et al., 2006).

Filamentous fungi have been isolated and were identified as efficient lignocellulose degrading enzyme producers. Development of cost-effective lignocellulolytic enzymes for degradation of plant biomass needs continuous screening of filamentous fungi. Until now powerful and efficient enzyme coctail has not been discovered yet for total hydrolysis of lignocellulosic biomass (Martinez et al., 2004).

1.4.1. The Genus Trichoderma

Trichoderma species are some of the most abundant filamentous fungi in nature. They are well characterized and classified members of the Ascomycete brown-rot fungi group, which can be found in soils, on decaying wood and vegetable materials. All of these strains maintain the anamorph form of *Hypocrea* however, the *Hypocrea* teleomorph form exists only on wood or on other fungi, while strains of *Trichoderma* cause diseases of living plants as well and also mushroom. (Bailey et al., 1998; Harman et al., 1998; Seaby et al., 1998). Several studies provide an extensive review of the biology, enzyme production and biological control of *Trichoderma* species. More than one hundred *Trichoderma* species have been isolated and documented in the last few years (Druzhinina et al, 2006; Samuels et al., 2012).

Commercial application of enzymes of *Trichoderma* species is widely accomplished. The *Trichoderma reesei* (teleomorph *Hypocrea jercorina*) is used extensively in industry due to its facility to excrete high levels of cellulase, hemicellulases and other useful enzymes (Martinez et al., 2008).

1.4.1.1. Trichoderma Longibrachiatum Cluster

Members of the genus *Trichoderma* are present in soils and decaying plant materials. Recently**,** the range of *˝Longibrachiatum˝* has become one of the most compelling parts of genus *Trichoderma* due to the industrial production of lignocellulose degrading enzymes. The number of species belonging to the *˝Trichoderma longibrachiatum* clade˝ is continuously increasing (Samuels et al., 2012).

The most essential species in the *Longibrachiatum* group is *Trichoderma reesei* (*Hypocrea jecorina*) due to the efficient production of lignocellulose degrading enzymes and recombinant proteins (Mäntylä et al., 1998; Nakari-Setala et al., 2009; Peterson et al., 2012). All genetically developed mutants of *T. reesei* have been related from a single wild-type isolate named *T. reesei* QM6a. Originally**,** a rotting canvas fabric sample was collected in the Solomon Islands during World War II and a cellulolytic fungus was isolated later from this army tent (Mandels et al., 1957). Originally, the strain QM6a was incorrectly identified as *T. viride.* Its cellulolytic enzymes have been extensively studied from the 60’s at Natick Laboratories (Mandels et al., 1969). Later on, QM6a was recognized not as a *T. viride* but a new species namely *T. reesei* in honour of Elwyn T. .

The significance of *T. reesei* genome was investigated previously, analysis of its genome indicated that despite its production efficiency, *T. reesei* possesses the lowest amount of cellulolytic and hemicellulolytic enzymes among *Sordariomycetes* at its disposal. Therefore**,** the search for additional isolates in *Trichoderma* section *Longibrachiatum* seems to be significant and may conclude in new and efficient lignocellulose degraders (Martinez et al., 2008; Schuster et al., 2012).

Application of *Trichoderma* strains in biotechnology has been available for 30 years now. Primarily, they used their cellulases in the food industry to clarify the juices and it was also used extensively in the animal feed industry (Harbak et al., 2002). Afterwards, they recognized their positive affects in the textile, pulp and paper industry as well (Mäntylä et al., 2007). Production of cellulases and hemicellulases by *Trichoderma* strains allows for biodegradation of waste materials and bioconversion of lignocellulosic agricultural wastes (e.g. wheat straw, corn cob, wood junk) to biofuels (Martinez et al., 2008).

In addition to the cellulases and hemicellulases production they also excrete other enzymes, especially chitinases, glucanases and antifungal toxics. Several plant diseases have been controlled by antifungal enzymes related to *Trichoderma* strains. Consequently, these organisms have considerable potential for a range of commercial applications (Mandels et al., 1969).

1.4.2. Penicillium Species

Various lignocellulolytic enzyme systems of the *Penicillium* species have been investigated in the last 30 years (Liu et al., 2013). They produce high-effective cellulose degrading enzyme complexes, therefore they have improved the ability to degrade plant biomass. Numerous *Penicillium* cellulase complexes act more efficiently than *Trichoderma reesei* cellulases in digestion of different lignocellulosics materials (Thygesen et al., 2003; Krogh et al., 2004; Adney et al., 2008; Martins et al., 2008).

Penicillium strains are extensively applied in biotechnology, not only for biodegradation of agricultural waste materials–but their other enzymes (e.g. amylases, lipases, phosphatases) have been studied as well (Petruccioli et al., 1988). In addition to their usage of enzyme production they are commonly used in medical section for producing different antibiotics. Their antibiotics are able to degrade the cell walls of bacteria, making it unable to grow and multiply (Fawcett et al., 1973).

1.5. The Aim of This Thesis

This research work is based on the DISCO project that aims to discover and develop enzymes that break down lignocellosic biomass more efficiently and more cost-effectively, making the bioethanol production process commercially viable in this respect.

The aim of the current PhD research work is to investigate novel hemicellulolytic fungal enzyme systems by a new screening method using up-to-date instruments, by which specific xylan degrading enzymes can be detected. The goal of screening selected lignocellulose degrading enzyme systems was to develope an improved hydrolysis of lignocellulosic biomass to fermentable sugars.

Furthermore, this screening method was applied for a smaller subgroup of strains belonging to the *Trichoderma* section *Longibrachiatum* by diverse chromatography methods and a mass spectrometry method as well.

The hydrolytic potential of novel *Penicillium* cellulases and new fast growing *Trichoderma* cellulases was performed. The strains were cultivated on several lignocellulosic carbon sources and microcrystalline cellulose in order to produce efficient lignocellulose degrading enzyme supernatants.

The secreted enzyme mixtures were tested in different hydrolysis experiments. Cellulase enzyme mixture optimas were analysed on different temperature and pH levels. Sensitivity of cellulases has been studied during hydrolysis in presence of softwood and herbaceous lignins. Lignin inhibition of enzyme cocktails has been investigated on both lower and higher hydrolysis temperature.

2. Materials and Methods

2.1. Microorganisms

Microorganisms were stored as freeze-dried cultures in glass ampoules for long term storage in the TUB (Technical University of Budapest) culture collection (www.tub-collection.hu). Out of them, mesophilic lignocellulolytic fungi have been screened to produce cellulose and/or xylan degrading enzymes. These fungi have been isolated from soil samples and decaying plant materials have also been collected worldwide. A few taxonomically identified fungi were introduced into the study as well. They were obtained from known culture collections such as ATCC, NRRL, CBS, IFO, etc.

Revitalization of freeze-dried fungi from ampoules was performed on Potato-Dextrose-Agar (PDA) medium in Petri dishes at 30°C and the sporulated cultures were used for inoculation of shake flask fermentation.

Fungal strains belonging to the *Trichoderma* section *Longibrachiatum* are listed in **Table 1.** Identifications were performed by classical taxonomy (Samuels et al., 2012) and by molecular tools (G.J. Samuels, and C.P. Kubicek, separately in their facilities) (see corresponding papers in Table 1). *T. reesei* QM 6a was purchased from the QM collection (Prof. E.G. Simmons). Strain RUT C30 has been kindly donated by Prof. D.E. Eveleigh (Rutgers University, New Brunswick, N.J., USA).

Two promising non-*Trichoderma* fungi were also found as efficient cellulase producers and subjected to detailed studies. They were identified as *Penicillium pulvillorum* TUB F-2220 and *Penicillium cf simplicissimum* TUB F-2378 at the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. Strains F-2220 and F-2378 were isolated from soil, in close proximity to river Danube, Vac, Hungary and from soil under *Parrotia persica* (Persian ironwood), Botanical Garden, Szarvas, Hungary, respectively. Both lignocellulolytic *Penicillium* strains are deposited in the TUB culture collection.

Table 1 Strains of Trichoderma section Longibrachiatum used in this study

Species	**Strain No.**	**Identity in other culture collection**	**Origin**	**References**
T.citrinoviride	TUB F-1299	GJS 10-270	Maldives	**§**
	TUB F-1345	GJS 10-272	Brazil	**§**
T.effusum	TUB F-354	DAOM 230007 ATCC MYA-4837	India	**Bissett et al., 2003**
T.ghanense	CPK 72	CBS 489.78	Colombia	**§**
T.konilangbra	CPK 132	CBS 100808	Uganda	**§**
T.longibrachiatum	TUB F-1417	GJS 10-198	Zimbabwe	**§**
	TUB F-2531	GJS 10-242	Brazil	**§**
T.parareesei	TUB F-2535	GJS 10-243	Malaysia	**§**
T. reesei	CPK 155	GJS 86-404	French Guyana	**Kubicek et al., 1996**
	QM 6a	ATCC 13631	Solomon Islands	**Mandels and Weber, 1969**
	RUT C30	ATCC 56765	Mutant of QM 6a	**Montenecourt and Eveleigh, 1979**
T.saturnisporum	ATCC 18903		Georgia, USA	**Turner et al., 1997**
T.gracile	TUB F-2543	GJS 10-263	Malaysia	**Samuels et al., 2012**
T.sinense	TUB F-1047	DAOM 230003	Taiwan	**Bissett et al., 2003**
	TUB F-1058	**DAOM 230004 ATCC MYA-4850**	**Taiwan**	**Bissett et al., 2003**

ATCC: American Type Culture Collection, Manassas, Virginia;
CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands;
CPK: collection of Christian P. Kubicek, Technical University of Vienna, Austria;
DAOM: Plant Research Institute, Department of Agriculture, Mycology, Ottawa, Canada;
GJS: collection of Gary J. Samuels, USDA, ARS, Beltsville, MD, USA;
TUB: Technical University of Budapest culture collection
§ These isolates have been identified by G.J. Samuels

2.2. Isolation of lignocellulolytic fungi

Apparently, two types of agar media were used to isolate the cellulase and hemicellulase producing fungi. The medium contained either 30 g/L cellulose powder (Sigmacell Type 20, Sigma Aldrich) or 30 g/L finely milled wheat straw (<0.3 mm) as carbon source. The other components were similar for both media (in g/L); $NaNO_3$, 3; $(NH_4)_2SO_4$, 1; KH_2PO_4, 1; $(NH_4)_2HPO_4$, 0.5; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; Difco yeast extract, 0.3; Bacto agar, 20; and (in mg/L) $FeSO_4.7H_2O$, 5; $MnSO_4$, 1.6; $CoCl_2.6H_2O$, 2; $ZnSO_4.7H_2O$, 3.45. The pH (before sterilization) was set to 6.5 use sulfuric acid. Media were autoclaved routinely (30 min, 121°C). The isolation agar media were supplemented with 100 µg/mL doxycycline hyclate (Sigma Aldrich) to suppress bacterial growth. Following incubation at 30°C primary fungal colonies were inoculated in Petri plates by streaking twice on potato-dextrose agar (PDA) supplemented with 1 g/L Triton X-100 in order to obtain single colonies. The fungal isolates were freeze-dried for long term storage.

Approximately 950 mesophilic lignocellulolytic filamentous fungi were isolated from soil samples and decaying plant materials (worldwide) along with being tested in shake flask fermentations. Most of these strains were *Trichoderma* species. However**,** several other identified and non-identified strains (e.g. *Penicillium*, *Aspergillus*) were tested, too. After the screeningwork a few promising strains were selected for further in-depth analysis.

2.3. **Enzyme Production**

Two types of shake flask cultivation media (LC-3 and LC-4) were used (**Table 2 and 3**). The properly sporulated Petri plate cultures were used for inoculation. Of all media 150 mL was sterilized in 750 mL cotton-plugged Erlenmeyer flasks. They were inoculated with 3 loopful of spores per flask. Shake flask fermentation was carried out at 30°C for 5 days on a rotary shaker at 220. The pH value was adjusted to 5.0 before sterilization. Crude supernatants (after centrifugation of fermentation broths) were stored at -18°C for further analysis.

Table 2. Composition of shake flask fermentation media

Macrocomponents (g/L)		
	LC-3	**LC-4**
Carbon source	20*	20*
KH_2PO_4	1.5	1
$(NH_4)_2HPO_4$	2	1
soybean meal, defatted	1	1
corn steep liquor, 50%	1	1
NaCl	0.5	0.5
$CaCO_3$	1	0.5
urea	0.3	-
$MgSO_4.7H_2O$	0.3	-
$CaCl_2$	0.3	-
$(NH_4)_2SO_4$	-	1
Microcomponents (mg/L)		
$FeSO_4.7H_2O$	5	5
$MnSO_4$	1.6	1.6
$CoCl_2.6H_2O$	2	2
$ZnSO_4.7H_2O$	3.45	3.45

* Quantity of Avicel PH-101 (microcrytalline cellulose) was 13 g/L.

Table 3 describes the carbon sources in the fermentation media. All carbon sources of lignocellulose origin were milled in a coffee grinder into powder (<0.3 mm) before use. LC-8, LC-10 and LC-12 media were similar to LC-3 while LC-9 and LC-11 were similar to LC-4 media except carbon sources.

Table 3. Carbon sources during the enzyme production

Media	Carbon source
LC-3	wheat straw (Hungarian origin)
LC-4	wheat straw (Hungarian origin)
LC-8	Avicel PH-101 (Fluka)
LC-9	Avicel PH-101 (Fluka)
LC-10	Eucalyptus wood (Spanish origin)
LC-11	corn fiber (Hungarian origin)
LC-12	pre-treated spruce (Swedish origin)

2.4. Enzyme Assays

Cellulase activity (FPA) was determined as described by Ghose et al. (1987). The reaction mixture contained 1x6 cm Whatman No.1 filter paper , 1.0 mL 50 mM citrate buffer (pH=4.8) and 0.5 mL appropriately diluted supernatant. Standard curve was prepared with glucose (Reanal, Budapest, Hungary). Overall cellulase activity was expressed as Filter Paper Unit (FPU) per ml of supernatant.

β-glucosidase activity was determined using 1.0 mL 5mM 4-nitrophenyl-β-D-glucopyranoside (Sigma Aldrich, USA) dissolved in 50 mM citrate buffer (pH=4.8) and 0.1 mL appropriately diluted supernatant. Standard curve was made with 4-nitrophenol at 400 nm (Hägerdal et al., 1979).

Endo-xylanase activity was assayed using 1.8 mL 1.0% birchwood glucuronoxylan Art 7500.1 (Carl Roth GmbH, Karlsruhe, Germany) in 50 mM citrate buffer (pH=5.0) and 0.2 mL appropriately diluted supernatant according to Bailey et al. Standard curve was prepared with xylose (Reanal, Hungary) (Bailey et al., 1992).

β-xylosidase activity was determined using 1.0 mL 5mM 4-nitrophenyl-β-D-xylopyranoside (Sigma Aldrich, USA) dissolved in 50 mM citrate buffer (pH=4.8) and 0.1 mL appropriately diluted supernatant. Standard curve was made with 4-nitrophenol at 400 nm (Herr et al., 1978).

α-L-arabinofuranosidase activity was assayed using 1.0 mL 2mM 4-nitrophenyl-α-L-arabinofuranoside (Sigma Aldrich, USA) dissolved in 50 mM citrate buffer (pH=4.8) and 0.1 mL properly diluted supernatant according to Poutanen et al. (1987). 4-Nitrophenol was used for the standard curve at 400 nm.

Protein precipitation was performed with acetone according to Wessel et al. (1984). Protein concentrations were measured with a commercial kit (DC Protein Assay) based on the Lowry method (1951), using bovine serum albumin (BSA) (Bio-Rad Laboratories Inc., Hercules CA, USA) as standard protein.

Fermentation experiments and enzyme assays were performed in Laboratory of Industrial Microbiology, Budapest University of Technology and Economics, Hungary.

2.5. Materials and Methods Applied in Xylanase Experiments

2.5.1. Substrates

Altogether four xylan-rich substrates (two water soluble and two water insoluble materials) have been used in the hydrolysis experiments (Kormelink et al., 1993a; Christov et al., 2000). The water-soluble materials were wheat arabino-xylan (WAX), medium viscosity (Megazyme, Wicklow, Ireland) and eucalyptus xylan hydrolysate (EXH) produced by hydrothermal treatment (Garrote et al., 1999), provided by Prof. Dr. Parajo of the University of Vigo-Ourense, Spain.

One of the insoluble substrates in the hydrolysis was wheat straw water unextractable solids (WS WUS). It was produced by mixing of 60 ± 0.1 g of wheat straw (PärnuJaagupi, Estonia – harvested in winter 2009, kindly provided by Andres Käsper, Biogold Estonia) with an excess of Millipore water (70 °C) in a shaker for one hour-long incubation at 60 °C and 150 rpm. After this, the material was filtered (110 mm Ø, Schleicher & Schuell, Dassel, Germany), The residue was collected, again mixed with water at 70 °C under the same conditions. After filtration the residues were collected and freeze-dried. The corn fiber alcohol insoluble solids (CF AIS) were produced as described by Kabel et al. (2002). In the first step starch was removed using α-amylase and amyloglucosidase then all low molecular weight materials were removed by washing with 70% ethanol. The final alcohol insoluble solids were washed with acetone and dried in the air. The corn fiber originated from a wet milling process (Archer Daniels Midland, Decatur, IL, USA). These two subtrates were prepared previously and kindly provided by Dr. Martine Van Gool of the Wageningen University, the Netherlands.

2.5.2. Sugar Composition of Substrates

Analysis of sugar composition of substrates begun with a total hydrolysis first using 72% (w/w) sulfuric acid at 30 °C for one hour and then another hydrolysis with 1 M sulfuric acid at 100 °C for three hours. The neutral monosaccharides were assayed as their alditol acetates with inositol as internal standard. A Focus gas chromatograph (Thermo Scientific, Waltham MA, USA) equipped with a Supelco SP 2380 column was used for determination.

The uronic acid content was analysed by the colorimetric m-HDP assay (Ahmed and Labavitch, 1978) using an auto-analyser (Skalar Analytical, Breda, The Netherlands) and calibration was performed by galacturonic acid (0–100 µg/mL). Sugar composition and other content of substrates are in **Table 4**. Analysis of sugar composition of substrates, hyrolysis experiments and analitycal measurements were performed in Laboratory of Food Chemistry, Wageningen University, the Netherlands. **Figure 8** represents schematical view of structure of WAX and EXH substrates.

Table 4. Sugar composition of xylan-rich hydrolysis model substrates

	Sugar content (w/w%)						
Substrate	**Ara**	**Xyl**	**Gal**	**Glu**	**UA**	**Ac**	**Total**
WAX	32	58	0	0	1	0	91
EXH	2	39	5	2	6	8	59
WS WUS	4	36	1	53	3	0	68
CF AIS	14	25	4	21	6	0	70

Ara= arabinose; Xyl=xylose; Gal=galactose; Glu=glucose; UA = uronic acid content; Ac = *O*-acetyl groups

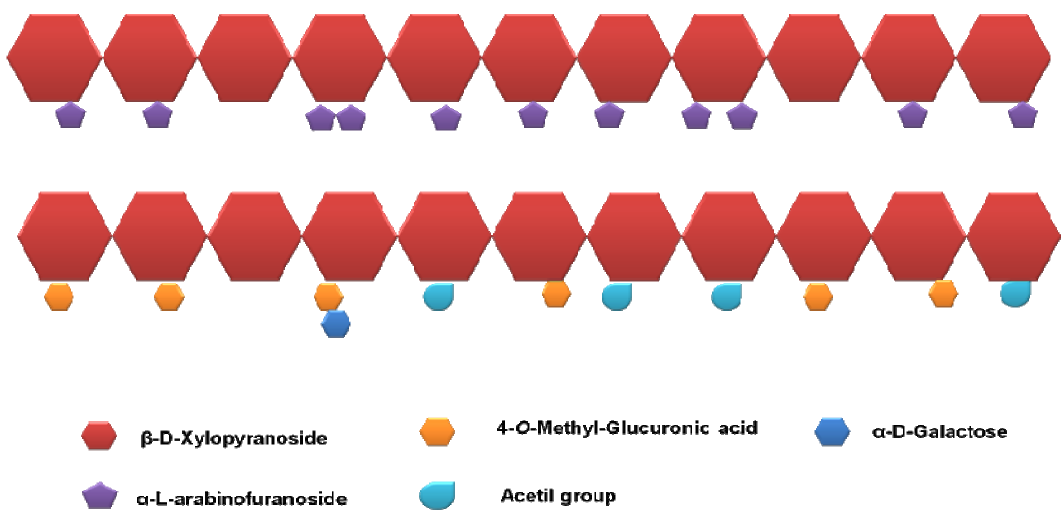


Figure 8. Schematical structure models for xylans extracted from wheat (upper) and eucalyptus wood (lower)

2.5.3. Hydrolysis

Four xylan-rich soluble and insoluble substrates were applied in hydrolysis experiments: WAX, EXH, wheat straw WUS and corn fiber AIS. Incubations were carried out with 5 mg/mL substrate and 1% (v/v) of fermentation supernatants, in 0.05 M Na-acetate buffer at pH=5.0, 500 rpm and 37°C for 24 h. In case of EXH hydrolysis we used Na- citrate buffer with the same conditions as earlier described. Incubation was stopped by boiling for 10 min.

Aspergillus awamori endo-(1,4)-β-D-xylanase-I (GH10) was used in the hydrolysis as reference. Purification and performance of endo-xylanase-I have been described earlier (Kormelink et al., 1993b).

2.5.4. *Analytical Characterization of Hydrolysis Products*

Characterization of Oligo- and Monomers by HPAEC

Clear hydrolysis supernatants were 100× diluted with milliQ water and analysed by High Performance Anion Exchange Chromatography (HPAEC) using an ICS3000 HPLC system (Dionex, Sunnyvale, CA), equipped with a CarboPac PA-1 column (2 mm ID × 250 mm; Dionex) in combination with a CarboPac PA guard column (2 mm ID × 25 mm) and a ISC3000 ED PAD-detector (Dionex). The flowrate of oligomer analysis was 0.3 mL/min using the following gradient of 0.1 M sodium hydroxide (NaOH) and 1 M sodium acetate (NaOAc) in 0.1 M NaOH: 0–45 min, 0–500 mM NaOAc in 0.1 M NaOH; 45–48 min washing step with 1 M NaOAc in 0.1 M NaOH; 48–60 min, equilibration with 0.1 M NaOH.

The elution profile of monomer analysis was: 20 minutes isocratic on 16 mM NaOH; 20-20.1 min, linearly 16- 100 mM NaOH; 20.1- 22.0 min isocratic at 0.1 M NaOH; 22.0-22.1 min linearly from 0-1 M NaOAc in 0.1 M NaOH; 22.1-26 min, isocratic in 1M NaOAc in 0.1 M NaOH; 26-26.1 min linearly from 1-0 M NaOAC in 0.1 M NaOH; 26.1-30 min isocratic at 0.1 M NaOH; followed by reconditioning of the column for 15 minutes at the starting gradient.

Twenty µl of samples were injected into the column. Quantification is based on the response factor of the standard solutions of xylose to xylotetraose (X1 Sigma Aldrich, Steinheim, Germany, X2–4 Megazyme, Wicklow, Ireland), D-arabinose and D-glucuronic acid (Sigma Aldrich).

Characterization of Molecular Weight of Oligomers by HPSEC

Determination of molecular weight distribution of polysaccharides and large oligosaccharides were performed by High Performance Size Exclusion Chromatography (HPSEC) on an Ultimate 3000 UPLC system (Dionex, Sunnyvale, CA, USA) equipped with three TSKgel SuperAW columns (6.0 mmID × 15 cm/column; 4 µm) in series (SuperAW4000, SuperAW3000, SuperAW2500; Tosohaas Bioscience, Stuttgart, Germany), in combination with a guard column (Tosohaas Bioscience). Elution passed off at 55 °C with 0.2 M sodium nitrate ($NaNO_3$) at 0.6 mL/min. The eluate was followed by a Shodex RI-101 (Kawasaki, Japan) refractive index (RI) detector. The system was calibrated with a pullulan series (Sigma Aldrich, St. Louis, MO, USA).

Determination of Acetic Acid Content by HPLC

For the determination of total acetic acid content of the EXH substrate, the EXH blank was saponified by adding 50 µL 8 M sodium hydroxide (NaOH) to 0.45 mL of an EXH solution (5 mg/mL). The sample was incubated for one hour on ice and 2 hour at room temperature. This saponified EXH solution and all samples were analysed for acetic acid using an Ultimate 3000 system (Dionex) equipped with an Shodex RI detector and an Aminex HPX 87H column (300 mm × 7.8 mm) (Bio-Rad, Hercules, CA). Twenty µL of samples were injected. Elution was performed with 0.005 M H_2SO_4 at a flow rate of 0.6 mL/min and a column oven temperature of 40 °C. The amount of acetic acid released was calculated as percentage of the total amount of acetic acid present in the samples.

Characterization of Oligomers by MALDI-TOF MS

For Matrix Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF MS) analysis an Ultraflex workstation (Bruker Daltonics, Bremen, Germany) equipped with a 337 nm laser was applied. The mass spectrometer was operated in the positive mode and calibrated with a mixture of maltodextrins (AVEBE, Veendam, The Netherlands; mass range 500–3500 Da). After a delayed extraction time of 120 ns, the ions were accelerated with a 25 kV voltage and subsequently detected using the reflector mode. One µL of desalted sample solution (AG 50 W-X8 resin; Bio-Rad, Hercules CA, USA) was mixed with 1 µL of matrix and dried under warm air. The matrix solution was prepared by dissolving 10 mg 2,5-dihydroxybenzoic acid (Bruker Daltonics) in a mixture of 700 µL water and 300 µL acetonitrile.

2.6. Materials and Methods Applied in Cellulase Experiments

2.6.1. Pretreatment of Lignocellulosic Materials

Natural wheat straw was obtained from local sources in Hungary and milled in a coffee grinder to powder (<0.3 mm particle size) before use. Hydrothermally pre-treated wheat straw samples were obtained from Biogold OU (Tallinn, Estonia). High pressure steam was used at 200°C for 20 min without any chemical additive as a pre-treatment method in Biogold. Steam pre-treated spruce (SO_2 catalyzed pre-treatment) was provided by SEKAB E-Technology (Örnsköldsvik, Sweden). Only the solid fractions of the pre-treated lignocelluloses were used in the experiments. Prior to using all the materials were washed with hot water (55°C, 2.2 l/kg lignocellulose). The washed pre-treated lignocelluloses were stored frozen (-20°C) or dried (45°C). Pretreatment experiments were performed in laboratory of Research Centre of Finland (VTT, Espoo, Finland).

2.6.2. Isolation of Spruce and Wheat Straw EnzHR lignins

The EnzHR lignins were isolated from hydrothermally pretreated wheat straw (Inbicon A/S (Kalundborg, Denmark) and steam pretreated spruce (Lund University, Sweden) using a combination of enzymatic hydrolysis of polysaccharides and protease mediated removal of residual cellulases as described in Rahikainen et al. (2011).

2.6.3. Temperature and pH Dependencies of Fungal Cellulase Mixtures

The impact of temperature and pH on the hydrolytic activity in the *Penicillium* species and *T. i* enzyme mixtures produced in shake flask fermentations (day 5) were assayed via 24 h hydrolysis of Avicel PH-101 (10 mg/ml) at 30-70°C (pH:5.0) and pH 3-8 (T=45°C). The buffers used in the experiments were 0.05 M sodium acetate (pH 3-6), and phosphate (pH 6-8) supplemented with 0.5 mg/ml Na-azide. The hydrolysis experiments were carried out in a rotatory shaker (150 rpm). The fungal culture supernatants were dozed to the hydrolysis on activity basis (10 FPU/g substrate). All the mixtures were supplemented with 6 U β-glucosidase (Novozyme 188)/g substrate (DM). The hydrolysis reactions were terminated by boiling for 10 min. The solid and liquid fractions in hydrolysis mixtures were separated by centrifugation and released sugars were quantified from the liquid fraction using DNS assay (Miller et al., 1959). All the hydrolysis experiments were carried out in triplicates. Background values from the mixtures lacking substrate (enzyme blank) or Avicel/lignin (substrate blank) were subtracted.

2.6.4. Hydrolysis Experiments

The hydrolytic activity of the *Penicillium* species and *T. i* enzyme mixtures produced in the shake flask fermentations on Avicel PH-101 (Fluka) were tested for Avicel PH-101, pre-treated wheat straw (Biogold) and pre-treated spruce (SEKAB E-Technology). The hydrolysis experiments were performed in 0.05 M Na-acetate buffer (supplemented with 0.5 mg/ml Na-azide) at pH 5.0 and 45°C in rotatory shaker (150 rpm) with 10 mg DM/ml substrate concentration. Enzyme loadings were 10 FPU cellulase/g DM and 6 U β-glucosidase (Novozym 188, Novozymes) /g DM substrate. Samples were taken from the hydrolysis mixture at 4, 24 and 48 h time-points and hydrolysis yields were quantified using DNS assay (Miller et al., 1959). All the hydrolysis experiments were carried out in triplicates. Background values from the mixtures lacking a substrate (enzyme blank) or Avicel/lignin (substrate blank) were subtracted.

2.6.5. Lignin-caused Inhibition of Hydrolysis

The impact of enzyme hydrolyzed spruce (SP EnzHR) and enzyme hydrolyzed wheat straw (WS EnzHR) lignins on the hydrolytic activity of *Penicillium* species and *Trichoderma i* enzyme mixtures produced in shake flask fermentations (with Avicel PH-101 as carbon source, day 5) were tested. The hydrolysis experiments were carried out with Avicel PH 101 (10 mg/ml) as substrate in the presence (5 mg/ml) or absence of lignins. The hydrolysis was carried out in 0.05 M sodium acetate buffer (pH 5.0) supplemented with 0.5 mg/ml Na-azide at 35°C and 45°C in rotatory shaker (150 rpm) for 24 hours. Enzyme loadings were 10 FPU/g

DM cellulase and 6 U/g DM Novozym 188 β-glucosidase. The hydrolysis reactions in the samples were terminated by boiling (10 min), after which solids and liquids were separated by centrifugation and released sugars were quantified from the liquid fraction with DNS assay (Miller et al., 1959). All the hydrolysis experiments were carried out in triplicates. Background values from the mixtures lacking substrate (enzyme blank) or Avicel/lignin (substrate blank) were subtracted.

2.6.6. Purification of *P. pulvillorum* Cellulases

Shake flask culture of TUB F-2220 *P. pulvillorum* after 7d cultivation on LC8 (with Avicel as carbon source) were centrifuged at 3000 × g, filtrated through 0.45µm glass fibre filters and concentrated and changed to 6 mM sodium phosphate buffer pH 7.0 by ultrafiltration (cut off 10,000 Da). The following protein purification steps were carried out with ÄktaPurifier (Pharmacia, Sweden). First, the concentrated and buffered enzyme sample was applied to a DEAE Sepharose HighTrap FF column (V = 1 ml, Pharmacia BioTech, Sweden). The bound proteins were eluted with increasing NaCl gradient (10 CV, 0–0.3 M). The unbound fraction was changed to 10 mM sodium acetate buffer pH 3.9 and applied to a CM Sepharose HighTrap FF column (V = 1 ml). The bound protein was eluted with a 0.05 M NaCl gradient. Avicelase and CMCase activity and SDS-PAGE (10% Tris–HCl gel) were analyzed from the pooled fractions. The pooled protein fractions were buffer changed to 25 mM sodium acetate buffer pH 5 by ultrafiltration (cut off 10,000 Da). Reference enzyme *T. i* Cel7A was purified from the culture of *T. i* RUTC30 as described previously (Rahkamo et al., 1996; Suurnäkkiet al., 2000). Purification experiments were performed in laboratory of Research Centre of Finland (VTT, Espoo, Finland).

2.6.7. Enzyme Assays in Protein Purification

Total protein concentrations in the enzyme fractions were analyzed with a Bio-Rad DC kit according to manufacturer's instructions using BSA as a standard. Avicel hydrolysing activity was assayed on 96 well plates in triplicates using 10 mg/ml microcrystalline celluloses (Serva) as a substrate in buffer (50 mM (pH 5) sodium acetate or McIlvaine pH 3–7). After 3 h incubation at 35–50 °C with mixing rate of 1000 rpm (ThermoMicroMixer, FinePCR), the hydrolysis was terminated by an addition of 10 M sodium hydroxide (final concentration 0.2 M). The liquid and solid fractions of the hydrolysis mixtures were separated by centrifugation (20,000 × g, 10 min) and released sugars were quantified using PAHBAH assay and glucose standards. The 1,4-β endoglucanase activity was analyzed using CMCase assay with 2% carboxymethyl cellulose (CMC, Sigma) as substrate in 50 mM sodium citrate pH 5 and 10 min incubation at 50 °C. Reducing sugars were quantified with DNS reagent (1.5 vol) and glucose standards. Beta-glucosidase activities were assayed using 1 mM p-nitrophenyl β-d-glucopyranoside (Sigma) as substrate in 50 mM sodium citrate buffer (pH5) with 10 min incubation at 50 °C. Xylanase activities were determined using 1% birch glucuronoxylan (Roth, Germany) as substrate in 50 mM sodium citrate (pH5) with 5 min incubation at 50 °C. Reducing sugars were quantified with DNS reagent and xylose standards (Viikari et al., 2007). Enzyme assays were performed in laboratory of Research Centre of Finland (VTT, Espoo, Finland).

2.6.8. Hydrolysis Experiments with P. pulvillorum Cellulases

The partially purified *P. pulvillorum* cellulases were tested in the hydrolysis of Avicel (Serva) at 45 °C and 55 °C at pH 5 with enzyme dosage 4 mg/g substrate and 4–48 h hydrolysis time in triplicates. The reactions were supplemented with 30 U of thermostable Thermoascus aurantiacus TaCel3a β-glucosidase/g DM kindly provided by ROAL OY (Rajamäki, Finland). Hydrolysis reactions were terminated by heating at 100 °C for 10 min. Hydrolysis yields were determined by measuring released sugars with a PAHBAH assay (Lever et al., 1972; Viikari et al., 2007).Hydrolysis experiments were performed in laboratory of Research Centre of Finland (VTT, Espoo, Finland).

2.6.9. De Novo Sequencing of the Purified P. pulvillorum Cellulases

The partially purified *P. pulvillorum* (TUB F-2220) cellulases were subjected to peptide sequencing in Protein Chemistry Unit at Helsinki University Institute of Biotechnology, Finland (http://www.biocenter.helsinki.fi/bi/protein/index.htm). The proteins were separated on 12% SDS-PAGE and stained with 0.1% Coomassie Brilliant Blue. The protein bands were excides from the gel, alkylated with iodoacetamide and digested with trypsin. The de novo sequencing of peptides was carried out using LC–MS/MS technique. The resulting peptide sequences were analyzed using Mascot (www.matrixscience.com) and FASTS (http://fasta.bioch.virginia.edu/fasta www2/fasta www.cgi) software (Mackey et al., 2002; Perkins et al., 1999). De novo sequencing of enzymes were performed in laboratory of Research Centre of Finland (VTT, Espoo, Finland).

2.6.10. Adsportion Assay

Adsorption of the purified *P. pulvillorum* and *T. i* cellulases to cellulose and lignins was tested by incubating 10 µg of protein/mg of Avicel (10 mg/ml) or EnzHR lignins (spruce and wheat straw origin, 10 mg/ml) in 50 mM sodium acetate (pH 5) 24 h at 4 °C (Avicel) or 35 °C (lignins) with mixing (1200 rpm) in duplicates. Enzymes without lignin and lignins without enzyme were used as controls. After incubation, the solids and liquids were separated by centrifugation (10 min, 14,000 × g). The enzymes in the liquid fraction were applied to SDS-PAGE (10% Tris–HCl gel, Criterion Stain Free, BioRad Laboratories Inc., USA) and the protein bands were quantified using the stain free visualization system and Image Lab software as described in Várnai et al. (2010). Impact of pH to the adsorption of the enzymes to spruce EnzHR lignin was tested in similar manner but using 50 mM Na-citrate pH 4–6 as buffer in duplicates. Influence of temperature to the adsorption of the enzymes to spruce EnzHR lignin was studied by incubation the enzymes with lignin at 35 °C and 45 °C (repeated 1–2 times). After separation of the liquid and solid fractions, the solid fraction was resuspended to equal to original volume of 50 mM sodium acetate pH 5 and both liquid and solid fractions were analyzed on SDS-PAGE. Adsorption assays were performed in laboratory of Research Centre of Finland (VTT, Espoo, Finland).

2.6.11. Cloning of TUB F-2220 P. pulvillorum cellulases

For isolation of genomic DNA, *P. pulvillorum* (TUB F-2220) strain was cultivated on potato dextrose agar plates for three days, after which the mycelia was freeze dried over night. Genomic DNA was isolated from the lyophilized cultures with CTAB extraction buffer. For isolation of RNA, the TUB-F-2220 was cultivated in LC8 medium in shake flasks for 5days, after which the total RNA was isolated from the mycelia using Qiagen RNA Easy Plant/Fungi

Mini Kit. The RNA was reverse transcribed to 5’ and 3’ RACE ready cDNA with SMARTerRace kit (Clontech). For amplification of *cbh* genes, degenerate oligos were designed against conserved regions of fungal CBH enzymes (5’-ACCAA[C,T]TGCTA[C,T]ACI[A,G]G[C,T]AA-3’ and 5’-GC[C,T]TCCCAIAT[A,G]TCCATC-3’). The internal *cbh* gene fragments were amplified from TUB F-2220 genomic DNA using the degenerate oligos as primers. The amplified PCR products were sequenced with Sanger sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The fragments matching the peptide sequences obtained in de novo sequencing of purified *P. pulvillorum* cellulases were selected for continuation. The 5’ end of the *cbh* gene was amplified using ligation mediated PCR. Digestion of genomic DNA (50 µg) was tested with eight restriction enzymes (*ScaI, StuI, HpaI, EcoRV, HincII, NruI, SspI, HaeIII*) and the PCR amplification was carried out using gene specific inner and outer primers 5’-CAAGACCTGCGCGTCCAACTG-3‘ and 5’- GATGGCGCCAGCTACGCAAGC-3’ using following conditions: 94 °C 3 min, 30× (94 °C 1 min, 60 °C 2 min, 72 °C 2 min), 72 °C 10 min. The 3’ end of the gene was amplified from the cDNA using gene specific primer ‘5-CGGACAGGCCAACGTCGAGG-3’ using SMARTerRace kit (Clontech) according to the manufacturers instruction: 5 × (94 °C 30 sec, 72 °C 3 min), 5 × (94 °C 30 sec, 70 °C 30 sec, 72 °C 3 min), 25 × (94 °C 30 sec, 68 °C 30 sec, 72 °C 3 min). The PCR products were sequenced with Sanger sequencing and full length gene was assembled using overlapping regions in the PCR fragments. The sequence analyses were carried out using Geneious Pro software (Biomatters Ltd., New Zealand), NCBI-BLAST and ClustalW available via EMBL-EBI (www.ebi.ac.uk), SignalP (http://www.cbs.dtu.dk/services/SignalP/) and protein analysis tools at SIB Bioinformatics Resource Portal Expasy (www.expasy.org) (Muller et al., 1989; Saghai-Maroof et al., 1984). Cloning experiments were performed in laboratory of Research Centre of Finland (VTT, Espoo, Finland).

3. Results and Discussion

3.1. Screening of Fermentation Supernatants on Soluble Xylan-rich Model Substrates (Paper #1)

Two kind of soluble xylan rich substrates were applied in hydrolysis: wheat arabinoxylan (WAX) and eucalyptus xylan hydrolysate (EXH). The sugar composition of xylans is shown in w/w% (**Table 4**). WAX is mostly substituted with arabinose. Xylan backbone of EXH is substituted with 4-*O*-methylglucuronic acid and acetic acid (Christov et al., 2000; Kormelink et al., 1993a). The lower amount of sugar in EXH is resulted by the hydrothermal treatment (Garrote et al., 1999).

3.1.1. Production and Selection of the Fermentation Liquids

Initial selection of filamentous fungi was based on the growing ability of the microorganisms on agar media containing cellulose and wheat straw as a sole source of carbon. The properly selected and purified lignocellulolytic fungi were then freeze-dried (long-term storage). A few strains originated from known culture collections (ATCC, NRRL, IFO, etc.) were also introduced in the study. In the next step revitalized fungi were tested in shake flask fermentation experiments. At the end of the fermentation the following xylan degrading enzyme activities were assayed from the supernatants by colorimetric methods: endo-xylanase, 1,4-β-xylosidase and α-L-arabinofuranosidase. Origin and enzyme activites of selected fermentation cultures are shown in **Table 5**.

Table 5. Information about the selected isolates and their enzyme activities.

Sample no.	**Fungal strains**	**Shake flask medium**	**Isolation origin of strain**	**Colorimetric methods**		
				Xyl (IU/ml)	**BX (IU/ml)**	**AF (IU/ml)**
B1	*Trichoderma sp. TUB F-1702*	LC-3	*Soil, Ho Chi Minh City (Saigon), Vietnam*	17.9	1.05	50.36
B4	*Non-identified TUB F-2292*	LC-4	*Soil, Sibelius park, Helsinki, Finland*	0.5	0.05	2.94
B7	*Non-identified TUB F-2346*	LC-3	*Decaying forest litter, Camlidere, Turkey*	2.3	0.13	11.7

B8	*Non-identified TUB F-2346*	LC-4	*Decaying forest litter, Camlidere, Turkey*	3.3	0.11	12.83
B12	*Non-identified TUB F-2342*	LC-4	*Soil, Horsetooth Mt., Ft.Collins, Colorado, USA*	31.2	0.15	5.95
B14	*Non-identified TUB F-2353*	LC-4	*Soil, Horsetooth Mt., Ft.Collins, Colorado, USA*	28.6	0.15	6.41
B27	*Non-identified TUB F-2348*	LC-4	*Soil under thuya tree, city park, Budapest, Hungary*	1.4	0.02	7.23
B29	*Trichoderma sp. TUB F-2350*	LC-4	*Meadow soil, 1180 m elevation, mountain near Yalta, Crimera, Ukraine*	1.7	0.07	2.66
B30	*Non-identified TUB F-2352*	LC-4	*Decaying forest litter, Camlidere, Turkey*	10.7	1.02	25.49
B34	*Non-identified TUB F-2385*	LC-4	*Soil, near Dead Sea, Jordan*	1.6	0.04	3.02
B36	*Non-identified TUB F-2388*	LC-4	*Soil in oasis, Syrian desert, Palmira, Syria*	0.3	0.04	2.49
B38	*Non-identified TUB F-2390*	LC-4	*Soil, Queensland, Australia*	5.9	0.52	11.81
B40	*Asp. oryzae NRRL 3485*	LC-4	*NRRL 3485 (=ATCC 46244)*	22.8	0.4	5.87
B41	*Asp. niger ATCC 10864*	LC-4	*ATCC 10864*	24.9	1.09	14.1
B42	*Asp. foetidus ATCC 14916*	LC-4	*ATCC 14916*	33.8	0.55	12.08
B43	*Non-identified TUB F-2386*	LC-4	*Soil, near Dead Sea, Jordan*	2.2	0.07	2.87
B45	*Trichoderma sp. TUB F-1647*	LC-4	*Dead bark of an unidentified tree with lichen, Embudu Village Island, South-Male Atoll, Maldives*	11	0.14	11.46
B49	*Non-identified TUB F-2372*	LC-4	*Soil under Parrotia persica (Persian ironwood), Arboretum, Szarvas, Hungary*	6.5	0.22	19.18
B52	*Asp. terreus OKI 16/5*	LC-4	*Cellulose pulp, Hungary*	3.5	0.22	5.6
B53	*Non-identified TUB F-2394*	LC-4	*Soil, New South Wales, Australia*	1.8	0.03	3.2
B54	*Non-identified TUB F-2361*	LC-4	*Garden soil under thorn-bush, Budapest, Hungary*	2.1	0.04	4.31
B62	*Cha. globosum OKI 270*	LC-4	*OKI 270*	0.7	0.02	3.09
B66	*Non-identified TUB F-2378*	LC-4	*Soil under Parrotia persica (Persian ironwood), Arboretum, Szarvas*	9.7	0.56	8.19
B68	*Pae.bacillisporus IFO 9387*	LC-4	*IFO 9387*	1	0.03	5.78
B72	*Myr. verrucaria NRRL 2003*	LC-4	*NRRL 2003 (=ATCC 9095) 1.2*	1.2	0.02	4.89
B73	*Non-identified TUB F-2341*	LC-4	*Soil, Horsetooth Mt., Ft.Collins, Colorado, USA*	2.7	0.12	5.63
B74	*Trichoderma sp. TUB F-2380*	LC-4	*Soil under Gynerium argenteum (pampas grass), Arboretum, Szarvas, Hungary*	8.4	0.36	25.25
B78	*Pae. varioti IFO 4855*	LC-4	*IFO 4855*	1.1	0.14	2.91

Xyl=endo-xylanase activity; BX=1,4-β-xylosidase activity; AF=1,4-α-L-arabinofuranosidase activity
Asp.=Aspergillus; Cha.=Chaetomium; Pae.= Paecilomyces; Myr.= Myrothecium

Strains ATCC 10864 and ATCC 14916 (two black *Aspergilli*) and three *Trichoderma* strains (F-1647, F-1702 and F-2380) are relatively good producers of endo-xylanase, β-xylosidase and arabinosidase enzymes. These two black *Aspergilli* were previously found to be good xylan decomposers (Bailey and Poutanen, 1989; Linden et al., 1994). Besides other, non-identified fungi have shown similar good secretion of xylan degrading enzymes. Nevertheless, traditional colorimetric assays do not allow us to draw conclusions concerning the presence of individual enzymes involved in the degradation of complex xylans. Also weak producers of xylanases may secrete significant hemicellulolytic enzymes required for total degradation. Consequently, the application and combination of further methods (HPAEC, HPLC and MALDI-TOF MS) are likely to result in new information about the complexity of fermentation supernatants.

3.1.2. Enzyme Recognition in WAX Digests

Fermentation supernatants were tested in hydrolysis of WAX. After the incubation the products of hydrolysis digests were analysed by HPAEC. The hydrolysis products of WAX degradation by the fermentation supernatants have given different elution patterns which are visualized in **Figure 9**. It gives an insight into the yielded monosaccharides and the substituted oligosugars too. As a reference *Aspergillus awamori* endo-xylanase-I (G10) WAX digest was used to identify the elution patterns of arabinoxylan oligoaccharides in the digests produced.

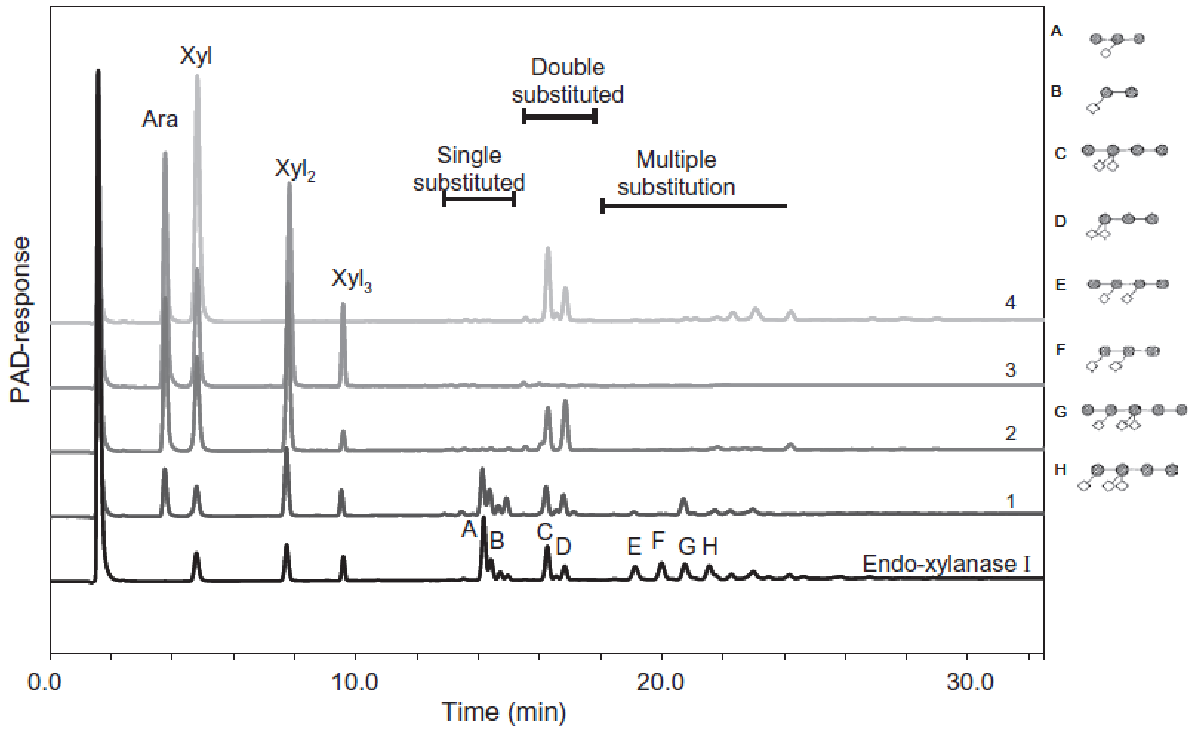


Figure 9. Endo-xylanase-I digest pattern and four typical HPAEC patterns as found for digests of fermentation liquid on WAX (line 1: degradation pattern of WAX after hydrolysis with sample B6, line 2: WAX after hydrolysis with sample B13, line 3: WAX after hydrolysis with sample B7, line 4: WAX after hydrolysis with sample B42).

A part of the fermentation liquids have produced monosaccharides, while others released substituted oligosugars. Comparing the elution profiles of the 78 fermentation supernatants we have demarked four different patterns. **Figure 9** shows the elution pattern of endo-xyl-I reference and the four types of the elution pattern as well. The intensity of different patterns can be used for quantification of xylan degrading enzyme activities. However, we can not draw conclusions about the specific enzyme activities, because the dosages of the enzymes were volumetric in the hydrolysis.

Type 1: Arabinose and xylose are present next to linear xylooligomers and (single, double and multiple) substituted arabinoxylooligomers.

In **Figure 9** line #1 represents the digests type 1. Elution pattern of this cluster is similar to the endo-xyl-I reference elution pattern however, a low amount of arabionse is also present in fermentation liquids. Fermentation liquids consist mostly of endo-xylanases. Most of the fermentation supernatants (42 from 78) belong to this group.

Type 2: Arabinose, xylose, linear xylooligomers and double substituted arabinoxylooligomers are present. Multiple and single substituted arabinoxylooligomers are not detected.

These fermentation supernatants mostly consist of endo-xylanase activity as well as arabinoxylan hydrolase activity (acting only on monosubstitued xyloses, but no action on double subtitued xylopyranosyl units). This kind of performaces was published earlier as well (Pouvreau et al., 2011; Sorensen et al., 2006). Presence of linear xylooligomers means that there is no β-xylosidase in fermentation supernatants. Twenty-two fermentation liquids are included in this cluster.

Type 3: Arabinose, xylose and linear xylooligomers are present. No substituted (single, double and multiple) arabinoxylooligomers are detected.

Fermentation liquids of this group have endo-xylanase activity and also arabinoxylan hydrolases activity on both mono and double substituted xylopyranosyl units. Presence of linear xylooligomers resulted in the absence of β-xylosidase in fermentation supernatants. Only 8 from the 78 digests belong to this group.

Type 4: Arabinose, xylose, double and multiple substituted arabinoxylooligomers are present. Linear xylooligomers and single substituted arabinoxylooligomers are not detected.

Four fermentation liquids concern this cluster. The high amount of xylose in the digests means that they have extremly high β-xylosidase activity. Additionally, arabinoxylan hydrolases activity is present in fermentation liquids as well (only on the single substitued xylooligomers).

Fermentation liquid number B63 after WAX digestion could not be classified in one of the four groups. This fermentation supernatant could release arabinose from double substituted oligomers although not from single substituted arabinoxylooligomers.

Quantitative information about the hydrolysis products of all fermentation liquids can be found in **Appendix Table A1**. The relative content of released materials (w/w%) were calculated from the knowledge of the total content of each monosaccharide components in the initial substrate.

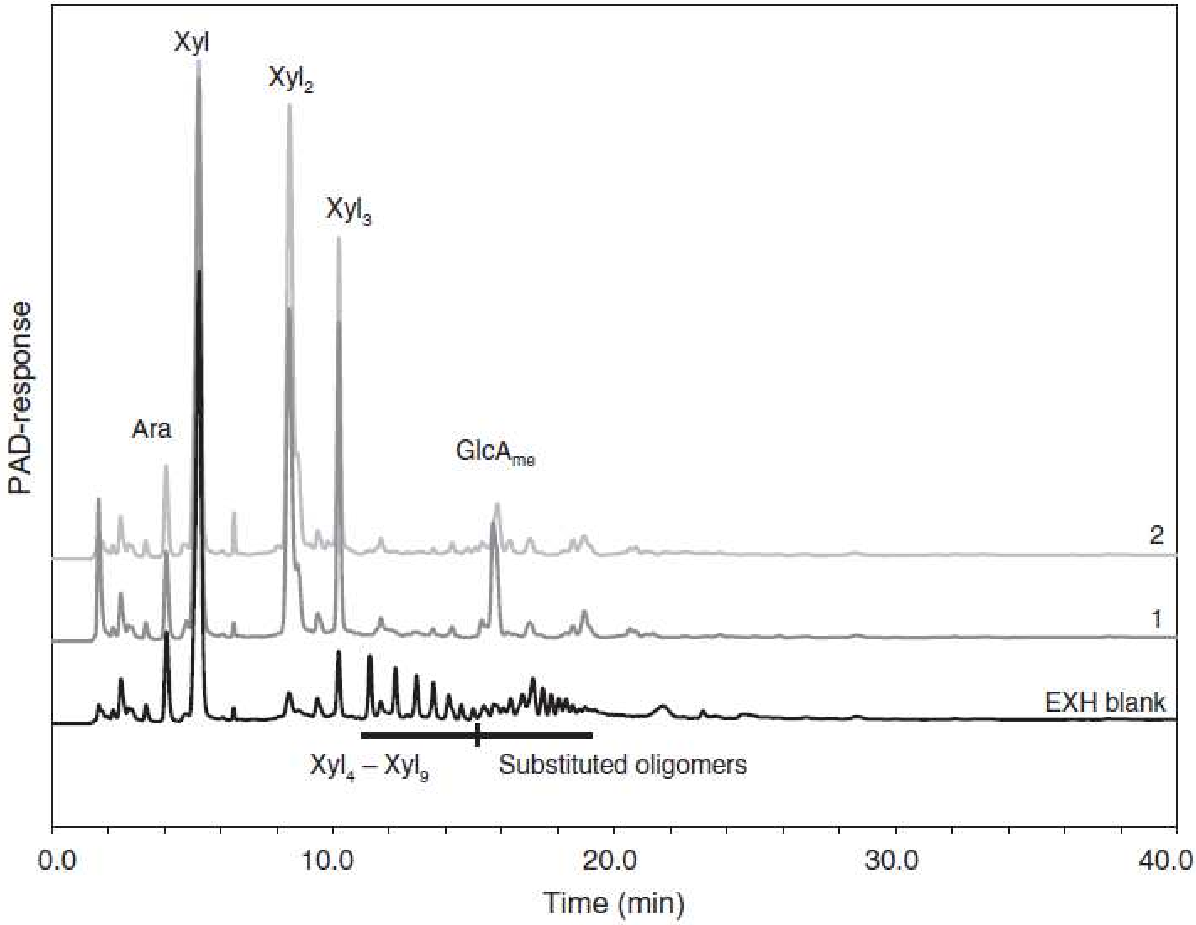
Appendix Table A1 represents that 10 out of 78 fermentation supernatants cloud release more than 50% of the arabinose. Three fermentation liquids have released more than 50% of the total xylose. Seven out of 78 fermentation liquids could hydrolyze more than 50% of the total WAX to monomers or oligomers. Besides, It is shown that only 5 out of 78 fermentation liquids could hydrolyze more than 25% of the total xylose present to monomer (they have β-xylosidase activity).

3.1.3. Enzyme Recognition by EXH Degradation

Eucalyptus xylan hydrolysate (EXH) as another complex soluble substrate was also applied for the hydrolysis as WAX does not consist of all substituents presented in a complex lignocellulose. The building blocks of EXH are xylose, galactose, 4-*O*-methylglucuronic acid and acetic acid (**Table 4**) (Paper #1; Kabel et al., 2007a). The degradation products were identified by HPLC and MALDI-TOF MS after hydrolysis of EXH by fermentation supernatants with the purpose of regognizing recognize acetyl xylan esterase activity and to detect the ability of fermentation supernatants to liberate galactose and 4-O-methylglucuronic acid (Paper #1). The exact position of any substituent (acetyl group, 4-O-methylglucuronic acid, galactose) is only hypothetical, based on the sugar content of EXH and literature (Kabel, 2007a).

Released Oligosaccharides

Figure 10 shows an expamle of the HPAEC pattern of EXH digest to represent the mono- and oligomers after hydrolysis. Line EXH blank maintains the HPAEC pattern of undigested EXH substrate, while Line 1 represents the digested EXH substrate by enzyme mixtures of endo-xyl-I, glucuronidase and acetyl xylan esterase. Line 2 is an example of HPAEC pattern for digested EXH by one of the fermentation liquids.



Fiugre 10. HPAEC patterns of undigested EXH blank, line 1: Example of the digest of EXH of reference enzyme mixture (Endo-xylanase-I, Glucuronidase and acetyl xylan esterase); line 2: Example of the digest of EXH of sample B35

The untreated sample still contains some xylose, monomeric arabinose, linear xylodoligomers and substituted oligomers. In the reference (**Figure 10**, line 1) digest 4-*O*-methylglucuronic acid, xylose, xylobiose and xylotriose are present and a part of substituted oligomers are demolished. Fermentation liquid B35 **(Figure 10**, line 2) could release arabinose, xylobiose, xylotriose and 4-*O*-methylglucuronic acid from EXH substrate. Results of all fermentation supernatnats are represented in **Appendix Table A1**.

Since the HPEAC evaluation method is not competent for analysis of galactosidase activity and acetyl xylan esterase activity, further techniques were used for evaluation. MALDI-TOF MS was used for identification of acetyl xylan esterase activity, glucuronidase activity and galactosidase activity. HPLC was applied for acetic acid content analysis as well.

Figure 11 presents the MALDI-TOF mass spectra of some samples. In each spectrum the main peaks were commented with hypotetical structures of xylooligomers in samples. The exact position of any substituent (acetyl group, 4-*O*-methylglucuronic acid, galactose) is only hypothetical, based on sugar content of EXH and literature (Christov, 2000). Presentation of structures based on the m/z value and the calculated presumptive structures of the main xylooligosaccharides, this gave an insight into the degree of substitution. Besides, the location of substituents is also hypotetical.

The blank EXH sample mainly contained acetylated glucuronoxylooligomers. The saponified sample only contains non-acetylated oligomers. Quite a few fermentation liquids were able to release the acetyl substituents. The example of fermentation liquid B41 shows that it typically releases the acetyl groups and degrades the oligomers to smaller molecules (**Figure 11**). The spectra of the blank sample and of fermentation liquid B41 are significantly different from each other and show that the release of side groups is necessary to allow xylanases to degrade the substrate to smaller molecules. Using HPLC it was shown that although most fermentation liquids had acetyl esterase activity, only 5% of the fermentation liquids could remove more than 75% of the acetyl substituents of the backbone (**Table A1**). Information on the positional specificity of the acetyl xylan esterases could only be obtained when using synthetical Ac-NPh-Xyl (Biely and Puchart, 2006).

Only 8 out of the 78 fermentation liquids have galactosidase activity (**Table A1**). Fermentation liquid B36 is an example of a fermentation liquid that is able to hydrolyze the galactose unit from the 4-*O*-methylglucuronic acid.

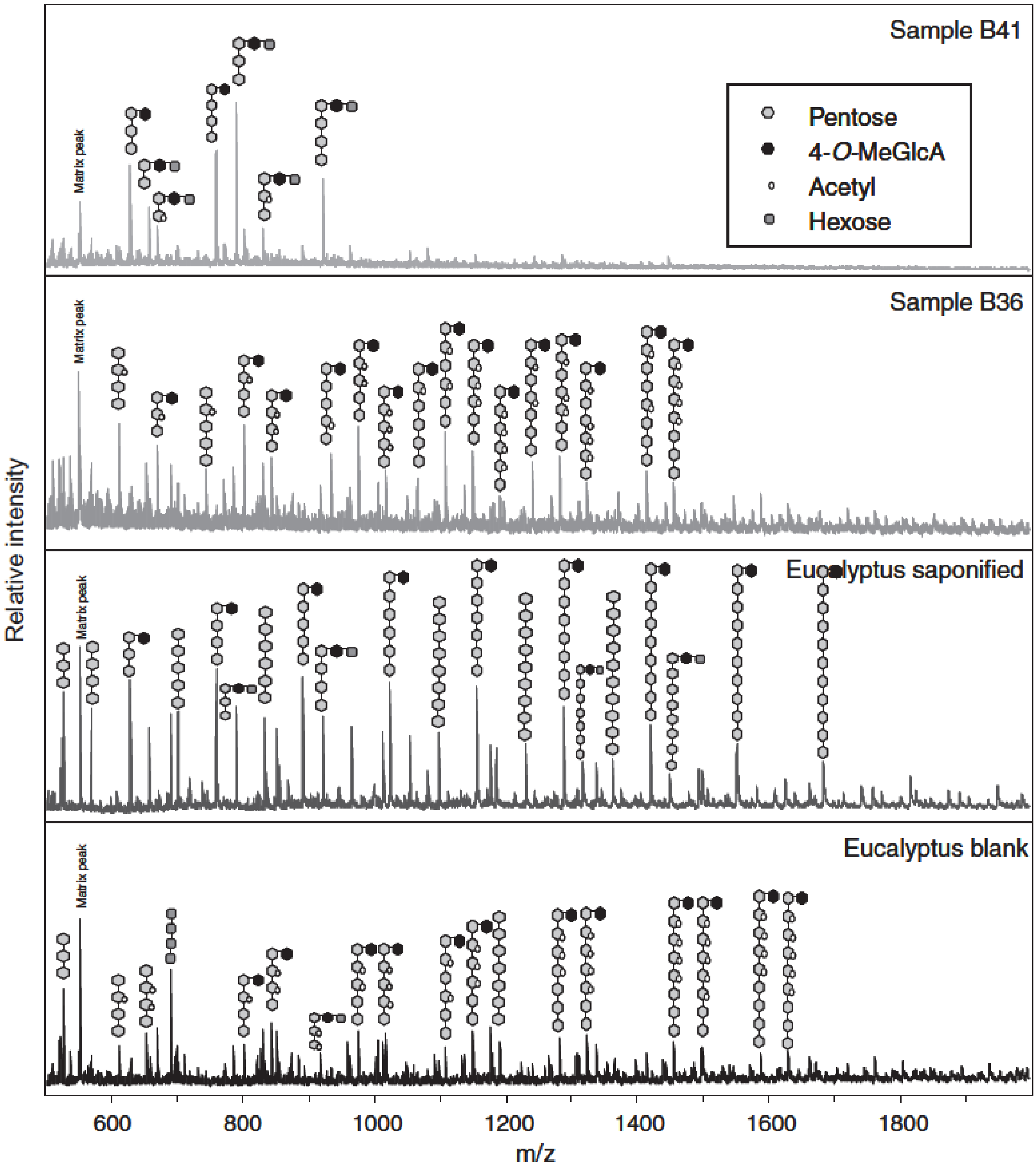


Figure 11. MALDI-TOF mass spectra with hypothetical structures of EXH before and after saponification and two fermentation liquids: example of digest strong in hexose release (B36), example of digest strong in acetyl and xylose release (B41).

All MALDI-TOF mass spectra have revealed typical resistant structures based on the five main peaks in each spectrum. The positions of the substituents are only hypothetical, based on the sugar composition of the EXH (**Table 4**) and literature (Christov et al., 2000; Garrote et al., 1999; Kabel et al., 2002). Eighty percent of the fermentation liquids degraded higher oligomers and accumulated $X^{2a}XU^{M2,4m2}$ (code according to (Fauré et al., 2009)) representing 2-*O*-Ac-β-D-(Xylp)2-α-D-Galp-(1→2)-4-*O*-Me-α-D-GlcpA-(1→2). This component was present as one of the five main peaks in 80% of the spectra. Next to this component, in 87% of the fermentation liquids the accumulation of $X^{2a}XU^{4m2}$ was seen, representing 2-*O*-Ac-β-D-(Xylp)2-4-*O*-Me-α-D-GlcpA-(1→2).

For each of the hydrolysis products formed, a top five was selected based on the amount of products present after digestion (**Table A1**). For 4-*O*-methylglucuronic acid release the top five ranged from 46–66% of the total amount of 4-*O*-methylglucuronic acid present in the initial substrate. Acetic acid release the top five ranged from 75–84% of all acetic acid present. Xylose and xylooligomers (DP:2–4) were released in much lower amounts, 1–13% and 0–11%, respectively. Furthermore, the qualitative results on galactosidase activity are given, based on the five main structures present in the MALDI-TOF mass spectra lacking any galactose moiety. The selection in **Table A1** represents that 17 fermentation liquids were active towards EXH. However, none of the fermentation liquids was able to hydrolyze more than 17% of the total substrate to monomers and xylooligomers (Sample B12, **Table A1**).

Table 6 presents the three best enzyme producing soil fungi per activity and/or method. Surprisingly, only a few fungi were selected as best producers for more than one enzyme activity. As example samples B12 and B14 are indicated as best endo-xylanase producers. Nevertheless, neither of the samples were able to degrade WAX substrate, indicating that the endo-xylanases are hindered by substitution of the xylan backbone. In contrast to sample B30, B41 and B74 are able to produce some quite different accessory enzymes but are not selected for their endo-xylanase expression.

Table 6. Top three fermentation supernatants per enzyme activity, based on traditional and novel methods

Sample no	**Top 3 per activity determined by classical colorimetric methods**			**Top 3 per activity determined by current screening method**					
	Endo-xylanase[a]	**β-Xylosidase**[b]	**α-Arabino furanosidase**[b]	**Endo-xylanase**[c]	**β-Xylosidase**[d]	**α-Arabino furanosidase**[e]	**Glucuronidase**[f]	**Acetyl xylan esterase**[g]
B1		X	X					
B7						X		
B8			X			X		
B12	X						X	
B14	X							
B27								X
B30		X	X		X			
B34							X	
B38						X		
B41		X			X			X
B42	X				X			
B45				X				
B52				X				
B62							X	
B72								X
B74			X	X		X		

a-Based on reducing sugar release from birch glucuronoxylan.
b-Based on liberated 4-nitrophenol from the respective PnP substrates.
c-Based on the release of xylooligomers (DP2-4) from WAX.
d-Based on the release of xylose from WAX/EXH.
e-Based on the release of arabinose from WAX (either at *O*-2 or *O*-3 position).
f-Based on the release of 4-*O*-methylglucuronic acid from EXH.
g-Based on the acetic acid release from EXH.

3.1.4. Conclusions

The new screening method yields valuable information concerning the enzyme activities present in 78 fungal fermentation liquids. In most cases, enzyme activity of fermentation liquids by an instrumental method and the result of a classical screening method differed. Our method combines various analytical tools being able to distinguish various specific enzyme activities in crude fermentation liquids using only soluble and insoluble model substrates. It is a precise method for a detailed screening of complex enzyme mixtures.

3.2. Performance of Hemicellulolytic Enzymes in Culture Supernatants from a Wide Range of Fungi on Insoluble Wheat Straw and Corn Fiber Fractions (Paper #2)

3.2.1. Substrate Composition

Table 4 displays that the WS WUS mainly contained xylose and glucose. The relatively simple structure of WS WUS hemicellulose can be displayed by the degree of substitution using the molar ratio of arabinose: xylose and uronic acid: xylose, which are only 1:8 and 1:16, respectively. Besides, the low abundance of other monomers in the substrate suggests that the hemicellulose is composed of a linear xylan backbone with minor amounts of substituents. This illustrates the presence of a predominantly 'linear' hemicellulose and a significant amount of cellulose. The level of acetylation of WS WUS was 1.8% (w/w), indicating a molar ratio of acetic acid: xylose of 1:4. The composition of this substrate is in agreement with that provided in the literature (Kabel et al., 2007b; Miron and Ben-Ghedalia, 1982). The CF AIS was more complex as can be concluded from the various monosaccharides present. Xylose, arabinose and glucose were the main sugars and the molar ratios of arabinose: xylose and uronic acid: xylose were 1:2 and 1:9, respectively. Moreover, the substrate was highly acetylated, 1:2 for acetic acid: xylose (mol based). The ratios of substitution illustrate a highly decorated xylan backbone. These findings are in line with the complexity of corn fiber xylan as described by others (Appeldoorn et al., 2010; Van Eylen et al., 2011). Compared to WS WUS, a lower amount of cellulose was present in CF AIS, indicated by the lower amount of glucose. Together with the complex xylan structure, this lower cellulose content contributes to a different architecture of insoluble CF AIS compared to WS WUS. Both substrates had a total sugar content of 70% (w/w), which could be explained by the presence of other components like lignin, proteins and/or salts.

3.2.2. Enzyme Screening on Insoluble WS WUS

Figure 12 shows examples of HPAEC elution patterns of untreated WS WUS (blank) and WS WUS after incubation with three culture supernatants (#13, 36, 57). HPSEC results showed that no polymeric soluble material was present in any of the samples (data not shown). The blank sample confirms that the substrate only contains insoluble material. Culture supernatant #36, together with 21 other culture supernatants, hardly released any monomers or oligomers, indicating that those culture supernatants could not hydrolyze the insoluble substrate. Fifty-six out of 78 hydrolyzed >5% of the substrate to monomers or oligomers. The wheat straw WUS digest of culture supernatant #13 showed the release of glucuronic acid, a high release of arabinose and other monomers. Culture supernatant #57 also released relative large amounts of xylobiose and xylotriose~~,~~ but was lacking a glucuronic acid releasing enzyme. To define the peak of co-eluted xylose, glucose and/or galactose, a more specific gradient was applied to HPAEC. Using this gradient, the different monomers could be separated and quantified as displayed in **Table A2** (Appendix) for selected culture supernatants. A complete overview of the quantitative data of all culture supernatants can be found in the **Appendix Table A2**. **Table A2** in Appendix shows that 13 out of 78 culture supernatants could solubilize more than 10% of the total insoluble substrate WS WUS. No more than 3 samples were able to solubilize 14% of the WS WUS. Typical values for total cellulose hydrolysis in WS WUS by various cellulase preparations are around 10– 20% (Koullas et al., 1992; Lamsal et al., 2011). Pretreatment enhanced total hydrolysis by opening up the cell wall structure (Pedersen and Meyer, 2009) and values up to 63% solubilization after pretreatment of wheat straw have been reported (Lamsal et al., 2011).

The enzymes in the culture supernatants releasing high amounts of monosaccharides are able to hydrolyze part of the complex hemicellulose and cellulose network. The maximum amount of glucose and xylose released from the WS WUS was reached by sample #38 (21% and 22% respectively). In general, both cellulases and xylanases are necessary for cell wall deconstruction (Wang, 2010) and these enzymes enhance the release of monosaccharides synergistically (Ghose and Bisaria, 1979). The release of other substituents e.g. arabinose, or galactose by accessory enzymes is crucial for the complete hydrolysis of the xylan backbone. Nevertheless, these substituents are only present in small quantities and thus, do not play a pivotal role within the degradation of WS WUS.

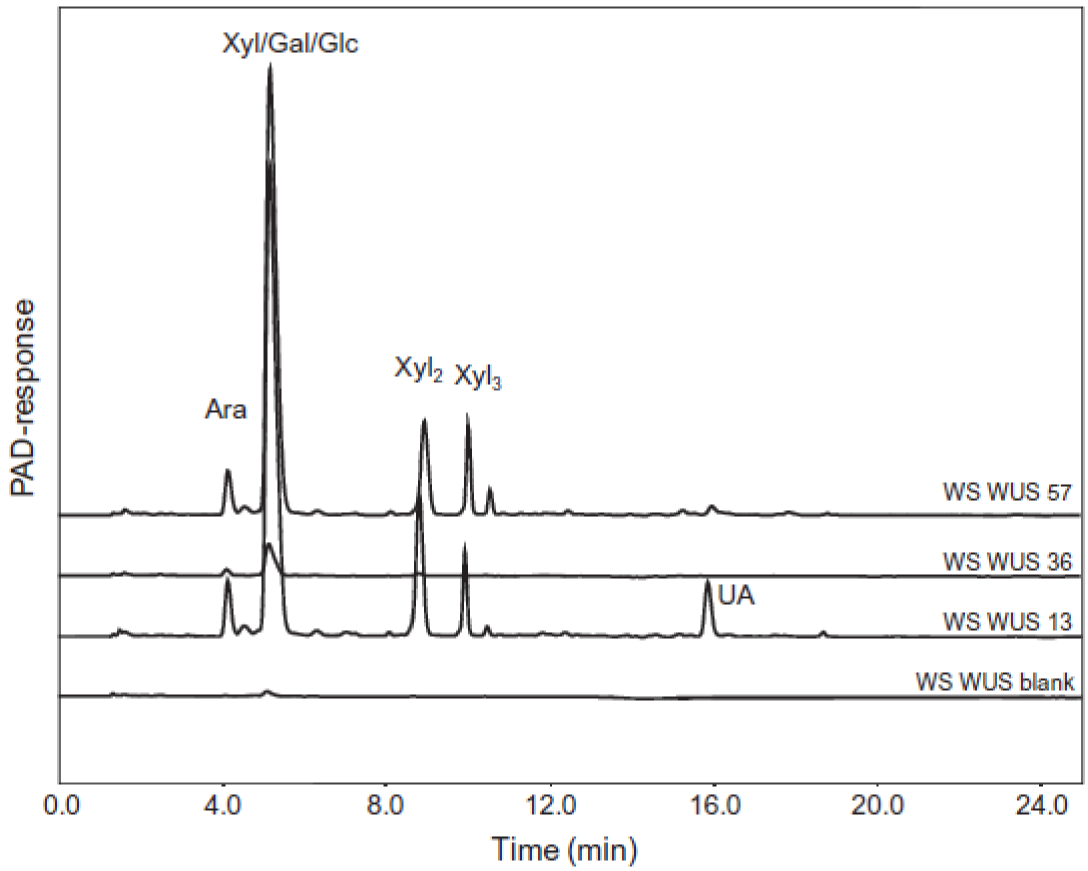


Figure 12. HPAEC patterns of digested WS WUS by fermentation liquids and the undigested WS WUS blank (Ara: arabinose; Xyl/Gal/Glc: xylose, galactose, glucose; Xyl2,3: xylobiose, xylotriose; UA: glucuronic acid.

It is remarkable that only two culture supernatants (samples #7 and 38) of the top five best-performing strains in hydrolysis of WAX (Paper #1) and WS WUS are the same. As expected from the sugar composition, xylanases and cellulases are necessary for the main degradation of WS WUS. WAX digestion has already indicated that active xylanases were present in many of the culture supernatants (Paper #1). However these were not effective in the degradation of WS WUS. This is visualized by culture supernatants #36 and #13. Culture supernatant #36 was selected previously for enzyme activities which were not tested by using WS WUS. Culture supernatant #13 was already active towards WAX but not selected as a top five best-performing sample (Paper #1). However, this culture supernatant seems to have better performance in WS WUS degradation which indicates that the substrate used in a screening procedure is significant to predict the efficiency of enzymes towards that particular substrate.

The differences between the culture supernatants hydrolyzing the WS WUS could be caused by both substrate and enzyme characteristics. One of the reasons could be enzyme adsorption onto lignin and/or cellulose, causing the hydrolysis rate towards xylan to decrease (Lee et al., 1995; Yang and Wyman, 2006). Other reasons could be product inhibition, substrate crystallinity or accessibility (Bansal et al., 2009; Van Eylen et al., 2011). Moreover, it has been mentioned that xylanase activity may depend on the presence and type of a substrate binding domain attached to the xylanase (Cuyvers et al., 2011; Sun et al., 1998; Ustinov et al., 2008). One should also note that even though the same conditions were applied (pH 5.0, 35 °C) to screen for efficient degradation of soluble and insoluble substrates, these conditions could be optimized for each individual enzyme preparation to obtain an even more enhanced degradation (Polizeli et al., 2005).

3.2.3. Enzyme Screening on Insoluble CF AIS

The results obtained using WS WUS gave an insight into the crude culture supernatants that being able to degrade a relatively simple xylan rich substrate. The hydrolysis of CF AIS provided an insight into the ability of the enzymes in the culture supernatants to degrade a complex insoluble substrate. HPSEC analysis of the CF AIS samples did not display any high molecular weight material being solubilized by the enzymes present in any of the culture supernatants (data not shown). The relative amount of each constituent monosaccharide in the substrate was calculated and is displayed in the **Appendix Table A2**. **Table 7** displays the top enzyme producers hydrolyzing CF AIS as expressed per component released. The blank sample contained a small amount of glucose (**Appendix, Table A2**), which originated from starch removal (Van Eylen et al., 2011). Fifteen culture supernatants were selected as containing the best enzymes for hydrolyzing CF AIS. Seven of these 15 supernatants were not selected for their performance on WS WUS and thus seem to be efficient specifically for CF AIS hydrolysis.

Table A2 also shows that three out of 78 culture supernatants were able to solubilize 30–34% of the total CF AIS. Compared to WS WUS, a significantly larger part of the cell wall polysaccharides in CF AIS was solubilized. Since CF AIS xylan is reported to be extremely complex (Appeldoorn et al., 2010; Van Eylen et al., 2011), which is likely to be caused by the architecture of the substrate. It has been reported that more substituted xylans, like corn fiber xylan, absorb much less cellulose than a more linear xylan, like wheat straw xylan. This may lead to a tighter and denser structure of WS WUS, thereby making it less accessible for enzymes (Kabel et al., 2007b).

The main building blocks of CF AIS are xylose and glucose substituted with arabinose. It can be seen that the β-1→4 linked xylan backbone of this substrate must have been less available for the enzymes since only up to 10% of the xylose available in the initial substrate was hydrolyzed to monomer, compared to up to 20% for WS WUS. Sample #7 did not release much xylose from CF AIS, while it demonstrated to contain endoxylanases and β-xylosidases active on insoluble WS WUS by the release of xylose. This confirms that the β-1→4 linked xylan backbone in the substrate was less available for the enzymes present in this culture supernatant.

Table A2 also shows that six out of 78 culture supernatants were able to release 30% of arabinose from the insoluble CF AIS. The release of arabinose substituents is necessary to allow endoxylanases to hydrolyze the backbone (Kormelink et al., 1991). However, in all cases no synergy between arabinofuranosidases and xylanases was observed, since an increased level of released arabinose was not always correlated to an increased level of xylose (oligomers) released, e.g. samples #14, 73 and 74.

Since the digests had a high content of ‘other oligomers’ indicating the presence of an endoxylanase, they may have lacked an efficient b-xylosidase hydrolyzing the oligomers to monomers, or the b-xylosidase was hindered by substitution of the oligomers (Kormelink et al., 1993c). The hydrolysis of WS WUS by these culture supernatants did also result in higher amounts of released xylooligomers but not in high xylose release which makes the assumption of a lack in efficient b-xylosidase activity possible.

The glucuronic acid seems to have been difficult to release. The maximum amount of glucuronic acid released was 9%. The lack of enzymes being able to release this glucuronic acid group from the backbone of xylooligomers in corn fiber was described by others as well (Appeldoorn et al., 2010). The best-performing culture supernatants on CF AIS were #14, 73 and 74, which were not selected for their performance on WS WUS. Again this confirms the importance of the selection of the substrate used in a screening procedure.

3.2.4. Importance of the Choice of Substrate in Screening for Enzyme Activity

In total 21 samples were selected as top hydrolyzers of either WS WUS or CF AIS (**Table A2**). Out of these 21 samples, fourteen were selected for their performance on WS WUS, as well as 15 for their performance on CF AIS. Eight of the samples were selected for efficient solubilization of both substrates. Five samples originated from the *Trichoderma* genus, of which especially the *Trichoderma reesei* is known for efficient production of cellulases and hemicellulases (Banerjee et al., 2010; Gusakov, 2011). Furthermore, four *Penicillium* strains were selected as good producers of enzymes degrading insoluble xylan-rich material. The efficient production of cellulases and hemicellulases by various *Penicillium* species was reported by others (Gusakov, 2011; Jorgensen et al., 2005). Sample #70 originated from *Phanerochaete chrysosporium*. This fungus is one of the best-studied fungi for bioconversion of plant biomass and is known to produce hemicellulolytic enzymes (Hori et al., 2011). Eleven selected strains remained unidentified.

Based on the results for soluble substrates (Paper #1) and insoluble substrates in this study, the five best fungi can be selected for each substrate. The selection was based on the total amount of main building blocks released. **Table 7** displays that for each substrate, different culture supernatants were selected as possessing the top five enzyme contents. Only supernatant #7 was selected for three out of four substrates, indicating the presence of highly active and/or efficient enzymes in this sample. The supernatant was derived from a *Penicillium* species previously described as a effective producer of cellulases and hemicellulases (Jorgensen et al., 2006). In addition, *Trichoderma* and *Aspergillus* species were selected. Sample #74 and #13 were selected twice for different substrates. Sample #74 was derived from a *Trichoderma* species; the fungus producing sample #13 remains unidentified. The selection of different culture supernatants for each substrate proves the need of using different substrates for identification of efficient enzymes within crude culture supernatants. This study demonstrates even more efficiently that the choice of a substrate to be degraded is of importance to screen for specific enzyme activity.

The novelty of the enzymes present in the culture supernatants will be established by identification of the strains, purification and characterization of the enzymes as well as using optimized conditions for each enzyme.

Table 7. Top enzyme producers for the different substrates, based on the release of the main building blocks in the substrates: wheat arabinoxylan (WAX); eucalyptus xylan hydrolysate (EXH); wheat straw water unextractable solids (WS WUS); corn fiber alcohol insoluble solids (CF AIS).

Ranking	**Top five producers on different substrates**			
	WAX[a]	**EXH[b]**	**WS WUS[c]**	**CF AIS[d]**
1	38 (Penicillium sp.)	41 (Aspergillus sp.)	13 (non-identified)	73 (non-identified)
2	74 (Trichoderma sp.)	40 (Aspergillus sp.)	7 (Penicillium sp.)	74 (Trichoderma sp.)
3	49 (Penicillium sp.)	42 (Aspergillus sp.)	57 (non-identified)	14 (non-identified)
4	45 (Trichoderma sp.)	27 (Bionectria sp.)	38 (Penicillium sp.)	13 (non-identified)
5	7 (Penicillium sp.)	7 (Penicillium sp.)	12 (non-identified)	44 (non-identified)

a-Main building blocks: arabinose, xylose, xylo-oligomers DP2-4.
b-Main building blocks: xylose, xylo-oligomers DP2-4, acetyl.
c-Main building blocks: glucose, xylose, xylo-oligomers DP2-4.
d-Main building blocks: arabinose, xylose/glucose, oligomers.

3.2.5. Conclusions

The screening for specific enzyme activity within crude lignocellulolytic enzyme mixtures is only effective when using the correct substrate. Solubility, polymer chemical fine structure and the cell wall architecture of the substrates used in screenings will influence enzyme performance and thus the outcome of the screening. Especially *Penicillium, Aspergillus* and *Trichoderma* species produced high hemicellulolytic activity. The use of targeted substrates allows the recognition of several efficient enzymes within crude culture supernatants.

3.3. Diversity in Production of Xylan Degrading Enzymes Among Species Belonging to the *Trichoderma* Section *Longibrachiatum* (Paper #3)

3.3.1. Enzyme Production in Shake Flask Fermentation

Wheat straw, corn fiber and eucalyptus wood as xylan rich fermentation carbon sources were applied to 15 selected fungal isolates belonging to *Trichoderma* section *Longibrachiatum* in shake flask experiments. After 5 days of fermentation enzyme activities were assayed from the supernatants by traditional colorimetric methods to have an initial indication if enzyme production was succesful. Volumetric enzyme activities are summarized in **Appendix Table A3** (top 3 samples are bolded).

T. reesei strains (e.g., wild-type QM 6a, mutant RUT C30) are good producers of overall cellulase activity as having been found earlier by many authors (Peterson, 2012; Mandels, 1969; Montenecourt, 1979). In this study *T. reesei* CPK 155 exhibited similar filter paper activity with reference QM 6a on wheat straw fermentation medium. However**,** the endo-xylanase activity of CPK 155 was higher than QM 6a fermented on all three carbon sources~~,~~ and even better than mutant RUT C30 grown on wheat straw and corn fiber. Strain *T. reesei* CPK 155 (=GJS 86-404) have been found earlier as a outstanding cellulase producer (Kubicek, 1996). *T. parareesei* F-2535, and *Trichoderma gracile* (F-2543) are also efficient in endo-xylanase production. On the contrary**,** *Trichoderma reesei* strains (QM 6a and RUT C30) have been found to be the best among tested fungi in volumetric β-xylosidase and α-L-arabinofuranosidase secretion. Soluble protein secretion was the highest in case of mutant RUT C30 strain fermented on all three carbon sources (**Table A3**).

Apart from the traditional colorimetric enzyme assays, the samples were subjected to an in-depth analysis of hemicellulose degrading activities by HPLC and mass spectrometry.

3.3.2. Evaluation of WAX Degradation Products

WAX is mostly substituted with arabinose (Kormelink, 1993a). The hydrolysis products of WAX released by fermentation supernatants were analysed by HPAEC and HPSEC. The HPAEC elution patterns gave an insight to the mono- and xylooligomers released, while the remaining polymers were seen by HPSEC. The results of HPAEC and HPSEC were compared and a remarkable correlation was found (data not shown).

The enzyme content of the fermentation supernatants strongly depends on both the microorganism as well as on the carbon source applied in the shake flask fermentation. Detailed results are summarized in **Appendix, Table A4**. Due to large number of fermentation samples (45 in total) however, only the most interesting digests were selected for a more detailed evaluation. Three groups of samples (fermented on three different carbon sources) were compared to each other within one group. The top 3 samples are bolded from each group in **Table A4**.

WAX digest hydrolyzed by supernatants fermented on wheat straw contained much arabinose next to the double substituted arabinoxylooligomers. The most effective enzyme producers were *T. reesei* RUT C30 and QM 6a, *T. parareesei* F-2535 and also *T. gracile* F-2543, as they have excreted an efficient α-L-arabinofuranosidase and endo-xylanase, displayed by a high arabinose and xylooligosaccharide content.

Supernatants from shake flask fermentations produced on eucalyptus wood as carbon source could release smaller linear and single substituted oligomers from WAX, which means that these supernatants did not contain a sufficient quantity of β-xylosidase and α-L-arabinofuranosidase enzymes that would be capable to release single substituated xylooligomers. These include *T. reesei* QM 6a, *T. parareesei* F-2535, *T. gracile* F-2543, *T. citrinoviride* F-1299 and *T. konilangbra* CPK 132.

Supernatants belonging to the third group (fermented on corn fiber as carbon source) were able to degrade the WAX substrate into monomers especially xylose and linear, single and double substituted xylooligomers. They include *T. saturnisporum*, both *T. sinense* isolates, *T. konilangbra* and *T. i* strains, they have produced endo-xylanase and a lower amount of α-L-arabinofuranosidase and β-xylosidase in contrast to the eucalyptus wood group.

The amount of total released sugars from WAX was 36-49% in case of supernatants fermented on wheat straw, 18-27% if supernatant was produced on eucalyptus wood and 39-47% when the carbon source of fermentation was corn fiber. The results indicate that fermentation supernatants produced on wheat straw and corn fiber were the most effective in the degradation of wheat arabinoxylan. Both wheat straw and corn fiber contain a high amount of xylose, glucose and arabinose, the stucture of these carbon sources might affect the production of endo-xylanase, β-xylosidase, β-glucosidase, α-L-arabinofuranosidase enzymes.

In **Figure 13** HPAEC patterns of four strains are compared regarding their action on WAX substrate. The intensity is correlated to the amount of the detected sugars. ATCC 18903 (**Figure 13**. 1d) has high intensity meaning that it produced a high level of xylosaccharides in contrast to the TUB F-2535 strain (**Figure 13**. 1b). F-2535 could not release single substituted oligomers fermented on wheat straw and corn fiber. The supernatant sample of *T. gracile* F-2543 (**Figure 13.** 1c) has produced a similar amount of mono- and oligosaccharides as *T. reesei* RUT C30 (**Figure 13.** 1a) however, corn fiber fermentation supernatant could not release multiple substitutions. Also it can be seen that supernatant of *T. saturnisporum* fermented on eucalyptus wood could not degrade the WAX substrate, while corn fiber supernatant have not release single substituted xylooligomers and multiple substitutions (**Figure 13**. 1d).

To compare this evaluation method with traditional colorimetric assays both similarities and differencies could be observed. For example RUT C30 resulted in the best values with both analytical tools. However, RUT C30 is a mutant with enhanced enzyme secretion while all other isolates (including reference culture *T.i* QM 6a) are wild-type strains selected directly from nature.

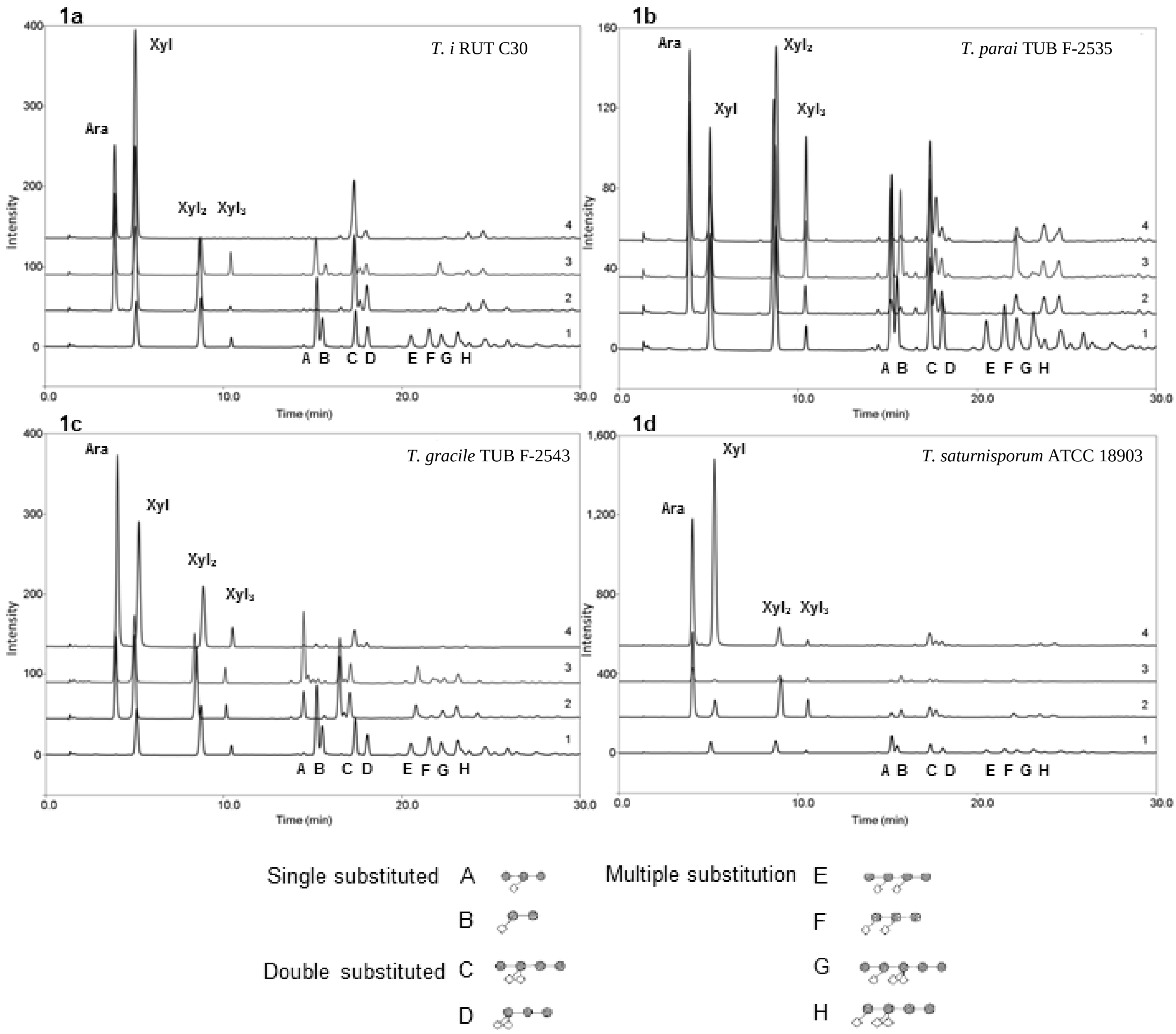


Figure 13. HPAEC profiles of hydrolysis product from digested wheat arabinoxylan (24h, 37°C, 500 rpm, pH:5.0) by four Trichoderma isolates fermented on three different fermentation carbon sources (wheat straw, eucalyptus wood, corn fiber). Line 1: endo-xylanase-I (reference); line 2: wheat straw fermentation supernatants; line 3: eucalyptus wood fermentation supernatants; line 4: corn fiber fermentation supernatants. Ara: arabinose; Xyl: xylose; Xyl_2: xylobiose; Xyl_3: xylotriose. 1a: T. reesei RUT C30; 1b: T. parareesei TUB F-2535; 1c: T. gracile TUB F-2543; 1d: T. saturnisporum ATCC 18903. Internal control (background, residual reducing sugars in fermentation supernatants) were considered.

3.3.3. Evaluation of EXH Hydrolysis

Eucalyptus xylan hydrolysate (EXH) as a complex soluble substrate was also applied for the hydrolysis. The building blocks are xylose, galactose, 4-*O*-methylglucuronic acid and acetic acid (**Table 4**) (Christov et al., 2000). Shake flask fermentation supernatants prepared on three carbon sources (wheat straw, eucalyptus wood, corn fiber) were used for the hydrolysis. The degradation products were identified by HPLC and MALDI-TOF MS after the hydrolysis of EXH by fermentation supernatants in order to recognize acetyl xylan esterase activity and to detect the ability of fermentation supernatants to liberate galactose and 4-*O*-methylglucuronic acid.

The exact position of any substituent (acetyl group, 4-*O*-methylglucuronic acid, galactose) is only hypothetical, based on the sugar content of EXH and literature (Christov 2000). Evaluation of mass spectra of samples was based on the 5 main peaks in each spectrum (Paper #1).

Table A5 (Appendix) gives a qualitative appraisal on different EXH digests. Only the best activities are emphasized as positive cases in **Table A5**. A few isolates have been identified as good enzyme producers for more than one activity. In the ˝wheat straw group˝ *T. parareesei* F-2535 was highly efficient when it comes to endo-xylanase, galactosidase and glucuronidase activity. Fermentation liquids of all three *T. reesei* strains have endo-xylanase and acetyl-xylan-esterase activity, while *T. citrinoviride* F-1299 supernatant was remarkably effective in galactosidase and glucuronidase activity.

From the ˝eucalyptus wood group˝ *T. saturnisporum* ATCC 18903 proved to be effective in endo-xylanase, galactosidase and glucuronidase activity. Moreover, *T. reesei* RUT C30 fermentation liquid had endo-xylanase or acetyl-xylan-esterase activity and *T. effusum* showed galactosidase and glucuronidase activity.

˝Corn fiber group˝ supernatants of all *T. i* samples proved to be outstanding when releasing the acetyl group. RUT C30 and CPK 155 were highly efficient in endo-xylanase activity while QM 6a had glucuronidase activity.

T. reesei isolates, *T. parareesei* and *T. gracile* were efficient in the total enzyme production. These three species are genetically very close to each other ("sister-species") as it turns out from recent findings (Samuels et al., 2012). Fermentation on wheat straw and corn fiber as carbon sources produced higher endo-xylanase and glucuronidase enzyme activities. High acetyl xylan esterase activities were measured from samples digested by supernatants from fermentation on eucalyptus wood and corn fiber. Eucalyptus wood consists of acetylated xylooligomers that may effect the production of acetyl xylan esterase (Christov, 2000).

Comparison of EXH results to WAX results it can be concluded that degradation depends on the structure of the substrate and the substituents could inhibit endo-xylanase in degradation of the xylan backbone (Biely, 2003).

3.3.4. Evaluation of Hydrolysis of WS WUS Substrate

WS WUS was selected as a relatively simple, xylan-containing insoluble hydrolysis substrate. The main components are linear xylan and a significant amount of cellulose (Gruppen et al., 1990). After hydrolysis the released mono- and oligosaccharides were analysed by HPEAC.

Table A6 (Appendix) summarizes the released substituents and other sugars of WS WUS by three different fermentation supernatants of each strain. Five out of 15 wheat straw fermentation liquids could solubilize more than 5 % (w/w) of the hydrolysis substrate, while the level is only 3 % if the strain was fermented on corn fiber and only 1 % if the carbon source in the fermentation was eucalyptus wood. Strain RUT C30 fermented on wheat straw has liberated 12 (w/w)% of total sugar content. Surprisingly, none of any other supernatant could reach this level. However, RUT C30 is a mutant and all other isolates are wild-type strains isolated directly from nature. Additional promising wild-type strains (*Trichoderma* and non-identified) were published earlier (Paper #2).

In order to release xylose and glucose the substrate needs to be degraded into smaller oligomers. The amount of xylooligosaccharides (DP2-4) were not significant in any sample however**,** low levels of arabinose, glucose and xylose were measured in some digests. By wheat straw fermentation supernatants of *T. reesei* RUT C30 and *T. parareesei* F-2535 there was a 13-17 w/w% arabinose and 9-13 % (w/w) glucose release from WS WUS. In contrast the liquids fermented on eucalyptus wood were not competitive enough to break down the structure of the same substrate. Shake flask supernatant samples of *T. reesei* QM 6a, *T. parareesei* F-2535 and *T. gracile* F-2543 fermented on corn fiber were relatively efficient in hydrolysis of wheat straw WUS as they could liberate 5-7 w/w% glucose. These strains were also outstanding in

degradation of wheat arabinoxylan. This correlation could be explained with the fact that these three species are genetically very close to each other (Nakari-Setala et al. , 2009).
From the results above it is evident that WS WUS is rather resistant against these enzymes as observed earlier by other authors testing various fungal enzymes (Kabel et al., 2007a).

3.3.5. Evaluation of Hydrolysis of CF AIS Substrate

Corn fiber AIS (another insoluble xylan-rich substrate) has a complex structure and is mostly composed of arabinose, xylose, glucose and a low amount of glucuronic acid (Appeldorn et al., 2010). CF AIS was also digested by the same 45 shake flask supernatants that were used formerly to hydrolyze WAX, EXH and WS WUS. Similar methods were applied to analyse the digests as used for the analysis of WS WUS hydrolyzates.
Table A6 (Appendix) represents the liberated mono- and oligosaccharides from CF AIS by different fermentation liquids. Eight out of the 15 fermentation supernatants propagated in shake flask on wheat straw as carbon source could solubilize 7-9 (w/w) % of the CF AIS hydrolysis substrate, while this number was 7 % if the fermentation carbon source was corn fiber and only 1 % when fermentation was performed on eucalyptus wood. The RUT C30 and F-2543 corn fiber liquids have liberated more than 10 % of total sugar content from CF AIS.
6-9 % (w/w) of total arabinose and 12-16 % of total glucose were released by *T. i* RUT C30, QM 6a, CPK 155 and *T. parareesei* F-2535 supernatants fermented on wheat straw from the CF AIS samples. The three *T. reesei* samples produced on corn fiber as carbon source could liberate 8-13 % arabinose and 13-17 % glucose. Furthermore, *T. gracile* F-2543 and *T. saturnisporum* ATCC 18903 corn fiber samples have released more than 13 w/w% arabinose and 7-8 % xylose and 4-*O*-methylglucuronic acid from the CF AIS substrate.
T. reesei isolates, *T. parareesei*, *T. gracile* and *T. saturnisporum* were effective in degradation of corn fiber AIS. These data correlate with the WAX results however, degradation of WAX resulted in higher level of products. The differences in structures of two substrates may serve as an explanation to these results. To compare the two insoluble substrates (WS WUS and CF AIS) were both hardly digestable for the supernatants~~,~~ however, higher amounts of total sugar were released from corn fiber AIS. Enzyme mixtures fermented on eucalyptus wood were less effective in hydrolysis experiments. The reason might have been that this fermentation substrate

have not induced the enzyme production properly or due to a higher lignin content of eucalyptus (Christov et al., 2000) the fungi were more inhibited in growth during the shake flask fermentation.

3.3.6. Conclusions

T. reesei CPK 155, *T. parareesei* TUB F-2535, *T. gracile* TUB F-2543 and *T. saturnisporum* ATCC 18903 isolates were equally valuable or better in hydrolysis of the xylan rich soluble and insoluble substrates than the well-known *T. reesei* QM 6a and RUT C30 strains. These new putative xylan-degrading enzymes may represent a potential for industrial applications. Enzyme production on wheat straw and corn fiber carbon sources was more effective than on eucalyptus wood. Interestingly, *T. longibrachiatum* and *T. citrinoviride* the two most common species in the *Longibrachiatum* section were not among the best xylanase producers.

3.4. Novel *Penicillium* Cellulases for Total Hydrolysis of Lignocellulosics (Paper #4)

3.4.1. Comparison of Hemicellulase Activities in Culture Supernatants of the Penicillium Species and Trichoderma i

Total cellulase and xylanase activities in the culture supernatants of the two *Penicillium* strains TUB F-2220 (*P.pulvillorum*) and TUB F-2378 (*P*. cf *simplicissimum*) and *Trichoderma reesei* RUT C30 during cultivation on cellulose (Avicel) are demonstrated in **Figure 14.** Maximal volumetric cellulase and xylanase activities in the cultures were obtained within 4-6 days of cultivation. The total volumetric cellulase activities (FPU) in the cultures of the two novel *Penicillium strains* were at a similar level as *T.i* RUT C30 (a mutant strain with a high level of protein secretion). On the contrary, volumetric xylanase activities in the two P. cultures were at a lower level than in the *Trichoderma* culture. Comparing the two *Penicillium* strains, strain TUB F-2378 was better xylanase producer than TUB F-2220.

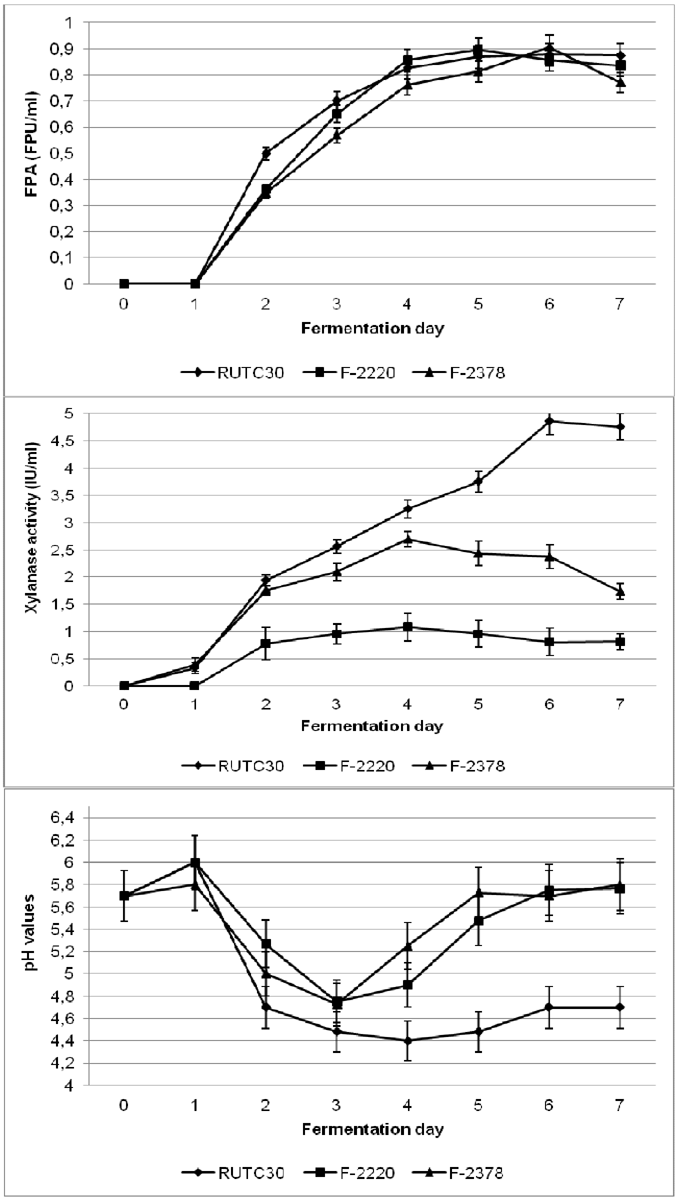


Figure 14. Cellulase (FPA) (a), xylanase (b) activities and pH values (c) of Penicillium pulvillorum TUB F-2220, Penicillium cf. simplicissimum TUB F-2378 and Trichoderma reesei RUT C30 in shake flask fermentation supernatants. Avicel PH-101 was used as a carbon source (medium LC-8). The presented values are mean of three replicates.

The temperature and pH optima of the cellulase activities in the cultures (grown on Avicel) of *Penicillium* strains and *T. reesei* RUT C30 strain are shown in **Figure 15**. The highest relative activities for all the three strains were obtained at 45°C and in pH 5.

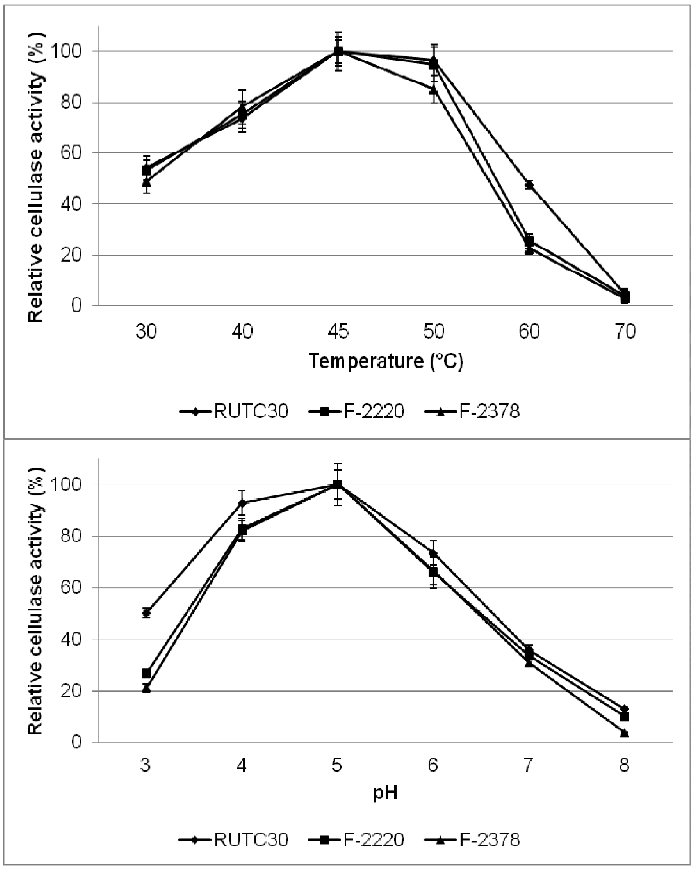


Figure 15. Temperature (a) and pH (b) dependency of cellulase activities in Penicillium pulvillorum TUB F-2220, Penicillium cf cimplicissimum TUB F-2378 and Trichoderma reesei RUT C30 shake flask fermentation supernatants grown on Avicel PH-101 as a carbon source. Hydrolysis substrate: Avicel PH-101 (10 mg/ml) at pH = 5.0, hydrolysis time: 24 h. Enzyme loading 10 FPU/g DM of substrate, supplemented with 6 U/g DM of substrate β-glucosidase (Novozym 188). The presented values are mean of three replicates.

In general, using Avicel as carbon source in the cultivations produced highest enzyme activities and total soluble proteins while using lignocelluloses as carbon source slightly delayed the (hemi)cellulase secretion (maximal activities were reached after 5-7d) (**Appendix, Table A7**).

Even though the cellulase activities in the cultures of *Trichoderma* and *Penicillium* strains were similar, the soluble protein concentration in the *T. reesei* RUTC30 culture supernatant was at least two times higher than in the cultures of the wild type strains, suggesting that the cellulase content in the culture supernatants of the wild-type strains was higher. In addition, the cultures of the novel *Penicillium* strains exhibited higher endoglucanase activity than the activities in the cultures of *T. reesei* QM6a as well the β-glucosidase activities. In contrast, highest xylanase and β-xylosidase activities (4.9 and 1.1 U/ml, respectively) were measured from *T. reesei* RUT C30 cultures.

3.4.2. Hydrolysis of Cellulose and Lignocelluloses by the Penicillium and Trichoderma Culture Supernatants

Hydrolytic performance of the culture supernatants enzymes of the two *Penicillum* strains and *T. reesei* RUTC30 are shown in **Figure 16**. Enzymes were dozed to the hydrolysis by an equal total cellulase activity (10 FPU/g DM) and supplemented by a β-glucosidase activity from Novozym 188 (6 U/g DM) in order to minimize the effect of variations in the β-glucosidase activities in the mixtures. Despite equal cellulase activity loading, culture supernatant enzymes of TUB F-2220 gave higher reducing sugar yields in hydrolysis of microcrystalline cellulose (Avicel) than TUB F-2378 or *T. reesei* RUT C30. In the hydrolysis of pre-treated spruce, TUB F-2220 and *T. reesei* RUT C30 enzymes gave similar yields, while TUB F-2378 enzymes were much less effective in the hydrolysis of this substrate. The hydrolysis yields from pre-treated wheat straw were similar to all the culture supernatants.

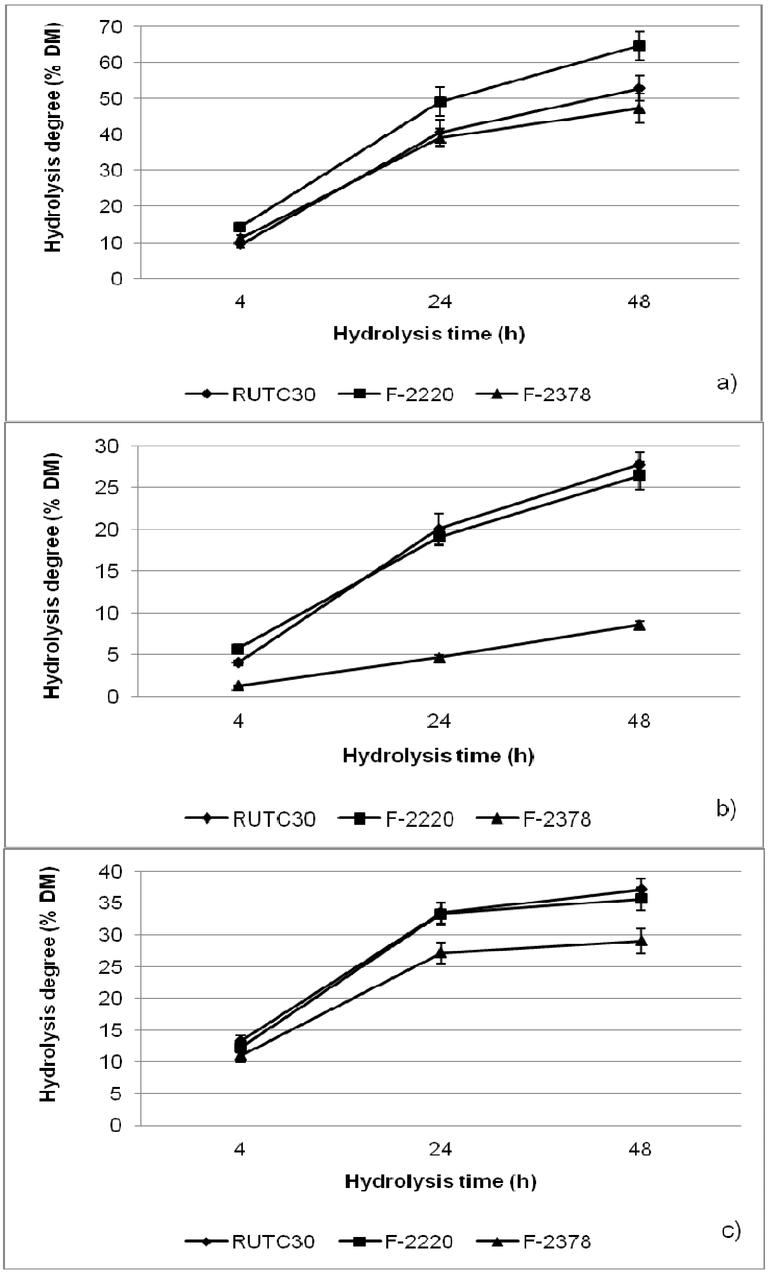


Figure 16. Hydrolysis of Avicel PH-101 (a), pretreated spruce (E-Tech) (b) and pretreated wheat straw (Biogold) (c) at pH = 5.0 and T = 45 °C by Penicillium pulvillorum TUB F-2220, Penicillium cf simplicissimum TUB F-2378 and Trichoderma reesei RutC30 shake flask fermentation supernatants grown on Avicel PH-101 as carbon source. Enzyme loading 10 FPU/g DM of substrate, supplemented with 6 U/g DM of substrate β-glucosidase (Novozym 188). The presented values are means of three replicates.

3.4.3. Partial Purification and Characterization of P. pulvillorum (TUB F-2220) Cellulases

Two of the major proteins (MW 72 and 70 kDa, according to SDS-PAGE) from *P. pulvillorum* (TUB F-2220) culture supernatant were separated using ion exchange chromatography (**Figure 17; Appendix, Table A8**). Analysis of the *de novo* amino acid sequences of the protein indicated that the 70 kDa and 72 kDa protein species contained glycosidic hydrolases belonging to families 6 and 7 with high similarity known as cellobiohydrolases within these families (**Table A8**).

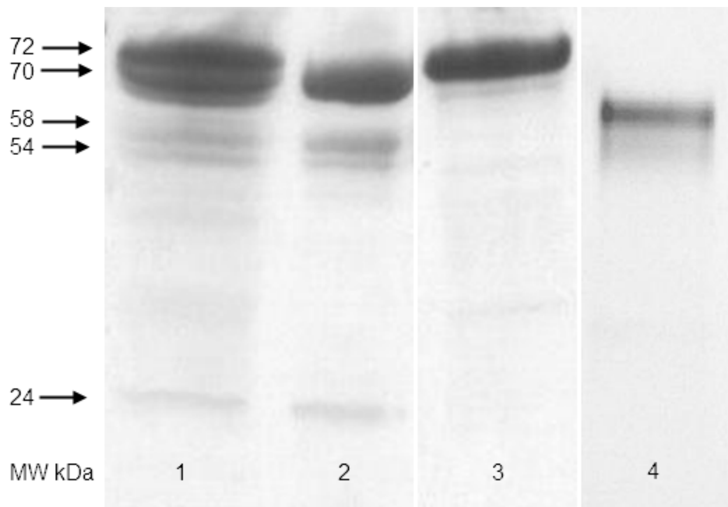


Fig. 17. SDS-PAGE image of the CBH preparations. Lane 1 Penicillium pulvillorum culture filtrate (cultivated on Avicel). Partially purified enzyme preparations PpCBH2 (lane 2) and PpCBH1 (lane 3) from Penicillium pulvillorum (TUB F-2220) and Trichoderma reesei reference TrCel7a (lane 4) on SDS-PAGE (10% gel).

All the peptide sequences obtained from the 72 kDa protein matched GH7 CBHs in Uniprot database, while the peptides from the 70 kDa protein showed high homology to GH6 CBHs and fungal glucoamylases. The putative glucoamylase peptides most likely originated from an impurity. The enzyme fractions were named *PpCBH1* (72 kDa) and *PpCBH2* (70 kDa). The pH and temperature profiles of Avicelase activity in the enzyme preparations were similar to the original culture supernatant (**Appendix, Figure A1)**. The partially purified TUB F-2220 cellulases *PpCBH1* and *PpCBH2* were further compared to *Trichoderma reesei Cel7a* (CBH1) in the hydrolysis of microcrystalline crystalline cellulose (Avicel) at 45°C and 55°C (**Appendix, Figure A2**). In contrast to *TrCel7a*, both *PpCBH1* and *PpCBH2* gave a lower final hydrolysis yield at 55°C than at 45°C, and especially the hydrolysis with *PpCBH2* that was interfered on the elevated temperature (55°C).

3.4.4. Lignin Sensitivity and Adsorption of the Cellulases to Lignin

Reason for the relatively lower performance of the culture supernatants of *Penicillium* species in the hydrolysis of lignocelluloses from pure cellulose was searched from the sensitivity of the culture supernatants enzyme to lignin. When Avicel was hydrolysed using the culture supernatants of *Penicillium* and *Trichoderma* strains in presence and absence of lignin at 45°C it was clear that exclusively the spruce EnzHR lignin was inhibitory to cellulose hydrolysis and that the F-2378 enzyme mixture was most sensitive to lignin (**Figure 18**). Interestingly, when the hydrolysis was repeated at 35°C, the inibitory effect of lignins was greatly dimished.

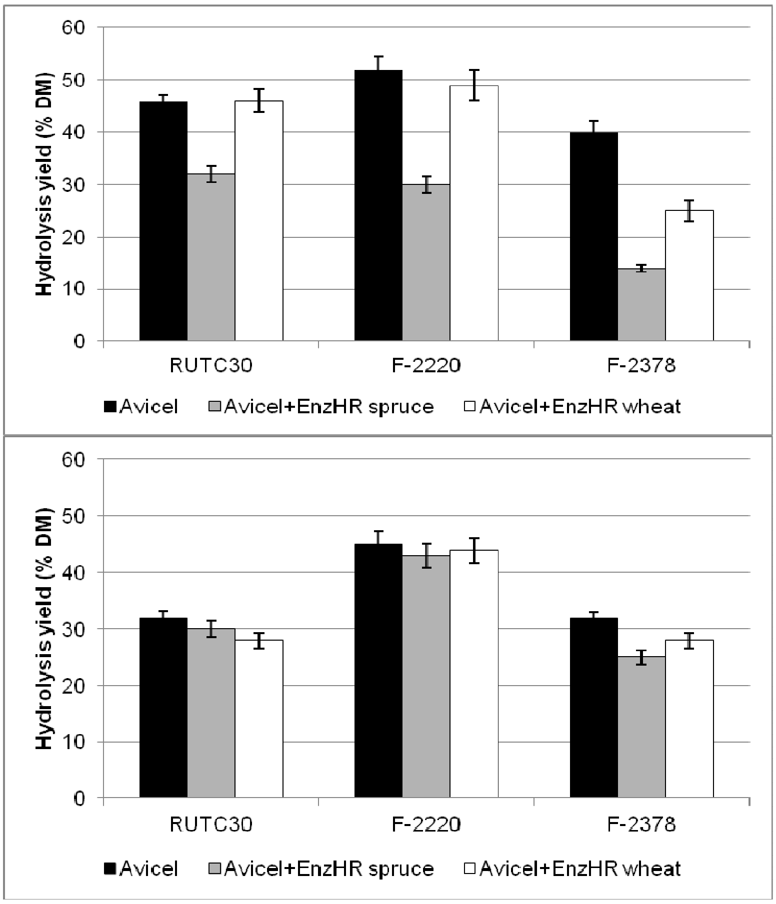


Figure 18. Inhibitory effect of lignins. Effect of EnzHR lignin isolated from pre-treated spruce or wheat straw on the hydrolysis of Avicel PH-101 by Penicillium pulvillorum TUB F-2220, Penicillium cf simplicissimum TUB F-2378 and Trichoderma reesei RUT C30 cellulase mixtures (shake flask fermentation supernatants grown on Avicel PH-101 as a carbon source). Hydrolysis was carried out at 45 °C (a) or 35 °C (b) in following conditions: Avicel PH-101: 10 mg/ml; EnzHR lignins: 5 mg/ml; pH = 5.0; hydrolysis time: 24 h. Enzyme loading 10 FPU/g DM of substrate, supplemented with 6 U/g DM of substrate β-glucosidase (Novozym 188). Released reducing sugars were quantified with DNS assay.

Since the lignin-caused inhibition of hydrolysis has been thought to be partially caused by the adsorption of the cellulases to lignin, adsorption behaviour of the *PpCBH1* and *TrCel7a* to microcrystalline cellulose (Avicel) and EnzHR lignins were isolated from pre-treated spruce and wheat straw were compared (**Appendix, Figure A3a)**. Both *PpCBH1* and *TrCel7a* efficiently adsorbed to Avicel (over 80% of the enzyme was adsorbed). However, adsorption of the *PpCBH1* to EnzHR lignins was clearly lower compared to *TrCel7a*. A higher amount of both enzyme preparations was adsorbed to the spruce EnzHR lignin than to the wheat straw EnzHR lignin. Impact of pH and temperature to the adsorption of the *PpCBH1* and *TrCel7a* was further studied. The adsorption of especially the *PpCBH1* was shown to be pH dependent: at pH 4 almost all the enzymes were adsorbed to the lignin but the adsorption clearly diminished at higher pHs (pH 5 and pH 6) (**Appendix, Figure A3b**). Increasing the incubation temperature in the adsorption experiment from 35°C to 45°C increased the amount of adsorbed enzyme especially for the *PpCBH1* and also reduced the amount of enzyme that could be released from the spruce EnzHR lignin particles to SDS-PAGE (**Appendix, Figure A3c**). The results clearly indicated that the increased temperature fortified the protein-lignin interactions and may be the reason for a higher inhibitory effect of lignins at a higher temperature.

3.4.5. Cloning of P. pulvillorum (TUB F-2220) CBHs

Amplification of the TUB F-2220 (*P. pulvillorum*) genomic DNA with degenerate primers designed for conserved regions in fungal CBH enzymes that yielded two specific amplicons of 500-600 bp (data not shown). Each amplicon represented a unique gene fragment, indicating that the TUB F-2220 genome contains at least two *cbhI* genes (*cbh1a* and *cbh1b*). The sequences of three tryptic peptides recovered from partially purified *PpCBH1* matched to the gene fragment *cbh1a*, indicating that the *cbh1a* gene fragment represents the gene for the enzyme identified in *PpCBH1*. Ligation mediated PCR of StuI and SsPI digested genomic DNA of TUB F-2220 with primers designed for the 5’ end of the *cbh1a* fragment resulted in 1500-2000 bp fragments containing complete 5’end of the gene. Ligation mediated PCR of the 3’ end of the *ppcbh1a* resulted in partial 3’ fragments of the gene and complete 3’ end of the *ppcbh1a* was amplified using 3’ RACE PCR using the specific primer designed for the 3’ end of the *cbh1a*.

Full length coding sequence of *PpCBH1* assembled from the PCR amplified fragments of TUB F-2220 genomic DNA and cDNA is shown in **Appendix, Figure A4.** Four of the *PpCBH1* de novo peptide sequences matched completely the translated amino acid sequence of coding the DNA sequence and two of them showed a 86-92% identity to the translated *ppcbh1a* sequence (**Appendix, Figure A4**). The closest BLAST neighbours of the enzyme were glycosyl hydrolase family 7 cellobiohydrolases from *Penicillium oxalicum*, *P. decumbens* and *P. janthinellum* (F6L7A1_PENOX, C9EI49_9EURO, GUX1_PENJA) with up to 83% sequence identity (**Appendix, Figure A5**). Signal prediction has indicated a presence of 25 amino acid long signal peptide in the N-terminus. Sequence homology to recognised GH7 enzymes indicated that the enzyme consisted of a catalytic domain of family 7 that is interlinked with carbohydrate binding domain (*CBM1*) by a linker peptide. Two conserved catalytic glutamate residues and four tryptophan residues were involved in glucan binding along with 18 cysteine residues in formation disulphide bridges that are found in the sequence of the catalytic domain (**Appendix, Figure A5**) [50, 51]. In *CBM*, three tryptophan residues are located in the same positions where tyrosine residues were associated to cellulose binding activity of *TrCel7a CBM* that are located (Lee et al., 2012).

3.4.6. Conclusions

Evaluation of the cellulase and hemicellulase systems of two novel lignocellulolytic *Penicillium* strains indicated that both strains secrete a high level of cellulase activities. Specific cellulase activities of the wild-type *P. pulvillorum* TUB F-2220 strain are approx. two times higher than being it is compared to *T. reesei* RUT C30. This attribute can make isolate F-2220 to an attractive biological starting material for strain improvement. The enzyme mixture secreted by *P. pulvillorum* (TUB F-2220) has exclusively high hydrolytic activity towards microcrystalline cellulose. Two of the main enzymes in the TUB F-2220 enzyme mixture were identified as GH7 and GH6 cellobiohydrolases most likely related to the high level of cellulose degrading activity. The inhibition of the hydrolytic activity of the *Penicillium* and *T. reesei* reference cellulases in the presence of lignin in the hydrolysis mixture was dependent on temperature and lignin and this is almost entirely absent in case of in the case of wheat straw lignin and at 35°C and is the highest with spruce lignin at 45°C. Based on adsorption assays the inhibition is influenced by temperature enhanced adsorption of the enzymes on lignin surfaces. The data indicates that the *P. pulvillorum* TUB F-2220 cellobiohydrolases have suitable properties for application in lower temperature lignocellulose conversion processes such as simultaneous saccharification and fermentation (SSF).

3.5. Novel Cellulases of Fast-growing *Trichoderma* Isolates for Degradation of Lignocellulosic Materials

Screening work (around 950 mesophilic filamentous fungi have been studied) of novel cellulases has resulted in two promising lignocellulolytic *Trichoderma s*trains. These isolates were tested in cellulase/hemicellulase enzyme production during fermentation experiments and in hydrolysis studies to compare them with well-known *Trichoderma reesei* RUT C30 and QM 6a.

Fermentation experiments were carried out on the microcrystalline cellulose carbon source (Avicel PH-101), where the growth of novel fast increasing *Trichoderma* strains were started earlier than *T. i* isolates and also the filter paper activities were more suitable for new strains. Novel *Trichoderma* isolates reached the same level of enzmye activity faster than *T. i* control strains. Likewise in β-glucosidase enzyme secretion was outstanding in case of fast growing *Trichoderma* strains.

Efficiency of new *Trichoderma* cellulases was better in *T. reesei* strains at a lower temperature. pH optima of novel enzymes were similar to control *T. reesei* cellulases. These positive results could be benefits in simultaneous saccharification and fermentation (Maurice, 2011).

The novel *Trichoderma* cellulases were equally efficient as control enzymes in hydrolysis of microcrystalline cellulose and pretreated herbaceous plant materials. However**,** in degradation of pretreated woody materials they could not overtake the cellulases from *T. reesei* RUT C30. The explanation could be that inhibition effect of wood lignin was more agressive than of herbaceous lignin.

Cellulases of fast growing *Trichoderma* were more resistant in lignin inhibition studies at a lower temperature than *T. reesei* cellulases. This behaviour is also favourable during simultaneous saccharification and fermentation (Maurice, 2011).

These results have inspired us for a further, in-depth analysis of novel *Trichderma* cellulases. At this moment, research works are still in progress for a possible patent, hence disclosures of results are unpermitted.

4. Summary

Filamentous fungi can be valuable sources of cellulosic and hemicellulsic enzymes used for bioconversion of lignocellulosic biomass to biofuels and other end-products. I assumed that unexplored microbial isolates may still exist in the earth, which are efficient in lignocellulose degradation and nobody has observed them yet. Consequently, I have performed a screening work in the microbial culture collection of Budapest University Technology and Economics in order to find outstanding lignocellulolytic enzyme producers. I have used both the mesophilic lignocellulolytic fungi stored in the TUB culture collection and we also isolated promising novel strains from soil and decaying plant materials collected in different parts of the world. The best selected filamentous fungi were investigated for further in-depth analysis.

This research is based on the 7th Framework EU-project, with the logo ’DISCO’ (’Targeted DISCOvery of novel cellulases and hemicellulases and their reaction mechanisms for hydrolysis of lignocellulosic biomass’). This project focuses on the development of efficient and cost-effective enzyme tools to degrade the lignocellulosic biomass to fermentable sugars, converting it to bioethanol and to investigate the performance of these enzymes.

Preliminary screening work was carried out approximately with 950 identified and non-identified filamentous fungi strains. Fermentation experiments were applied on different lignocellulosic carbon sources. Enzyme supernatants selection was performed by colorimetric cellulase and hemicellulase enzyme assays from a great number of filamentous fungi enzyme mixtures. Selected enzyme mixtures have been tested in hydrolysis of soluble xylan rich substrates xylan rich fractions (WAX, EXH), degradation products were analysed. Hydrolysis products were analysed by high performance liquid chromatography (HPLC, HPAEC and HPSEC) and mass spectrometry (MALDI-TOF MS). These methods help to prove the sufficiency of degradation that is dependent not only on the selected fungi enzyme preparation but also the characteristics of xylan rich substrates. Selection of the most efficient enzyme supernatants should be based on colorimetric assay results combined with up-to-date instrumental methods. Application of this process gives us a precise enzyme diagnostic tool.

Enzyme preparations of different fermentation supernatants were applied in the hydrolysis of insoluble wheat straw and corn fiber xylan rich fractions (WS WUS and CF AIS). Up to 14% of the carbohydrates in wheat straw and 34% of those in corn fiber were hydrolyzed by enzyme mixtures. The hydrolysis yield depended on the origin of the fungal isolate and on the complexity of the substrate to be degraded. *Penicillium, Trichoderma* or *Aspergillus* species and other non-identified fungi proved to be the best hemicellulolytic enzyme producers. The choice for an enzyme preparation to efficiently hydrolyse different xylan rich substrates is dependent on the xylan characteristics and could not be estimated by using model substrates.

Study of fungi genetically close to *T. reesei* in biodegradation of xylan may have a scientific value. Thus, fifteen strains belonging to *Trichoderma* section *Longibrachiatum* have been tested for extracellular xylan degrading enzyme production on three carbon sources (wheat straw, corn fiber and eucalyptus wood) in shake flask cultivation. The enzyme activities were assayed by traditional colorimetric enzyme assays and by novel instrumental methods. Hydrolysis of four different soluble and insoluble xylan-rich model substrates (WAX, EXH, WS WUS and CF AIS) was performed by fermentation supernatants. *T. reesei* CPK 155, *T. parareesei* TUB F-2535, *T. gracile* TUB F-2543 and *T. saturnisporum* ATCC 18903 isolates were equally effective or better in degradation of WAX and CF AIS than the well-known *T. reesei* QM 6a and RUT C30 strains. Conclusively**,** these fungi may be potential candidates for further experiments. Enzyme production on wheat straw and corn fiber fermentation carbon sources was more efficient than on eucalyptus wood carbon source.

The lignocellulolytic enzyme systems of two novel *Penicillium* strains (*P.pulvillorum* TUB F-2220 and *P.cf.simplicissimum* TUB F-2378) have been compared to well-known *T. reesei* QM 6a and RUT C30 strains. Comparison of the novel *Penicillium* and *T. reesei* secreted enzyme mixtures in the hydrolysis of lignocellulose substrates showed that the F-2220 enzyme mixture gave higher yields in the hydrolysis of crystalline cellulose. Yields are similar in the hydrolysis of pretreated spruce and wheat straw than enzyme mixture produced by *T. reesei* reference strains. The lignin sensitivity of the *Penicillium* cellulase complexes to softwood (spruce) and grass (wheat straw) lignins depended on lignin quality and hydrolysis temperature: lignin inhibition of cellulose hydrolysis in the presence of wheat straw lignin was lower at 35°C while at 45°C by spruce lignin a clear inhibition was observed. Thus, the cellulase complex of *Penicillium* *pulvillorum* TUB F-2220 seems to be valuable in the simultaneous saccharification and fermentation (SSF) process performed at a lower temperature.

Another finding of our experiments is that cellulases of two isolates identified as novel fast growing *Trichoderma* isolates are also valuable sources of lignocellulolytic enzymes. They are fast growing fungi and produce cellulase complexes more economically than well-known *Trichoderma reesei* strains. At the present moment they are under in-depth analysis, additional experiments will show in the future whether these strains and/or their cellulases are patentable. Until the end of the investigation I do not have permission to publish these results.

5. References

Adney WS, Baker JO, Decker SR, Chou Y-C, Himmel ME, Ding S-Y. 2008. Superactive cellulase formulation using cellobiohydrolase-1 from *Penicillium funiculosum*. US Patent 7,449,550 B2

Adsul MG, Bastawde KB, Varma AJ, Gokhale DV. 2007. Strain improvement of *Penicillium janthinellum* NCIM 1171 for increased cellulase production Biores Technol. 98: 1467-1473

Agbor VB, Cicek N, Sparling R, Berlin A, Levin DB. 2011. Biomass pretreatment: fundamentals toward application. Biotechnol Adv. 6:675-85

Ahmed AER, Labavitch JM, 1978. A simplified method for accurate determination of cell wall uronide content. J Food Biochem 1: 361–365

Appeldoorn MM, Kabel MA, Van Eylen D, Gruppen H, Schols H.A. 2010. Characterization of oligomeric xylan structures from corn fiber resistant to pretreatment and simultaneous saccharification and fermentation. J Agric Food Chem. 58: 11294-11301

Aro N, Pakula T, Penttila M. 2005. Transcriptional regulation of plant cell wall degradation by filamentous fungi. FEMS Microbiol Rev. 29: 719-739

Bailey MJ, Poutanen K. 1989. Production of xylanolytic enzymes by strains of *Aspergillus*. Appl Microbiol Biotechnol. 30: 5–10

Bailey MJ, Biely P, Poutanen K. 1992. Interlaboratory testing of methods for assay of xylanase activity. J Biotechnol. 23:257-270

Bailey BA, Lumsden RD. 1998. Direct efects of *Trichoderma* and *Gliocladium* on plant growth and resistance to pathogens, in *Trichoderma* & *Gliocladium*—Enzymes, G. F. Harman and C. P. Kubicek, Eds., vol. 2 of Biological Control and Commercial Applications, Taylor & Francis, London, UK. 327–342

Bamforth CW. 2009. Current perspectives on the role of enzymes in brewing, Journal of Cereal Science. 50(3): 353–357

Banerjee G, Scott-Craig JS, Walton JD. 2010. Improving enzymes for biomass conversion: A basic research perspective. Bioenergy Res. 3: 82-92

Bansal P, Hall M, Realff MJ, Lee JH, Bommarius AS. 2009. Modeling cellulase kinetics on lignocellulosic substrates. Biotechnol Adv. 27: 833-848

Beg QK, Kapoor M, Mahajan L,·Hoondal GS. 2001. Microbial xylanases and their industrial applications: a review. Appl Microbiol Biotechnol 56:326–338

Beguin P, Aubert JP. 1994. The biological degradation of cellulose. FEMS Microbiology Reviews. 13: 25-58

Berlin A, Gilkes N, Kurabi A, Bura R, Tu M, Kilburn D, Saddler J. 2005. Weak lignin-binding enzymes. Appl Biochem Biotechnol. 121-124: 163-170

Berlin A, Balakshin M, Gilkes N, Kadla J, Maximenko V, Kubo S, Saddler J. 2006. Inhibition of cellulase, xylanase and β-glucosidase activities by softwood lignin preparations. J Biotechnol. 125: 198-209

Bhat MK, 2000.Cellulases and related enzymes in biotechnology. Biotechnology Advances. 18(5): 355–383

Biely P, Vrsanská M, Tenkanen M, Kluepfel D. 1997. Endo-b-1, 4-xylanase families: differences in catalytic properties. J Biotechnol. 57: 151–166

Biely P. 2003. Diversity of microbial endo-beta-1,4-xylanases. In: Application of enzymes to lignocellulosics, ACS Symp Ser 855, Mansfield, SD and Saddler JN (eds) American Chemical Society, Washington DC, pp 361-380

Biely P, Puchart V. 2006. Recent progress in the assays of xylanolytic enzymes. J Sci Food Agric 86: 1636–1647

Bissett J, Szakacs G, Nolan CA, Druzhinina I, Gradinger C, Kubicek CP. 2003. New species of *Trichoderma* from Asia. Can J Botany. 81:570-586

Bura R, Mansfield S, Saddler J, Bothast, R. 2002. SO2-catalyzed steam explosion of corn fiber for ethanol production. Applied biochemistry and biotechnology. 98(1):59-72

Caffall KH, Campbell DM. 2009. The structure, function, and biosynthesis of plant cell wallpectic polysaccharides. Carbohydrate Res. 344: 1879–1900

Camacho NA, Aguilar O G. 2003. Production, purification, and characterization of a low-molecular-mass xylanase from *Aspergillus* sp. and its application in baking. Appl Biochem Biotechnol. 104(3):159-72

Chavez R, Bull P, Eyzaguirre J. 2006. The xylanolytic enzyme system from the genus *Penicillium*. J Biotechnol. 123: 413–433

Christov L, Biely P, Kalogeris E, Christakopoulos P, Prior BA, Bhat MK. 2000. Effects of purified endo-β-1, 4-xylanases of family 10 and 11 and acetyl xylan esterases on eucalypt sulfite dissolving pulp. J Biotechnol. 83:231–244

Cuyvers S, Dornez E, Delcour JA, Courtin CM. 2011. The secondary substrate binding site of the *Pseudoalteromonas haloplanktis* GH8 xylanase is relevant for activity on insoluble but not soluble substrates. Appl Microbiol Biotechnol. 1–11

Csiszar E, Losonczi A, Szakacs G, Rusznak I, Bezur L, Reicher J. 2001. Enzymes and chelating agent in cotton pretreatment. J Biotechnology. 89:271–279

da Silva AE, Marcelino HR, Gomes MCS, Oliveira EE, Nagashima T, Egito EST. 2012. Xylan, a PromisingHemicellulose for Pharmaceutical Use, Products and Applications of Biopolymers. Dr. Johan Verbeek (ed) InTech China. 61-84.

Davison A, Blaxter M. 2005. Ancient origin of gly-cosyl hydrolase family 9 cellulase genes. Molecular Biology Evolution. 22(5): 1273-1284

de Carvalho LMJ, de Castro IM, da Silva CAB. 2008. A study of retention of sugars in the process of clarification of pineapple juice (*Ananas comosus*, L. Merril) bymicro- and ultra-filtration. J Food Engineering. 78(4): 447–454 de Castro AM, Ferreira MC, da Cruz JC, Pedro KCNR, Carvalho DF, Leite SGF, Pereira Jr N. 2010. High-yield endoglucanase production by *Trichoderma harzianum* IOC-3844 cultivated in pretreated sugarcane mill byproduct. Enzyme Res. 2010:8

de Vries RP, Visser J. 2001. *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. Microbiol Mol Biol Rev. 65(4):497-522

Decker SR, Siika-Aho M, Viikari L. 2009. Enzymatic depolymerization of plant cell wall hemicelluloses in: biomass recalcitrance: deconstructing the plant cell wall for bioenergy. Himmel ME (ed) Blackwell Publishing Ltd Oxford, UK, pp. 352-373

Druzhinina IS,·Kopchinskiy AG, Kubicek CP. 2006. The first 100 Trichoderma species characterized by molecular data. Mycoscience. 47:55–64

Ebringerova A, Hromadkova Z. 1999. Xylans of industrial and Biomedical Importance. Biotechnol and Gen Engineering Rev. 16: 325-346

Eriksson T, Börjesson J, Tjerneld F. 2002. Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. Enzyme Microbiol Technol. 31(3): 353-364

Esposito E, da Silva M. 1998. Systematics and environmental application of the genus *Trichoderma*. Crit Rev Microbiol. 24(2):89-98

Fawcett PA, Loder PB, Ducan MJ, Abraham B, Abraham EP. 1973. Formation and Properties of Protoplasts from Antibiotic-producing Strains of *Penicillium chrysogenum* and Cephalosporium acremonium. J Gen Microb. 79: 293-309.

Fauré R, Courtin CM, Delcour JA, Dumon C, Faulds CB, Fincher GB, Fort S, Fry SC, Halila S, Kabel MA, Pouvreau L, Quemener B, Rivet A, Saulnier L, Schols HA, Driguez H, O'Donohue MJ. 2009. A brief and informationally rich naming system for oligosaccharide motifs of heteroxylans found in plant cell walls. Aust J Chem. 62: 533–537

Galbe M, Zacchi G. 2007. Pretreatment of lignocellulosic materials for efficient bioethanol production. Adv Biochem Eng Biotechnol. 108:41-65

Garrote G, Dominguez H, Parajó JC. 1999. Mild autohydrolysis: An environmentally friendly technology for xylooligosaccharide production from wood. J Chem Technol Biotechnol 74: 1101–1109

Ghose TK. 1987. Measurement of cellulase activities. Pure & Appl Chem. 59: 257-268

Ghose TK, Bisaria, VS. 1979. Studies on the mechanism of enzymatic hydrolysis of cellulosic substances. Biotechnol Bioeng. 21: 131-146

Gomez LD, Steele-King CG, McQueen-Mason SJ. 2008. Sustainable liquid biofuels from biomass: the writing's on the walls. New Phytologist 178: 473–485

Gruppen H, Marseille JP, Voragen AGJ, Hamer RJ 1990. On the large-scale isolation of water-insoluble cell-wall material from wheat-flour. Cereal Chem. 67:512-514

Guo R, Ding M, Zhang SL, Xu GJ, Zhao FK. 2008. Molecular cloning and characterization of two novel cellulase genes from the mollusk *Ampullaria crossean*. J Compressed Physiology [B]. 178(2): 209-215

Gupta R, Mehta G, Khasa YP, Kuhad RC. 2011. Fungal delignification of lignocellulosic biomass improves the saccharification of cellulosics. Biodegrad. 22(4): 797-804

Gusakov AV, 2011. Alternatives to *Trichoderma reesei* in biofuel production. Trends Biotechnol, 29, 419-425

Hägerdal B, Harris H, Pye EK. 1979. Association of β-glucosidase with intact cells of *Thermoactinomyces*. Biotechnol Bioeng. 21:345–355

Harbak L, Thygesen HV. 2002. Safety evaluation of a xylanase expressed in *Bacillus subtilis*. Food Chem Toxicol. 40(1):1-8

Harman GE, Björkman T. 1998. Potential and existing uses of *Trichoderma* and *Gliocladium* for plant disease control and plant growth enhancement. *Trichoderma* and *Gliocladium* Vol. 2. Edts: Harman GE and Kubicek CP Taylor&Francis Ltd. 1998 ISBN: 0-7484-0805-3

Henrissat B, Davies G. 1997. Structural and sequence-based classification of glycoside hydrolases. Current Opinion in Structural Biology. 7(5): 637–644

Herr D, Baumer F, Dellweg H. 1978 Purification and properties of an extracellular β-glucosidase from *Lenzites trabea*. Appl Microbiol Biotechnol. 5:29-36

Himmel ME, Ding SY, Johnson DK, Adney WS, Nimlos MR, Brady JW, Foust TD. 2007. Biomass recalcintrance: Engineering plants and enzymes for biofuel production. Science. 315(5813):804-807

Himmel ME, Xu Q, Luo Y, Ding SY, Lamed R, Bayer EA. 2010. Microbial enzyme systems for biomass conversion: emerging paradigms. Biofuels. 1(2): 323-341.

Hori C, Igarashi K, Katayama A, Samejima M. 2011. Effects of xylan and starch on secretome of the basidiomycete *Phanerochaete chrysosporium* grown on cellulose. FEMS Microbiol. Lett. 321: 14–23

Jorgensen H, Eriksson T, Borjesson J, Tjerneld F, Olsson L. 2003. Purification and characterization of five cellulases and one xylanase from *Penicillium brasilianum* IBT 20888. Enzyme Microb Technol. 32: 851-861

Jorgensen H, Morkeberg A, Krogh KBR, Olsson L. 2005. Production of cellulases and hemicellulases by three *Penicillium* species: effect of substrate and evaluation of cellulase adsorption by capillary electrophoresis. Enzyme Microb Technol. 36: 42-48

Jorgensen H, Olsson L. 2006. Production of cellulases by *Penicillium brasilianum* IBT 20888 – effect of substrate on hydrolysis performance. Enzyme Microb Technol 38: 381-390

Jovanovic I, Magnuson JK, Collart F, Robbertse B, Adney WS, Himmel ME, Baker SE. 2009. Fungal glycoside hydrolases for saccharification of lignocellulose: outlook for new discoveries fueled by genomics and functional studies. Cellulose. 16: 687-697

Kabel MA, Schols HA, Voragen AGJ. 2002. Complex xylo-oligosaccharides identified from hydrothermally treated *Eucalyptus* wood and brewery's spent grain. Carbohydr Polym. 50: 191–200

Kabel MA, Van den Borne H, Vincken JP, Voragen AGJ, Schols HA. 2007a. Structural differences of xylans affect their interaction with cellulose. Carbohydr Polymers. 69:94-105

Kabel MA, Bos G, Zeevalking J, Voragen AGJ, Schols HA. 2007b. Effect of pretreatment severity on xylan solubility and enzymatic breakdown of the remaining cellulose from wheat straw. Bioresour Technol. 98: 2034-2042

Kirk TK. 1983. Degradation and Conversion of Lignocelluloses. In: Smith JE, Berry DR, Kristiansen B. The filamentous fungi, version 4, Fungal technology. London: Edward Arnold. 266-295

Kormelink FJM, Searle-Van Leeuwen MJF, Wood TM, Voragen AGJ. 1991. Purification and characterization of a (1,4)-β-D-arabinoxylan arabinofuranohydrolase from *Aspergillus awamori*. Appl. Microbiol. Biotechnol. 35: 753–758

Kormelink FJM, Gruppen H, Vietor RJ, Voragen AGJ. 1993a. Mode of action of the xylan-degrading enzymes from *Aspergillus awamori* on alkali-extractable cereal arabinoxylans. Carbohydr Res. 249:355–367

Kormelink FJM, Gruppen H, Vietor RJ, Voragen AGJ. 1993b. Mode of action of (1→4)-β-D-arabinoxylanarabinofuranohydrolase (AXH) and α-L-arabinofuranosidases on alkali-extractable wheat-flour arabinoxylan. Carbohydr Res. 249: 345–353

Kormelink FJM, Hoffmann RA, Gruppen H, Voragen AGJ, Kamerling JP, Vliegenthart JFG. 1993c. Characterisation by 1H NMR spectroscopy of oligosaccharides derived from alkali-extractable wheat-flour arabinoxylan by digestion with endo-(1,4)-β-D-xylanase III from *Aspergillus awamori*. Carbohydr Res. 249: 369–382

Koullas DP, Christakopoulos P, Kekos D, Macris BJ, Koukios EG. 1992. Correlating the effect of pretreatment on the enzymatic hydrolysis of straw. Biotechnol Bioeng. 39: 113-116

Kristensen JB, Thygesen LG, Felby C, Jørgensen H, ElderNydetzky T. 2008. Cell-wall structural changes in wheat straw pretreated for bioethanol production. Biotechnol Biofuels. 1:5

Krogh KBR, Morkenberg A, Jorgensen H, Frisvad JC, Olsson L. 2004. Screening genus *Penicillium* for producers of cellulolytic and xylanolytic enzymes. Appl Biochem Biotechnol. 113-116: 389-401

Kubicek CP, Bolzlbauer UM, Kovacs W, Mach RL, Kuhls K, Lieckfeldt E, Borner T, Samuels GJ 1996. Cellulase formation by species of *Trichoderma* sect *Longibrachiatum* and of *Hypocrea spp*. with anamorphs referable to *Trichoderma* sect. *Longibrachiatum*. Fungal Genet Biol. 20:105-114

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

Kudah RC, Gupta R, Singh A. 2011. Microbial Cellulases and Their Industrial Applications. Enzyme Research 2011:10

Kulkarni N, Shendye A, Rao M. 1999. Molecular and biotechnological aspects of xylanases. FEMS Microbiology Reviews 23: 411-456

Kumar R, Wyman CE. 2009. Effect of xylanase supplementation of cellulase on digestion of corn stover solids prepared by leading pre-treatment technologies. Bioresource Technol. 100: 4203-4213

Lafond Saha M, Tauzin A, Desseaux V, Bonnin E, Ajandouz el-H, Giardina T. 2011 GH10 xylanase D from *Penicillium funiculosum*: biochemical studies and xylooligosaccharide production. Microb Cell Fact. 10:20

Lamsal BP, Madl R, Tsakpunidis K. 2011. Comparison of feedstock pretreatment performance and its effect on soluble sugar availability. Bioenergy Res. 4: 193-200

Laureano-Perez L, Teymouri F, Alizadeh H, Dale BE. 2005. Understanding factors that limit enzymatic hydrolysis of biomass. characterization of pretreated corn stover. Appl Biochem Biotechnol. 121-124

Lee D, Yu AHC, Saddler JN. 1995. Evaluation of cellulase recycling strategies for the hydrolysis of lignocellulosic substrates. Biotechnol Bioeng. 45: 328–336

Lee J, Jang Y, Lee HL, Kim G-H, Kim J-J. 2012. Screening for xylanase and β-xylosidase production from wood-inhabiting *Penicillium* strains for potential use in biotechnological applications. Holzforschung. 66:267–71

Lever M. A new reaction for colorimetric determination of carbohydrates. 1972 Anal Biochem. 47:273–9

Lin SY, Dence CW. 1992. Methods in lignin chemistry. Springer, Berlin Heidelberg New York, Lundquist K ISBN:3540502955. 289-300

Linden J, Samara M, Decker S, Johnson E, Boyer M, Pecs M, Adney W, Himmel M. 1994. Purification and characterization of an acetyl esterase from *Aspergillus niger*. Appl Biochem Biotechnol. 45-46: 383–393

Liu G, Qui Y, Li Z, Qu Y. 2013. Improving lignocellulolytic enzyme production with Penicillium: from strain screening to systems biology. Biofuels. 4 (5): 523-534

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem. 193:265-275

Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS. 2002. Microbial Cellulose Utilization: Fundamentals and Biotechnology. Microbiol Mol Biol Rev. 66(3): 506-577

Lynd LR, Laser MS, Bransby D, Dale BE, Davidson B, Hamilton R, Himmel M, Keller M, McMillan JD, Sheedan J, Wyman CE 2008. How biotech can transform biofuels. Nature Biotechnol 26:169-172

Mackey AJ, Haystead TAJ, Pearson WA. 2002. Getting more from less: algorithms for rapid protein identification with multiple short peptide sequences. Molec Cell Proteom 1:139–47

Mandels, M., Reese, E.T. 1957. Induction of cellulase in *Trichoderma viride* as influenced by carbon sources and metals. J Bacteriol. 73: 269-278

Mandels M, Weber J. 1969. The production of cellulases. In Cellulases and Their Applications, Hajny GJ and Reese ET (eds) Adv Chem Ser 95, American Chemical Society, Washington DC. 391-414

Mandels M, Kostick J, Parizek R. 1971. The use of adsorbed cellulase in the continuous conversion of cellulose to glucose. Journal of Polymer Science Part C: Polymer Symposia. 36(1): 445–459

Mäntylä A, Paloheimo M, Suominen P. 1998. Industrial mutant and recombinant strains of *Trichoderma reesei*. In: *Trichoderma* and *Gliocladium*, Vol 2, Harman GE & Kubicek CP (eds), Taylor & Francis Ltd London. 291-309

Mäntylä A, Paloheimo M, Hakola S, Lindberg E, Leskinen S, Kallio J, Vehmaanperä J, Lantto R, Suominen P. 2007. Production in *Trichoderma reesei* of three xylanases from *Chaetomium thermophilum*: a recombinant thermoxylanase for biobleaching of kraft pulp. Appl Microbiol Biotechnol. 76(2):377-86

Martinez D, Larrondo LF, Putnam N, Gelpke MDS, Huang K, Chapman J, Helfenbein KG, Ramaiya P, Detter JC, Larimer F, Coutinho PM, Henrissat B, Berka R, Cullen D, Rokhsar D. 2004. Genome sequence of the lignocellulose degrading fungus Phanerochaete chrysosporium strain RP78. Nature Biotechnol 22 (6): 695-700

Martinez D, Berka RM, Henrissat B, Saloheimo M, Arvas M, Baker SE, ChapmanJ,Chertkov O, Coutinho PM, Cullen D, Danchin EGJ, 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, Brettin TS. 2008. Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*). Nature Biotechnol 26 (5): 553-560

Martins LF, Kolling D, Camassola M, Dillon AJP, Ramos LP. 2008. Comparison of Penicillium *echinulatum* and *Trichoderma reesei* cellulases in relation to their activity against various cellulosic substrates. Bioresource Technol. 99: 1417-1424

Maurice LM. 2011. Factors Effecting Ethanol Fermentation Via Simultaneous Saccharification and Fermentation. Doctoral Thesis, Shanghai Jiao Tong University.

McMillan J. 1994. Selling Spectrum Rights. Journal of Economic Perspectives. 8:145-162

Merino ST, Cherry J. 2007. Progress and Challenges in Enzyme Development for Biomass Utilization. Adv Biochem Engin/Biotechnol 108: 95–120

Michelin M, Peixoto-Nogueira SC, Betini JH, da Silva TM, Jorge JA, Terenzi HF, Polizeli ML. 2010. Production and properties of xylanases from *Aspergillus terricola* Marchal and *Aspergillus ochraceus* and their use in cellulose pulp bleaching. Bioprocess Biosyst Eng. 33(7):813-21

Milala MA, Shugaba A, Gidado A, ENe AC, Wafar JA. 2005. Studies on the use of agricultural wastes for cellulase enzyme production by *Aspergillus niger,* Research J Agricul Bio Sci. 1:325–328.

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

Miron J, Ben-Ghedalia D. 1982. Effect of hydrolysing and oxidizing agents on the composition and degradation of wheat straw monosaccharides. Appl Microbiol Biotechnol. 15: 83-87

Mishra C, Rao M, Seeta R, Srinivasan MC, Deshpande V. 1984. Hydrolysis of lignocellulose by cellulase from *Penicillium funiculosum* cellulase. Biotechnol Bioeng. 26: 370-373

Montenecourt BS, Eveleigh DE. 1979. Selective screening methods for the isolation of high yielding cellulase mutants of *Trichoderma reesei.* In: Hydrolysis of Cellulose: Mechanisms of Enzymatic and Acid Catalysis, Adv Chem Ser 181, Brown R and Jurasek L (eds), American Chemical Society, Washington DC. 289-301

Muller PR, Wold B. 1989. In vivo footprinting of a muscle specific enhancer by ligation mediated PCR. Science 246:780–6

Mussatto SI and Teixeira JA. 2010. Lignocellulose as raw material in fermentation processes. Communicating Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology. 2: 907-907

Nakagame S, Chandra RP, Saddler JN. 2010. The effect of isolated lignins, obtained from a range of pretreated lignocellulosic substrates on enzymatic hydrolysis. Biotechnol Bioeng. 105: 871-879

Nakari-Setala T, Paloheimo M, Kallio J, Vehmaanpera J, Penttila M, Saloheimo M. 2009. Genetic modification of carbon catabolite repression in *Trichoderma reesei* for improved protein production. Appl Environ Microbiol. 75:4853-4860

Pan X, Arato C, Gilkes NR, Gregg D,Mabee W, Pye K, Xiao Z, Zhang X, Saddler JN. 2005. Biorefining of softwood using ethanol organosolv pulping: preliminary evaluation of process streams for manufacture of fuel-grade ethanol and co-products. Biotechnol Bioeng. 90:473–481

Pan X. 2008. Role of functional groups in lignin inhibition of enzymatic hydrolysis of cellulose to glucose. J Biobased Materials Bioenergy. 2: 25-32

Pedersen M, Meyer AS. 2009. Influence of substrate particle size and wet oxidation on physical surface structures and enzymatic hydrolysis of wheat straw. Biotechnol Prog. 25:399-408

Perkins DN, Pappin DJC, Creasy DM, Cottrell JS. 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophor 20:3551–67

Peterson R, Nevalainen H. 2012. *Trichoderma reesei* RUT C30 – thirty years of strain impovement. Microbiology–SGM. 158:58-68

Petruccioli M, Federici F. 1988. Extracellular Enzyme Production in Species of the Genus Penicillium. Mycologia. 80(5): 726-728.

Polizeli MLTM, Rizzatti ACS, Monti R, Terenzi HF, Jorge JA, Amorim DS. 2005. Xylanases from fungi: Properties and industrial applications. Appl Microbiol Biotechnol. 67: 577-591

Poutanen K, Ratto M, Puls J, Viikari L. 1987. Evaluation of different microbial xylanolytic systems. J Biotechnol. 6:49-60

Pouvreau L, Joosten R, Hinz SWA, Gruppen H, Schols HA. 2011. *Chrysosporium lucknowense* C1 arabinofuranosidases are selective in releasing arabinose from either single or double substituted xylose residues in arabinoxylans. Enzyme Microb Technol. 48(4-5):397-403

Ragauskas AJ, Williams CK, Davison BH, Britovsek G, Cairney J, Eckert CA, Frederick Jr WJ, Hallett JP, Leak DJ, Liotta CL, Mielenz JR, Murphy R, Templer R, Tschaplinski T 2006. The path forward for biofuels and biomaterials. Science 311 (5760):484-489

Rahikainen J, Mikander S, Marjamaa K, Tamminen T, Lappas A, Viikari L, Kruus K. 2011. Inhibition of enzymatic hydrolysis by residual lignins from softwood – study of enzyme binding and inactivation on lignin-rich surface. Biotechnol Bioeng. 108: 2823-2834

Rahkamo L, Siika-aho M, Vehviläinen M, Dolk M, Viikari L, Nousiainen P. 1996. Modification of hardwood dissolving pulp with purified Trichoderma reesei cellulases. Cellulose 3:153–63

Reese ET. 1976. History of the cellulase program at the U. S. Army Natick Development Center. Biotechnol Bioeng Symp. 6:9–20

Saghai-Maroof MA, Soliman KM, Jorgesen RA, Allard RW. 1984. Ribosomal DNA spacer-length polymorphisms in barly mendelian inheritance, chromosomal location and population dynamics. Proceedings of the National Academy of Science 81:8014–8

Saha CB. 2003. Hemicellulose bioconversion. J Ind Microbiol Biotechnol 30: 279-291

Samuels GJ, Ismaiel A, Mulaw TB, Szakacs G, Druzhinina IS, Kubicek CP, Jaklitsch WM 2012. The *Longibrachiatum* clade of *Trichoderma*: a revision with new species. Fungal Diversity. 55:77-108

Scheller HV, Ulvskov P. 2010. Hemicelluloses. Annu Rev Plant Biol. 61:263-289

Schuster A, Bruno KS, Collett JR, Baker SE, Seiboth B, Kubicek CP, Schmoll M. 2012. A versatile toolkit for high throughput functional genomics with *Trichoderma reesei*. Biotechnol Biofuels. 5:1

Seaby D. 1998. *Trichoderma* as a weed mould or pathogen in mushroom cultivation. *Trichoderma* and *Gliocladium* Vol. 2. Edts: Harman GE and Kubicek CP (eds) Taylor&Francis Ltd. Salisbury, UK. 267-285

Sewalt VJH, Ni W, Blount JW, Jung HC, Masoud SA, Howle PA. 1997. Reduced Lignin content and altered lignin composition in transgenic tobacco down-regulated in expression of L-phenylalanine ammonia-lyase or cinnamate 4-hydroxylase. Plant Physiol. 115: 41-50

Shrivastava B, Thakur S, Khasa YP, Gupte A, Puniya AK, Kuhad RC. 2011. White-rot fungal conversion of wheat straw to energy rich cattle feed. Biodegradation. 22(4): 823–831.

Sims REH, Mabee W, Saddler JN, Taylor M. 2010. An overview of second generation biofuel techologies. Bioresource Techno. 101:1570-158

Sizova MV, Izquierdo JA, Panikov NS, Lynd LR. 2011. Cellulose- and xylan-degrading thermophilic anaerobic bacteria from biocompost. Appl Environ Microbiol. 77(7): 2282-91

Sorensen HR, Jorgensen CT, Hansen CH, Jorgensen CI, Pedersen S, Meyer AS. 2006. A novel GH43 α-L-arabinofuranosidase from *Humicola insolens*: Mode of action and synergy with GH51 α-L-arabinofuranosidases on wheat arabinoxylan. Appl Microbiol Biotechnol. 73: 850–861

Sukumaran RK, Singhania RR,. Pandey A. 2005. Microbial cellulases-production, applications and challenges, J Sci Ind Res. 64(11): 832– 844

Sun JL, Sakka K, Karita S, Kimura T, Ohmiya K. 1998. Adsorption of *Clostridium stercorarium* xylanase A to insoluble xylan and the importance of the CBDs to xylan hydrolysis. J Ferment Bioeng. 85: 63–68

Suurnäkki A, Tenkanen M, Siika-Aho M, Niku-Paavola M-, Viikari L, Buchert J. 2000. *Trichoderma reesei* cellulases and their core domains in the hydrolysis and modification of chemical pulp. Cellulose 7:189–209

Tejirian A, Xu F. 2011. Inhibition of enzymatic cellulolysis by phenolic compounds. Enzyme Microb Technol. 48: 239

Thygesen A, Thomsen AB, Schmidt AS, Jorgensen H, Ahring BK, Olsson L. 2003. Production of cellulose and hemicellulose-degrading enzymes by filamentous fungi cultivated on wet-oxidised wheat straw. Enzyme Microb Technol. 32: 606-615

Twomey LN, Pluske JR, Rowe JB, Choct M, Brown W, McConnell MF, Pethick W. 2003. The effects of increasing levels of soluble non-starch polysaccharides and inclusion of feed enzymes in dog diets on faecal quality and digestibility. Anim Feed Sci Technol. 108(1–4): 71-82

Ustinov BB, Gusakov AV, Antonov AI, Sinitsyn AP. 2008. Comparison of properties and mode of action of six secreted xylanases from *Chrysosporium lucknowense*. Enzyme Microb Technol. 43:56-65

Van Eylen D, van Dongen F, Kabel M, de Bont J. 2011. Corn fiber, cobs and stover: Enzyme-aided saccharification and co-fermentation after dilute acid pretreatment. Biores Technol. 102: 5995-6004

Várnai A, Siika-aho M, Viikari L. 2010. Restriction of the enzymatic hydrolysis of steam-pretreated spruce by lignin and hemicellulose. Enz and Microb Technol. 46:185–93

Viikari L, Kantelinen A, Sundquistn J, Linko M. 1994. Xylanases in bleaching – from an idea to the industry. FEMS Microbiol Reviews. 13:335-350

Viikari L, Alapuranen M, Puranen T, Vehmaanperä J, Siika-aho M. 2007 Thermostable enzymes in lignocellulose hydrolysis. Adv Biochem Eng/Biotechnol 108:121–45

Wang K, Sun R-C. 2010. Chapter 7.5 - Biorefinery Straw for Bioethanol Cereal Straw as a Resource for Sustainable Biomaterials and Biofuels. Elsevier, Amsterdam. 267-287

Watanabe H, Tokuda G. 2001. Animal cellulases. Cellular Mol Life Sci 58(9):1167-1178

Wessel D, Flügge UI. 1984. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal Biochem. 138:141-143

Wilson DB, Irwin DC. 1999. Genetics and Properties of Cellulases. Adv Biochem Eng/Biotechnol. 65: 1-21

Wyman CE, Dale BE, Elander RT, Holtzapple M, Ladisch MR, Lee YY. 2005. Comparative sugar recovery data from laboratory scale application of leading pretreatment technologies to corn stover. Biores Technol. 96: 2026–2032

Xu Q, Singh A, Himmel ME. 2009. Perspectives and new directions for the production of bioethanol using consolidated bioprocessing of lignocellulose. Curr Opin Biotechnol 20: 364-371

Yang B, Wyman CE. 2006. BSA treatment to enhance enzymatic hydrolysis of cellulose in lignin containing substrates. Biotechnol Bioeng. 94: 611-617

Zhang PYH, Himmel ME, Mielenz JR. 2006. Outlook for cellulase improvement: screening and selection strategies. Biotechnol Adv. 24: 452–481

6. Figures and Tables

7. Appendix

Table A1. Hydrolysis products in WAX and EXH digests of fermentation liquids based on HPAEC, HPLC and MALDI-TOF MS

		Hydrolysis products formed of WAX (w/w %)				Hydrolysis products formed of EXH (w/w %)					
Sample number	Group[a]	Arabinose [b]	Xylose [b]	Xylooligomers[c] (DP2-4)	Total WAX[d]	4-O-Methyl Glucuronic Acid[b]	Acetyl[e]	Xylose [a]	Xylooligomers[c] (DP2-4)	Galactose [a]	Total EXH [d]
**		0	4	9	8	39	n.a.	2	10	-	15
B1	4	35	27	10	36	12	48	9	2	-	11
B2	2	36	18	19	36	18	55	4	2	-	8
B3	1	25	3	24	26	22	64	3	9	-	14
B4	1	12	1	12	12	15	54	1	9	+	11
B5	1	17	5	13	18	12	71	3	12	-	14
B6	1	14	4	14	16	8	56	2	13	-	14
B7	3	74	17	42	63	6	68	12	0	-	11
B8	3	51	17	23	43	5	58	13	0	-	12
B9	1	20	6	14	19	5	53	4	8	-	11
B10	1	27	8	19	27	3	57	3	8	-	10
B11	2	47	12	28	42	25	62	7	5	-	14
B12	2	36	10	25	34	47	63	5	7	-	17
B13	2	48	13	28	43	24	65	5	9	-	15
B14	2	43	12	26	40	47	66	5	6	-	16
B15	2	33	11	23	34	9	58	5	6	-	11
B16	2	32	9	24	32	10	58	4	7	-	11
B17	1	21	6	16	22	27	47	5	6	-	13
B18	1	28	7	22	28	21	42	4	6	-	11

		Hydrolysis products formed of WAX (w/w %)				Hydrolysis products formed of EXH (w/w %)					
Sample number	**Group[a]**	**Arabinose [b]**	**Xylose [b]**	**Xylooligomers[c] (DP2-4)**	**Total WAX[d]**	**4-O-Methyl Glucuronic Acid[b]**	**Acetyl[e]**	**Xylose [a]**	**Xylooligomers[c] (DP2-4)**	**Galactose [a]**	**Total EXH [d]**
B19	2	43	17	22	40	9	68	10	1	-	11
B20	2	31	9	22	31	7	64	6	7	-	10
B21	2	33	13	21	33	6	53	6	5	-	11
B22	2	34	13	22	34	8	66	5	5	-	10
B23	1	29	11	20	30	8	41	4	8	-	11
B24	1	40	8	24	34	25	45	6	6	-	13
B25	1	22	8	12	21	8	24	6	5	-	10
B26	1	35	10	25	34	8	51	4	7	-	11
B27	1	38	7	28	35	17	80	3	10	-	14
B28	1	14	4	13	16	13	55	4	8	-	12
B29	1	13	4	8	12	14	19	3	6	+	10
B30	4	37	34	4	36	3	68	11	0	-	10
B31	1	51	12	28	44	13	64	7	6	-	13
B32	1	33	5	23	29	12	58	4	9	-	13
B33	1	22	6	15	21	12	56	4	9	-	13
B34	1	42	6	32	39	47	54	2	8	-	15
B35	1	30	3	26	29	19	58	1	13	-	14
B36	1	8	1	7	8	12	34	1	7	+	9
B37	1	21	7	17	23	13	55	3	7	-	10
B38	3	87	23	44	73	7	60	11	2	-	11
B39	2	63	23	28	54	20	63	11	3	-	14
B40	2 or 4	54	25	22	49	19	79	10	1	-	13

		Hydrolysis products formed of WAX (w/w %)				Hydrolysis products formed of EXH (w/w %)					
Sample number	Group[a]	Arabinose [b]	Xylose [b]	Xylooligomers[c] (DP2-4)	Total WAX[d]	4-O-Methyl Glucuronic Acid[b]	Acetyl[e]	Xylose [a]	Xylooligomers[c] (DP2-4)	Galactose [a]	Total EXH [d]
B41	4	32	31	2	32	17	84	12	0	-	12
B42	4	33	35	1	34	23	75	11	0	-	13
B43	1	4	4	8	9	22	47	3	7	+	12
B44	1	36	3	31	32	31	69	2	8	-	13
B45	3	70	16	52	64	31	57	10	3	-	15
B46	2	26	8	24	28	20	56	6	8	-	14
B47	2	27	10	24	29	4	39	6	6	-	11
B48	2	29	2	30	29	3	59	3	10	-	11
B49	3	72	19	49	64	27	60	10	3	-	15
B50	2	34	6	33	34	23	40	5	8	-	14
B51	1	22	8	19	23	38	56	6	5	-	15
B52	3	68	8	55	60	3	58	3	8	-	10
B53	1	25	2	25	25	3	55	3	10	+	11
B54	1	22	4	23	24	46	76	3	8	-	16
B55	1	12	8	9	14	14	55	5	7	-	12
B56	2	30	13	25	33	23	47	5	7	-	13
B57	2	46	9	33	40	13	62	3	8	-	11
B58	1	27	3	22	24	13	71	3	11	-	14
B59	1	25	5	23	25	7	66	6	9	-	14
B60	1	24	7	22	25	38	60	4	8	-	16
B61	1	28	6	27	29	8	66	5	8	-	13
B62	1	26	1	21	22	66	41	1	1	+	11

		Hydrolysis products formed of WAX (w/w %)				Hydrolysis products formed of EXH (w/w %)					
Sample number	Group[a]	Arabinose [b]	Xylose [b]	Xylooligomers[c] (DP2-4)	Total WAX[d]	4-O-Methyl Glucuronic Acid[b]	Acetyl[e]	Xylose [a]	Xylooligomers[c] (DP2-4)	Galactose [a]	Total EXH [d]
B63	x	47	9	32	40	29	66	3	8	-	14
B64	1	24	5	24	25	18	70	3	9	-	13
B65	1	40	4	35	36	6	51	3	6	-	9
B66	3	69	19	46	62	4	56	5	8	-	12
B67	1	25	8	23	27	4	44	4	9	-	12
B68	1	20	4	20	21	16	42	2	11	+	13
B69	2	27	11	22	29	22	62	5	8	-	14
B70	1	31	5	25	28	18	61	2	10	-	13
B71	1	10	6	14	15	29	64	3	9	-	14
B72	1	20	5	20	21	28	79	3	7	-	12
B73	2	54	7	44	48	15	62	4	9	-	13
B74	3	77	22	51	69	7	56	4	7	-	11
B75	1	48	10	32	41	21	72	4	8	-	13
B76	1	16	2	15	15	13	72	3	11	-	14
B77	1	22	6	22	24	25	61	6	4	-	12
B78	1	13	4	10	13	20	18	6	4	+	11

[a] Based on degradation pattern on HPAEC after hydrolysis of WAX
[b] Amount as percentage of total amount of that component present in initial substrate (w/w %)
[c] Amount of xylooligomers as percentage of xylose present in initial substrate (w/w %)
[d] Monomers and linear xylooligomer products as percentage of total amount of these components present in initial substrate (w/w %)
[e] Based on combined HPLC & Maldi-TOF MS results, expressed as percentage of total amount of that component present in the initial substrate
[f] Based on Maldi-TOF MS results, qualitative result
**Endoxylanase-I-(WAX)/AGU,endoxylanase-I (AXE)EXH

Table A2: Sample information and quantitative results of all fermentation supernatants after hydrolysis of insoluble WS WUS and CF AIS, based on HPAEC results.

	Fungi		**Hydrolysis products formed of WS WUS (w/w %)**							**Hydrolysis products formed of CF AIS (w/w %)**						
Sample number	**Strain**	**Taxonomical posistion**	**Arabinose [a]**	**Galactose [a]**	**Glucose [a]**	**Xylose [a]**	**Glucuronic acid (+/- 4OMethyl) [a]**	**Xylooligomers (DP2-4) [b]**	**Total WS WUS [d]**	**Arabinose [a]**	**Galactose [a]**	**Glucose [a]**	**Xylose [a]**	**Glucuronic acid (+/- 4OMethyl) [a]**	**Other oligomers [c]**	**Total CF AIS [d]**
Blank			0	0	0	0	0	0	0	0	0	2	0	0	0	0
1	TUB F-1702	*Trichoderma sp.*	35	12	15	20	3	1	11	14	5	16	6	1	10	18
2	TUB F-1702	*Trichoderma sp.*	27	7	14	15	1	1	9	9	2	11	3	0	8	13
3	TUB F-2292	Non-identified fungus	6	4	3	1	0	1	2	5	2	4	1	0	12	14
4	TUB F-2292	Non-identified fungus	6	2	3	1	0	1	2	3	1	3	0	0	7	8
5	TUB F-2293	*Coniochaeta ligniaria*	6	3	4	3	1	3	3	4	2	5	1	3	12	14
6	TUB F-2293	*Coniochaeta ligniaria*	8	7	9	2	1	3	5	5	2	8	1	3	10	13
7	TUB F-2346	*Penicillium sp.*	38	47	19	23	1	3	14	14	12	14	8	1	8	15
8	TUB F-2346	*Penicillium sp.*	26	37	14	16	1	1	10	11	12	11	5	1	7	13
9	QM6a	*Trichoderma i*	22	10	11	8	2	3	7	8	2	11	2	1	8	13
10	QM6a	*Trichoderma i*	20	5	15	8	1	6	9	8	2	12	1	1	8	12
11	TUB F-2342	Non-identified fungus	21	15	10	10	3	8	8	26	11	20	8	6	15	26
12	TUB F-2342	Non-identified fungus	32	17	17	13	3	11	13	24	10	18	6	3	16	26
13	TUB F-2353	Non-identified fungus	41	22	17	15	5	16	14	24	10	17	6	9	19	29
14	TUB F-2353	Non-identified fungus	24	15	14	11	2	8	10	29	10	19	7	7	20	31
15	TUB F-2358	*Trichoderma harzianum*	21	7	8	7	1	7	7	15	4	19	4	0	13	20
16	TUB F-2358	*Trichoderma harzianum*	21	4	13	7	0	4	8	10	1	15	2	0	8	13
17	TUB F-2360	*Trichoderma sp.*	16	7	6	7	1	1	4	6	1	9	3	0	8	11
18	TUB F-2360	*Trichoderma sp.*	17	4	7	7	1	2	5	8	2	13	2	0	7	11
19	RUT C30	*Trichoderma i*	38	10	19	21	5	1	13	29	9	20	11	6	13	26
20	RUT C30	*Trichoderma i*	34	5	19	11	2	8	12	15	1	14	3	1	15	21

21	TUB F-1598	*Trichoderma harzianum*	26	12	17	15	1	1	11	12	4	17	4	0	8	14
22	TUB F-2294	*Trichoderma sp.*	30	10	18	13	1	2	11	9	2	15	3	0	7	12
23	TUB F-2295	*Trichoderma sp.*	20	5	10	5	1	2	6	7	2	12	1	0	5	9
24	TUB F-2335	*Trichoderma sp.*	20	6	7	9	1	2	6	20	3	13	6	2	4	12
25	TUB F-2343	*Trichoderma sp.*	18	5	7	7	1	1	5	7	1	9	2	1	10	13
26	TUB F-2344	*Trichoderma sp.*	27	6	18	10	0	5	11	10	2	17	2	0	8	14
27	TUB F-2348	*Bionectria ochroleuca*	23	10	9	6	2	5	7	18	8	13	3	4	19	26
28	TUB F-2349	Non-identified fungus	6	3	3	1	0	0	2	7	2	4	1	0	5	7
29	TUB F-2350	*Trichoderma sp.*	11	8	3	4	0	0	2	4	1	4	1	0	3	5
30	TUB F-2352	Non-identified fungus	14	10	4	5	1	0	3	9	0	12	3	0	4	8
31	TUB F-2356	Non-identified fungus	23	18	10	11	2	1	7	25	8	18	10	7	14	25
32	TUB F-2357	Non-identified fungus	14	8	2	2	1	2	2	16	4	9	2	2	15	21
33	QM6a	*Trichoderma i*	27	9	11	7	0	4	7	12	2	16	2	0	10	15
34	TUB F-2385	*Corynascus verrucosus*	17	14	2	5	1	3	3	10	5	4	1	1	8	11
35	TUB F-2387	Non-identified fungus	17	6	5	3	1	3	4	12	3	11	1	1	7	12
36	TUB F-2388	Non-identified fungus	6	3	1	1	0	0	1	8	2	5	1	0	5	7
37	TUB F-2389	Non-identified fungus	7	4	3	2	0	0	2	6	2	4	1	0	4	6
38	TUB F-2390	*Penicillium sp.*	37	31	21	22	1	1	14	22	13	21	10	1	8	19
39	NRRL1808	*Aspergillus oryzae*	18	17	10	10	2	1	7	11	5	14	6	3	5	11
40	NRRL3485	*Aspergillus oryzae*	19	9	7	13	2	1	6	10	4	11	6	5	4	10
41	ATCC10864	*Aspergillus niger*	18	8	9	9	1	0	6	9	5	14	5	4	5	11
42	ATCC14916	*Aspergillus foetidus*	16	13	8	7	1	0	5	9	4	15	5	2	5	11
43	TUB F-2386	Non-identified fungus	5	6	4	2	0	0	2	1	1	3	0	0	3	4
44	TUB F-2382	Non-identified fungus	22	4	11	4	0	4	7	23	3	12	1	1	21	28
45	TUB F-1647	*Trichoderma sp.*	33	21	20	14	1	7	13	26	10	24	8	0	14	26
46	TUB F-2369	*Trichoderma sp.*	27	8	15	9	1	6	10	13	2	17	3	0	9	16
47	TUB F-2370	Non-identified fungus	30	10	8	12	1	2	7	9	5	11	3	1	7	11
48	TUB F-2371	Non-identified fungus	9	3	3	1	0	1	2	13	5	5	1	0	8	12
49	TUB F-2372	*Penicillium ochrochloron*	31	22	19	15	2	2	12	28	11	22	11	1	14	26

50	TUB F-2374	Non-identified fungus	21	11	11	5	1	2	6	15	6	15	4	0	12	19
51	QM 6a	*Trichoderma i*	26	11	13	8	1	4	8	13	2	18	3	1	11	17
52	OKI 16/5	*Aspergillus terreus*	32	4	17	10	0	6	11	26	2	19	4	0	15	24
53	TUB F-2394	*Humicola grisea/Humicola nigrescens*	8	7	9	1	1	1	4	15	2	9	1	0	17	22
54	TUB F-2361	*Bionectria ochroleuca*	16	5	7	3	1	3	5	13	3	10	1	0	16	21
55	TUB F-2375	*Trichoderma sp.*	14	4	8	6	1	7	6	10	2	13	2	0	15	20
56	TUB F-2376	*Trichoderma sp.*	27	11	15	9	1	6	10	15	3	20	3	0	12	20
57	TUB F-2377	Non-identified fungus	38	19	18	12	3	14	13	20	5	16	3	0	13	20
58	QM 7721	*Paecilomyces terricola, Synonym: Acremonium implicatum ; Fusidium terricola*	19	9	9	4	1	4	6	15	3	8	1	1	15	20
59	ATCC 11282	*Cladosporium herbarum*	8	5	2	3	1	1	2	11	4	6	2	0	10	13
60	RUT C30	*Trichoderma i*	22	3	14	6	1	5	9	11	1	10	1	0	15	20
61	BSUM	*Epicoccum nigrum*	20	9	6	3	1	1	4	15	4	8	3	0	9	14
62	OKI 270	*Chaetomium globosum*	8	5	4	0	1	0	2	9	1	3	0	0	4	6
63	TUB F-2291	Non-identified fungus	29	14	17	10	3	11	12	19	5	17	3	5	16	23
64	TUB F-2303	Non-identified fungus	27	7	12	6	1	5	8	17	5	11	1	0	16	21
65	TUB F-2305	Non-identified fungus	22	6	13	6	1	4	8	12	1	11	1	0	13	18
66	TUB F-2378	Non-identified fungus	30	17	18	7	1	6	11	28	8	23	6	2	15	26
67	TUB F-2379	*Trichoderma sp.*	18	6	10	4	1	4	6	11	1	16	1	0	8	14
68	IFO 9387	*Paecilomyces bacillisporus, Synonym: Acremonium bacillisporum*	14	7	8	2	1	3	5	14	4	9	1	0	13	18
69	RUT C30	*Trichoderma i*	32	9	16	9	1	9	11	16	3	20	2	1	13	21
70	NCAIM F-00740	*Phanerochaete chrysosporium*	19	6	12	6	1	7	8	17	2	8	1	1	20	25
71	VKM F-1767	*Phanerochaete chrysosporium*	14	7	15	6	1	6	9	4	3	8	1	1	11	14

72	NRRL 2003	*Myrothecium verrucaria*	13	6	9	4	1	3	5	15	3	10	2	2	14	20
73	TUB F-2341	Non-identified fungus	32	20	16	8	2	3	10	32	8	23	5	2	23	34
74	TUB F-2380	*Hypocrea lixii (Trichoderma harzianum)*	29	13	11	9	1	12	10	35	6	23	8	0	21	34
75	TUB F-2381	Non-identified fungus	33	16	18	11	3	11	13	25	5	21	4	1	17	27
76	TUB F-2383	Non-identified fungus	14	3	4	2	0	4	3	14	1	6	1	0	10	13
77	TUB F-2384	Non-identified fungus	4	2	2	2	0	0	1	6	2	6	2	1	3	6
78	IFO 4855	*Paecilomyces varioti*	15	6	3	4	1	0	3	13	3	7	3	1	5	9

[a] Amount as percentage of total amount of that component(s) present in initial substrate (w/w%)
[b] Amount of xylooligomers as percentage of xylose present in initial substrate (w/w%)
[c] Unidentified oligomers as percentage of total amount of sugars in initial substrate
[d] Monomers and linear xylooligomer products as percentage of total amount of these components present in initial substrate (w/w%)

Origin of strains: ATCC: American Type Culture Collection, Manassas, Virginia, USA; BSUM: Botany School, University of Melbourne, Parkville, Australia; IFO: Institute for Fermentation, Osaka, Japan; NRRL: Northern Regional Research Center, USDA, Peoria, Illinois, USA; OKI: National Institute for Public Health, Budapest, Hungary; QM: Quartermaster Research and Development Center, U.S. Army, Natick, Massachusetts; RUT: Rutgers University, New Brunswick, New Jersey, USA; TUB F-…: Technical University of Budapest, culture collection of fungi; VKM: Russian Collection of Microorganisms, Moscow, Russia

Table A3. Volumetric cellulase and hemicellulase enzyme activities and total soluble protein concentrations of Trichoderma section Longibrachiatum strains after 5 days of fermentation grown in shake flasks on wheat straw, eucalyptus wood and corn fiber as carbon sources

Strain	Wheat straw as carbon source in fermentation						Eucalyptus wood as carbon source in fermentation						Corn fiber as carbon source in fermentation					
	FPA (FPU/ml)	Xyl activity (IU/ml)	β-glu activity (IU/ml)	β-xyl activity (IU/ml)	α-ara activity (IU/ml)	Protein conc (mg/ml)	FPA (FPU/ml)	Xyl activity (IU/ml)	β-glu activity (IU/ml)	β-xyl activity (IU/ml)	α-ara activity (IU/ml)	Protein conc (mg/ml)	FPA (FPU/ml)	Xyl activity (IU/ml)	β-glu activity (IU/ml)	β-xyl activity (IU/ml)	α-ara activity (IU/ml)	Protein conc (mg/ml)
Rut C30	**0.54**	**6.06**	0.42	**0.14**	**0.38**	**1.21**	**0.22**	**2.68**	0.09	0.04	**0.06**	**0.19**	**0.99**	**5.48**	**0.45**	**0.64**	**1.37**	**1.44**
QM6a	**0.42**	5.68	**0.48**	**0.14**	**0.31**	**0.67**	0.02	1.31	0.04	0.02	**0.05**	0.08	**0.34**	**3.38**	0.24	**0.25**	**0.53**	0.52
CPK 155	**0.44**	**23.93**	**0.83**	0.13	**0.26**	**0.69**	0.02	2.43	**0.11**	0.02	0.03	0.09	0.27	**9.09**	0.18	0.08	0.24	**0.62**
TUB F-2535	0.21	5.65	0.17	0.05	0.12	0.36	**0.05**	**2.74**	0.05	0.02	**0.05**	0.03	0.18	2.25	0.08	0.06	0.18	0.36
TUB F-2543	0.36	5.88	0.20	**0.11**	0.14	0.45	**0.09**	**3.48**	0.05	0.03	0.04	**0.11**	**0.30**	3.37	0.09	0.11	**0.52**	0.38
ATCC 18903	0.00	0.88	0.00	0.02	0.02	0.18	0.00	0.32	0.00	0.01	0.01	0.01	0.15	1.34	0.02	**0.21**	0.12	0.25
TUB F-1345	0.16	4.05	0.35	0.07	0.03	0.33	0.01	0.69	**0.16**	0.03	0.01	0.06	0.08	0.77	0.22	0.05	0.03	0.44
TUB F-2531	0.11	**7.46**	0.11	0.08	0.03	0.33	0.00	0.91	0.03	0.03	0.01	**0.15**	0.18	3.01	0.13	0.09	0.05	**0.69**
TUB F-1299	0.07	3.10	0.39	0.05	0.01	0.28	0.00	1.31	**0.18**	0.02	0.02	0.06	0.09	1.05	**0.25**	0.14	0.11	0.46
TUB F-1417	0.01	1.36	0.08	0.07	0.04	0.28	0.00	1.32	0.04	0.03	0.04	0.04	0.01	0.34	0.05	0.05	0.04	0.29
TUB F-1047	0.03	2.06	0.13	0.04	0.02	0.30	0.00	0.68	0.04	0.01	0.01	0.03	0.09	0.95	0.18	0.06	0.03	0.46
TUB F-1058	0.03	2.49	0.13	0.04	0.02	0.31	0.00	0.53	0.03	0.01	0.01	0.05	0.09	2.49	0.23	0.05	0.04	0.57
CPK 72	0.00	0.33	0.00	0.01	0.02	0.18	0.00	0.20	0.00	0.00	0.01	0.08	0.05	0.29	0.00	0.09	0.09	0.23
CPK 132	0.09	1.21	0.08	0.02	0.01	0.40	0.00	0.42	0.00	0.00	0.01	0.04	0.04	0.91	0.17	0.12	0.06	0.50
TUB F-354	0.00	0.19	**0.59**	0.05	0.01	0.27	0.00	0.15	0.01	0.02	0.01	0.04	0.00	0.14	**1.40**	0.02	0.01	0.28

FPA: filter paper activity; Xyl: xylanase; β-glu: β-glucosidase; β-xyl: β-xylosidase; α-ara: α-L-arabinofuranosidase; Protein conc: total soluble protein concentration

All shake flask experiments were performed in duplicate and mean values are presented in the table. Standard deviation is less than ±5 %

Table A4 Released mono- and oligosaccharides (w/w%) from wheat arabinoxylan (WAX) with Trichoderma section Longibrachiatum supernatants after 24h incubation at pH 5.0, 37°C and 500 rpm as determined by HPAEC.

Strain	**Wheat straw as carbon source in fermentation**				**Eucalyptus wood as carbon source in fermentation**				**Corn fiber as carbon source in fermentation**			
	Ara[a]	**Xyl[b]**	**Oligomers (DP2-4)[c]**	**Total[d]**	**Ara[a]**	**Xyl[b]**	**Oligomers (DP2-4)[c]**	**Total[d]**	**Ara[a]**	**Xyl[b]**	**Oligomers (DP2-4)[c]**	**Total[d]**
Rut C30	**41±2.0**	**33±1.2**	**15±0.6**	**49**	**18±0.8**	**9±0.3**	0	**22**	**33±1.4**	**42±1.8**	**31±1.2**	**39**
QM6a	**38±1.4**	**19±0.9**	**16±0.7**	**46**	**16±0.5**	4±0.2	14±0.5	**18**	41±1.8	**37±1.7**	18±0.9	**47**
CPK 155	18±0.8	10±0.5	13±0.5	24	8±0.3	5±0.2	19±0.8	15	23±1.0	11±0.5	3±0.1	28
TUB F-2535	**30±1.3**	**15±0.6**	**19±1.0**	**36**	**22±1.0**	**10±0.3**	**30±1.2**	**27**	26±1.1	5±0.2	21±0.9	32
TUB F-2543	12±0.5	9±0.3	13±0.6	21	8±0.2	**8±0.2**	**27±1.0**	16	**30±1.1**	12±0.5	16±0.7	**35**
ATCC 18903	14±0.7	3±0.1	9±0.3	25	9±0.4	1±0.1	13±0.6	10	**32±1.4**	**30±1.2**	4±0.2	**39**
TUB F-1345	4±0.1	2±0.1	10±0.5	5	5±0.2	2±0.1	19±0.7	10	19±0.7	5±0.2	9±0.3	22
TUB F-2531	13±0.5	11±0.5	1±0.1	17	2±0.1	3±0.1	2±0.1	3	16±0.4	16±0.6	5±0.2	18
TUB F-1299	19±0.9	7±0.3	11±0.5	23	12±0.4	4±0.1	**21±0.8**	14	23±1.1	11±0.5	6±0.3	29
TUB F-1417	3±0.1	3±0.1	5±0.1	7	3±0.2	3±0.1	6±0.2	7	2±0.1	1±0.1	18±0.8	5
TUB F-1047	0	9±0.4	7±0.2	10	0	6±0.3	9±0.3	8	1±0.1	15±0.8	4±0.2	15
TUB F-1058	0	7±0.3	4±0.2	8	0	4±0.1	3±0.2	5	5±0.2	29±1.5	**29±1.4**	22
CPK 72	2±0.1	1±0.1	6±0.2	4	4±0.2	1±0.1	3±0.1	6	7±0.3	0	14±0.6	5
CPK 132	18±0.7	7±0.2	10±0.4	20	1±0.1	3±0.1	11±0.4	8	3±0.1	8±0.3	**25±1.2**	13
TUB F-354	0	2±0.1	1±0.1	5	0	0	0	0	0	0	7±0.2	0

[a] arabinose; [b] xylose; [c] xylooligosaccharides; [d] total amount of released mono- and oligosaccharides from the substrates (w/w%)

All hydrolysis experiments were performed in duplicate and mean values are presented. Standard deviation is less than ±5 %

Table A5 Qualitative evaluation of Maldi-TOF MS and HPLC results of eucalyptus xylan hydrolysate (EXH) digests after 24h incubation at pH 5.0, 37°C and 500 rpm.

Strain	**Wheat straw as carbon source in fermentation**				**Eucalyptus wood as carbon source in fermentation**				**Corn fiber as carbon source in fermentation**			
	Xyl	**Gal**	**AXE**	**UA**	**Xyl**	**Gal**	**AXE**	**UA**	**Xyl**	**Gal**	**AXE**	**UA**
Rut C30	+		+		+		+		+		+	
QM6a	+		+					+			+	+
CPK 155	+		+				+		+		+	
TUB F-2535	+	+		+					+			
TUB F-2543	+						+				+	
ATCC 18903					+	+		+	+			
TUB F-1345	+											
TUB F-2531	+					+						
TUB F-1299		+		+				+				
TUB F-1417		+								+		
TUB F-1047									+			
TUB F-1058	+											
CPK 72		+				+				+		
CPK 132						+						
TUB F-354		+				+		+				

Xyl: endo-xylanase activity; Gal: galactosidase activity; AXE: acetyl-xylan-esterase activity; UA: 4-*O*-methyl-glucuronidase activity.

Table A6 Released mono- and oligosaccharides (w/w%) from wheat straw water unextractable solids (WS WUS) and corn fiber alcohol insoluble solids (CF AIS) substrates during hydrolysis by Trichoderma section Longibrachiatum shake flask fermentation supernatants after 24h incubation at pH 5.0, 37°C and 500 rpm.

Strain	Released products from WS WUS (w/w%)																		Released products from CF AIS (w/w%)																	
	Wheat straw as carbon source in fermentation						Eucalyptus wood as carbon source in fermentation						Corn fiber as carbon source in fermentation						Wheat straw as carbon source in fermentation						Eucalyptus wood as carbon source in fermentation						Corn fiber as carbon source in fermentation					
	Ara[a]	Glu[b]	UA[c]	Xyl[d]	Oligomers[e]	Total[f]	Ara[a]	Glu[b]	UA[c]	Xyl[d]	Oligomers[e]	Total[f]	Ara[a]	Glu[b]	UA[c]	Xyl[d]	Oligomers[e]	Total[f]	Ara[a]	Glu[b]	UA[c]	Xyl[d]	Oligomers[e]	Total[f]	Ara[a]	Glu[b]	UA[c]	Xyl[d]	Oligomers[e]	Total[f]	Ara[a]	Glu[b]	UA[c]	Xyl[d]	Oligomers[e]	Total[f]
Rut C30	**17**	**13**	2	**7**	**3**	**12**	**8**	**6**	1	**3**	**2**	**6**	10	**7**	**2**	**4**	1	**6**	**9**	**16**	0	**3**	1	**9**	**6**	**10**	0	1	1	**6**	11	**17**	**2**	**3**	1	**10**
QM6a	9	6	1	**4**	2	6	2	1	0	0	1	1	**16**	**5**	**4**	**4**	1	**6**	**9**	**15**	0	**4**	1	**9**	4	4	0	0	1	3	**13**	**13**	**2**	**3**	**2**	**9**
CPK 155	**10**	**7**	1	**6**	**3**	**8**	**3**	**2**	0	1	1	2	7	4	0	1	**2**	4	6	**15**	0	0	1	**7**	**5**	6	0	1	1	**4**	8	**13**	0	2	**2**	8
TUB F-2535	**13**	**9**	0	3	**5**	**8**	**8**	**4**	0	**2**	**2**	**4**	**13**	**5**	1	1	**2**	4	**9**	12	0	2	1	**7**	**6**	**7**	0	1	1	**4**	7	5	0	1	1	4
TUB F-2543	7	6	1	**4**	2	6	4	**2**	1	**2**	**2**	**3**	**17**	**5**	**3**	**4**	1	**5**	6	11	0	2	1	6	4	**7**	0	1	1	**4**	**15**	8	**8**	**8**	**3**	**11**
ATCC 18903	5	2	1	1	1	2	1	1	0	0	0	1	12	2	**2**	2	0	3	8	2	1	1	2	4	2	1	0	0	0	1	**13**	4	**7**	**7**	**2**	8
TUB F-1345	4	3	1	2	0	3	1	1	0	0	0	1	3	2	1	1	1	2	4	10	0	2	0	5	2	2	0	0	0	1	4	9	0	2	1	5
TUB F-2531	5	3	1	2	1	3	1	1	0	1	1	1	4	3	1	1	1	2	5	10	0	2	1	6	1	2	0	0	1	1	5	11	0	2	1	6
TUB F-1299	4	3	0	1	2	3	2	1	0	1	1	1	3	2	1	1	0	2	5	10	0	1	1	6	3	4	0	1	0	2	5	10	0	1	0	5
TUB F-1417	3	2	0	1	1	2	2	1	0	1	1	1	2	2	0	0	0	1	2	2	0	0	1	1	2	2	0	0	1	1	2	2	0	0	0	1
TUB F-1047	3	2	0	1	1	2	1	1	0	1	0	1	3	3	1	1	0	2	0	3	0	0	1	1	0	1	0	0	0	1	1	9	0	1	1	4
TUB F-1058	3	2	0	1	1	2	1	1	0	1	0	1	5	4	1	1	**2**	4	0	3	0	0	0	1	0	0	0	0	0	0	1	10	0	1	1	4
CPK 72	2	1	0	0	0	1	1	1	0	0	0	0	4	1	1	0	0	1	2	1	0	0	0	1	1	1	0	0	0	1	3	1	0	0	0	1
CPK 132	5	2	1	1	1	2	1	0	0	0	0	1	2	2	1	1	1	2	6	2	1	2	1	3	0	1	0	0	0	1	1	4	0	0	0	2

Strain	Released products from WS WUS (w/w%)																		Released products from CF AIS (w/w%)																	
	Wheat straw as carbon source in fermentation						Eucalyptus wood as carbon source in fermentation						Corn fiber as carbon source in fermentation						Wheat straw as carbon source in fermentation						Eucalyptus wood as carbon source in fermentation						Corn fiber as carbon source in fermentation					
	Ara[a]	Glu[b]	UA[c]	Xyl[d]	Oligomers[e]	Total[f]	Ara[a]	Glu[b]	UA[c]	Xyl[d]	Oligomers[e]	Total[f]	Ara[a]	Glu[b]	UA[c]	Xyl[d]	Oligomers[e]	Total[f]	Ara[a]	Glu[b]	UA[c]	Xyl[d]	Oligomers[e]	Total[f]	Ara[a]	Glu[b]	UA[c]	Xyl[d]	Oligomers[e]	Total[f]	Ara[a]	Glu[b]	UA[c]	Xyl[d]	Oligomers[e]	Total[f]
TUB F-354	1	1	0	0	0	1	0	1	0	0	0	0	1	1	0	0	0	1	0	1	0	0	0	1	0	1	0	0	1	1	0	1	0	0	0	1

[a] arabinose; [b] glucose; [c] 4-O-methylglucuronic acid; [d] xylose; [e] xylooligosaccharides; [f] total amount of released mono- and oligosaccharides from the substrates (w/w%)

All hydrolysis experiments were performed in duplicate and mean values are presented. Standard deviation is less than ±5 %

Table 1 *Carbohydrate compositions of the pre-treated lignocellulosic substrates.*

Sample	Arabinose % dm	Galactose % dm	Glucose % dm	Xylose % dm	Mannose % dm
Hydrothermally pre-treated wheat straw (Biogold)	<0.5	<0.5	55	5	<0.5
Steam pre-treated spruce (E-Tech)	<0.5	<0.5	54	1.2	2.7

Table 2 *Carbohydrate (% DM) and nitrogen (% DM) content in the spruce and wheat straw EnzHR lignins.*

EnzHR lignin	Glucose % dm	Xylose % dm	Mannnose % dm	Total carbohydrates % dm	Nitrogen % dm
Spruce	7.5	<0.1	0.3	7.8	0.3
Wheat	4.5	0.3	<0.1	4.8	1.4

Table A7. Maximal enzyme activities for Trichoderma i QM6a (wild-type), Trichoderma i RutC30 (mutant), Penicillium pulvillorum TUB F-2220 and Penicillium cf. simplicissimum TUB F-2378 strains in shake flask fermentation supernatants on Avicel PH-101 (Fluka), pretreated spruce (E-Tech, Sweden) and pretreated wheat straw (Biogold, Estonia) as a carbon source. In parenthesis the day of fermentation when the maximal activity was reached. The presented values are mean of three replicates.

Enzyme activities	**Avicel PH 101 (medium LC-8)**				**Pretreated spruce (medium LC-12)**				**Pretreated wheat straw (medium LC-16)**			
	QM6a	**RutC30**	**F-2220**	**F-2378**	**QM6a**	**RutC30**	**F-2220**	**F-2378**	**QM6a**	**RutC30**	**F-2220**	**F-2378**
FPA (FPU/ml)	0.54±0.04 (6)	0.88±0.06 (6)	0.90±0.07 (5)	0.91±0.06 (6)	0.10±0.01 (7)	0.49±0.02 (7)	0.35±0.01 (6)	0.27±0.01 (5)	0.56±0.03 (7)	0.94±0.06 (6)	0.74±0.06 (6)	0.62±0.06 (5)
Xylanase (IU/ml)	2.53±0.12 (6)	4.86±0.36 (6)	1.09±0.10 (4)	2.69±0.21 (4)	0.36±0.03 (7)	0.81±0.07 (7)	0.36±0.03 (6)	0.49±0.04 (6)	2.69±0.24 (6)	2.49±0.23 (6)	0.76±0.07 (5)	1.72±0.11 (6)
β-glucosidase (IU/ml)	0.79±0.01 (6)	0.47±0.01 (6)	1.71±0.04 (4)	1.45±0.02 (6)	0.08±0.01 (5)	0.10±0.01 (5)	0.14±0.01 (6)	0.16±0.01 (7)	0.44±0.02 (6)	0.27±0.02 (7)	1.34±0.05 (6)	0.80±0.04 (6)
β-xylosidase (IU/ml)	0.50±0.02 (6)	1.11±0.03 (6)	0.12±0.01 (5)	0.19±0.01 (6)	0	0	0.02 (7)	0	0.03 (6)	0.18±0.01 (7)	0.32±0.01 (6)	0.15±0.01 (5)
Endoglucanase (IU/ml)	7.32±0.71 (6)	15.48±1.53 (6)	11.67±1.02 (5)	10.23±0.99 (6)	0.42±0.04 (5)	3.05±0.29 (7)	2.32±0.21 (5)	2.51±0.23 (6)	3.53±0.30 (6)	6.44±0.59 (6)	3.19±0.31 (6)	2.98±0.26 (6)
Soluble protein (mg/ml)	0.56±0.02 (6)	1.53±0.04 (6)	0.62±0.02 (5)	0.82±0.03 (6)	0.12±0.01 (5)	0.61±0.02 (7)	0.26±0.01 (6)	0.29±0.01 (6)	0.46±0.02 (6)	0.84±0.02 (6)	0.34±0.01 (6)	0.20±0.01 (6)

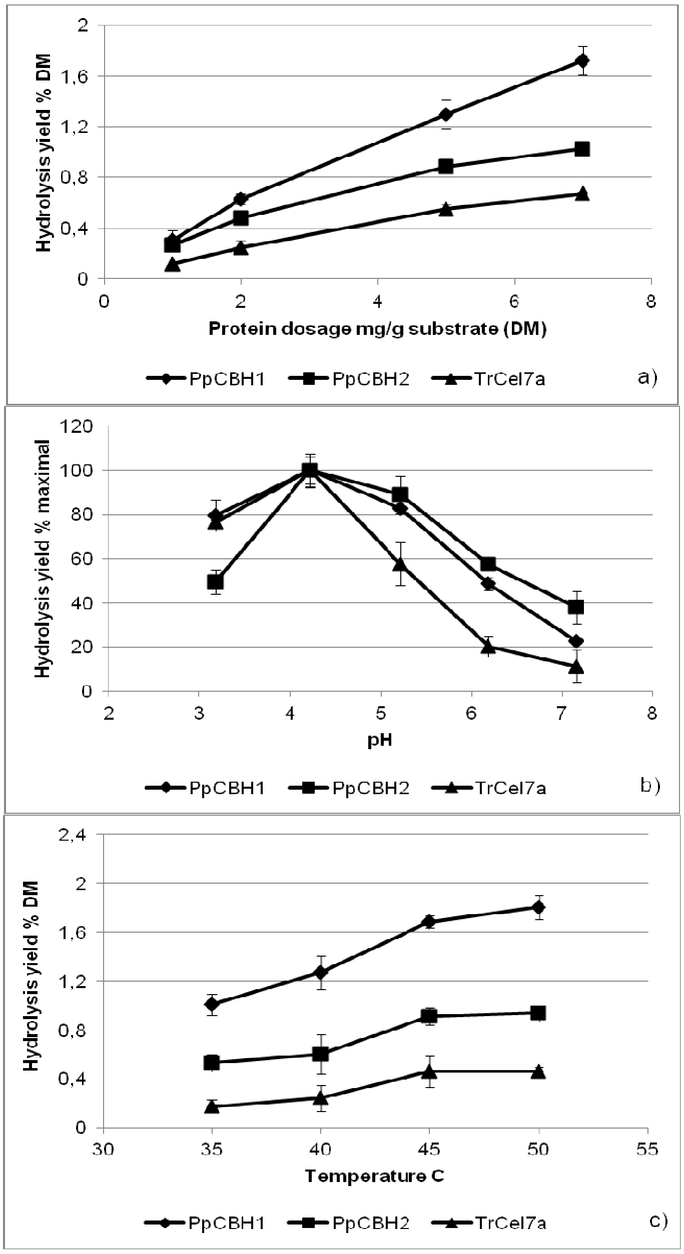


Figure 5. Avicelase activity in the CBH preparations. Protein load (a), pH (b) and temperature responses (c) of Avicelase activity in the Penicillium pulvillorum (TUB F-2220) PpCBH1 and PpCBH2 partially purified enzyme preparations and purified Trichoderma i reference TrCel7a.

Table 4 *Tryptic peptide sequences of the major protein in Penicillium pulvillorum (TUB F-2220) protein preparations PpCBH1 and PpCBH2.*

	Sequence	Homology	Accession number
PpCBH1	L/INFVTTASQK	CBH	CHB_ASPAC
	TAFGDL/IDDFTK	CBH	GUX1A_NEUCR
	GTGYCDSQCPR	CBH	CBHC_ASPTC
	TDDGTSTLSEL/IK/QR	CBH	GUX1_PENJA
	NFYGSGL/ITVDTK	CBH	CBHC_ASPNC
	SNTCGGTYSTDR	CBH	CBHA_ASPOR
PpCBH2	A Y L/I D S L/I R	Glycosyl hydrolase family 6 protein	XP_001269265
	VAGSAFAAR	Glucoamylase	AMYG_ASPKA
	VPSFVWL/IDTAAK	CBH	CBHC_ASPNC
	VPTMGTYL/IADL/IEAK	CBH	CBHC_NEOFI
	D/LD/LTWSAAL/IL/ITANMR	Glucoamylase	AMYG_ASPOR
	GDWCNVL/IGTGFGVR	CBH	CBHC_NEOFI
	VGTTTATQDDTWR	Glucoamylase	AMYG_ASPAW
	PSYTQGDS N	CBH	CBHC_NEOFI
	TCDDL/ITFQNMVSR	Glucoamylase	AMYG_ASPOR

Table 5 *Side-activities (U/mg protein) in the partially purified PpCBH1 and PpCBH2 from Penicillium pulvillorum (TUB F-2220) and Trichoderma i reference TrCel7a.*

	CMCase (U/mg prot)	**β-glucosidase (U/mg prot)**	**Xylanase (U/mg prot)**
PpCBH1	0.9	0.6	0.4
PpCBH2	2.2	0.4	0
TrCel7a	0.1	0.1	0

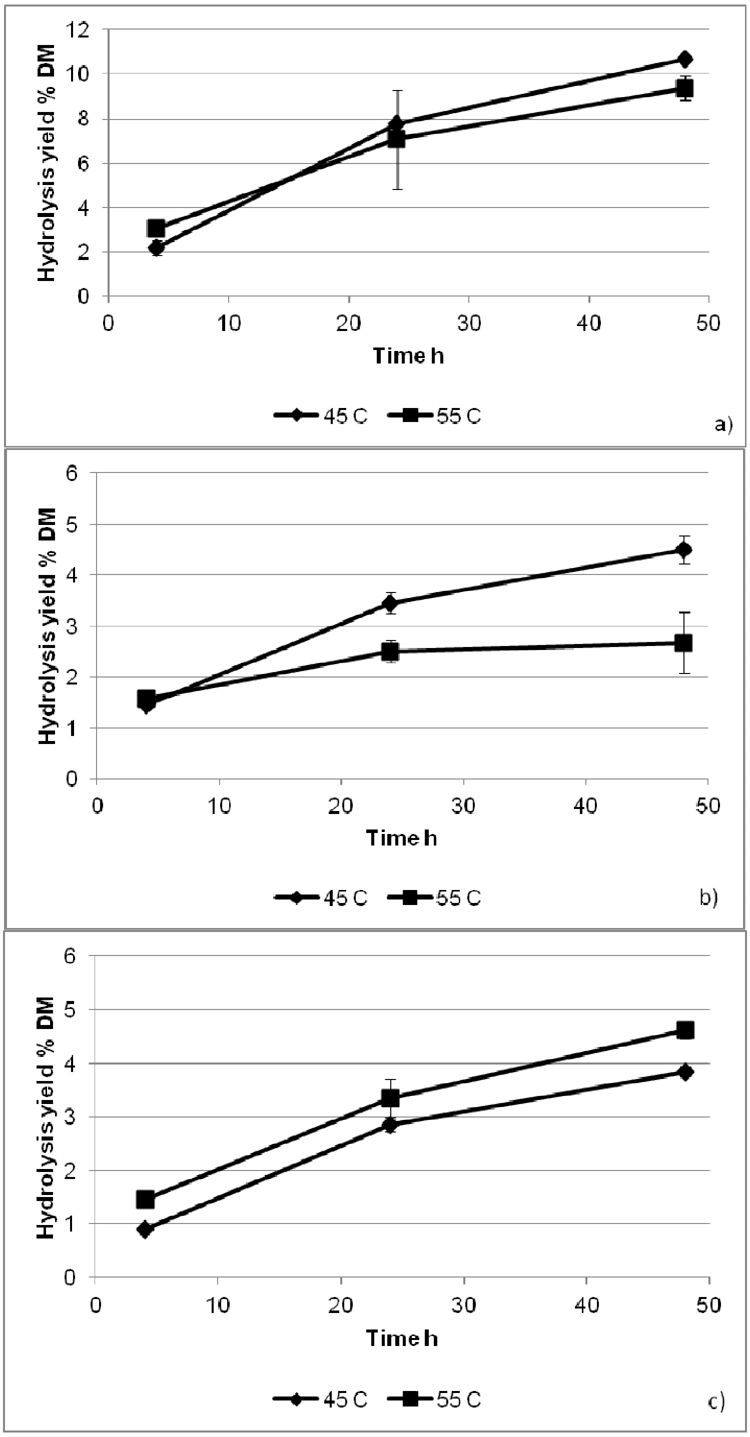


Figure 6. Hydrolytic performance of the CBH preparations at different temperatures. PpCBH1 (a), PpCBH2 (b) and TrCel7a (c) reference at 45 ◦C and 55 ◦C. Substrate: 10 mg/ml Avicel, pH 5, enzyme dosage 4 mg/g substrate supplemented with TaCel3a β-glucosidase 30 U/g substrate. Hydrolysis yields were quantified by reducing sugar assay (PAHBAH method) with a glucose standard.

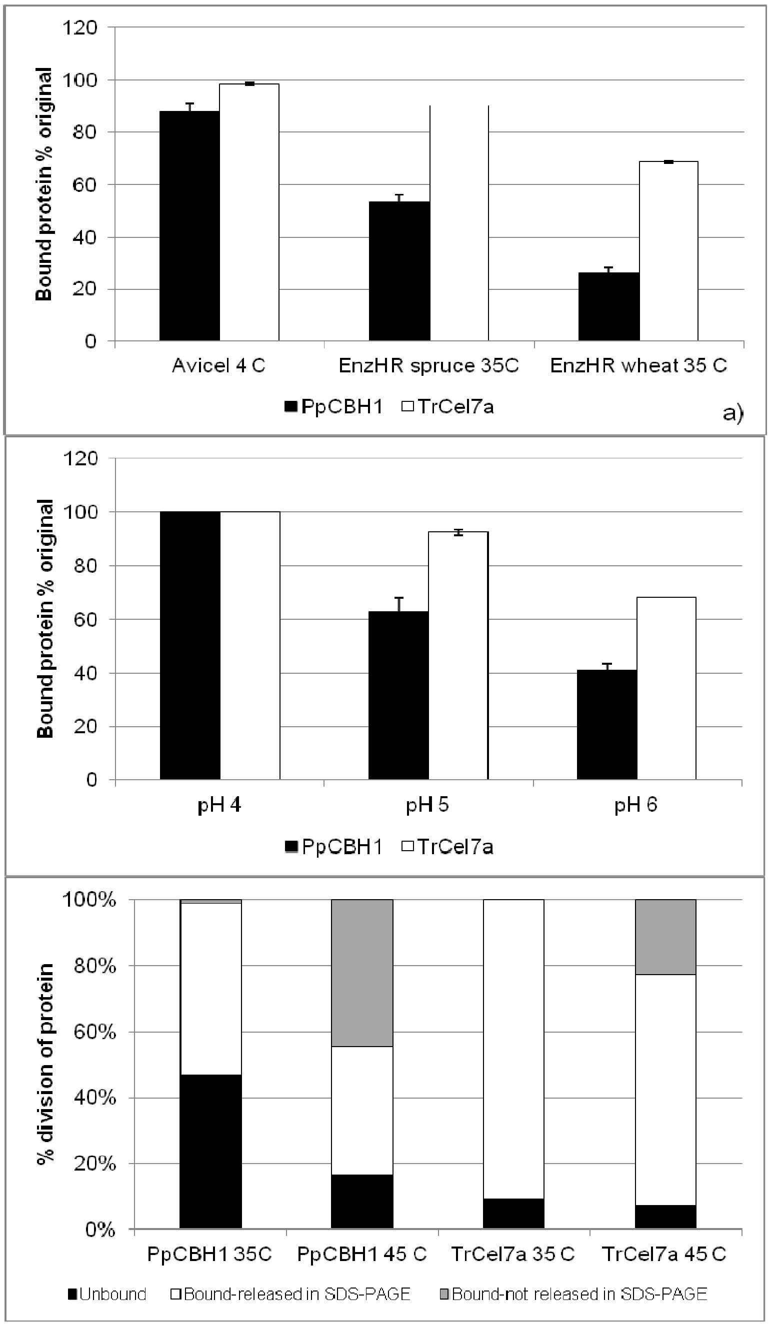


*Figure 8. Adsorption of the CBHs to cellulose and lignins. (a) Adsorption of PpCBH1 and TrCel7a to Avicel and EnzHR lignins isolated from spruce and wheat straw. (b) Adsorption of PpCBH1 and TrCel7a to EnzHR lignin from spruce at pH 4–6. (c) Adsorption of PpCBH1 and TrCel7a to EnzHR lignin from spruce at 35 ◦C and 45 ◦C. Conditions (unless otherwise specified): 10 mg/ml Avicel or lignin, 10 mg protein/g Avicel or lignin, pH 5, T = 4 ◦C (Avicel) or 35 ◦C (lignins), incubation time 24 h. Adsorption was followed using protein band intensity quantification with BioRad Criterion Stain Free SDS-PAGE and Image Lab software. In (a) and (b) Bound protein % original = (Ie − If)/Ie*100, where Ie is band intensity of the enzyme control incubated without lignin and If is band intensity after incubation with lignin.*

atgaagggatcaatctcctatcaaatctacaagggtgccctgctcctttcggcactcttg

M K G S I S Y Q I Y K G A L L L S A L L

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G S V Q A Q Q A G T L A T E T H P S L T

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W S K C T A G G S C T A V S G S V V I D

gccaactggcgctgggttcactccaccagtggctcgaccaactgctacacgggcaacacg

A N W R W V H S T S G S T N C Y T G N T

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W D A T L C P D D K T C A S N C A V D G

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A S Y A S T Y G V T T T G N S L R I N F

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V T T A S Q K N I G S R L Y L L E N D T

acctatcagaagttcaacctgctgaaccaggagtttaccttcgatgtggatgtctccaac

T Y Q K F N L L N Q E F T F D V D V S N

ctgccctgtggtctcaacggcgccctctactttgtggacatggatgccgatggtggcatg

L P C G L N G A L Y F V D M D A D G G M

accaagtaccctaccaacaaggccggagccaagtacggaactggttactgcgacagccag

T K Y P T N K A G A K Y G T G Y C D S Q

tgccctcgtgatctcaagttcatcaacggacaggccaacgtcgagggctggaccccttcc

C P R D L K F I N G Q A N V E G W T P S

accaccgatgccaactccggcattggcaaccacggctcatgctgtgctgagatggatatc

T T D A N S G I G N H G S C C A E M D I

tgggaggccaactccgtctccgaggctcttactccccacccttgcgatactcccggacaa

W E A N S V S E A L T P H P C D T P G Q

actatgtgcactggaaatacctgcggtggtacctacagcactgaccgctatggtggtact

T M C T G N T C G G T Y S T D R Y G G T

```
tgcgatcccgatggctgtgatttcaacccgtaccgccagggcgttactaacttctacggt
 C  D  P  D  G  C  D  F  N  P  Y  R  Q  G  V  T  N  F  Y  G
tctggcctgaccgttgacaccaagtccccttcaccgttgtgactcaattcctcaccgac
 S  G  L  T  V  D  T  K  S  P  F  T  V  V  T  Q  F  L  T  D
gacggtacctccaccggtaccctatctgagatcaagcgcttctacgtgcagaacggcaag
 D  G  T  S  T  G  T  L  S  E  I  K  R  F  Y  V  Q  N  G  K
gttatcggccagccgcagtccaccatcgttggcaactccggaaactccatcaccgacgcc
 V  I  G  Q  P  Q  S  T  I  V  G  N  S  G  N  S  I  T  D  A
ttctgcacggcccaaaagaccgctttcggcgacatcgacgacttcaccaagcacggcgcc
 F  C  T  A  Q  K  T  A  F  G  D  I  D  D  F  T  K  H  G  A
ttggccggtatgggtgccgctttcgccgagggcatggttctcgtcatgagtctctgggat
 L  A  G  M  G  A  A  F  A  E  G  M  V  L  V  M  S  L  W  D
gaccacaactccaacatgctctggctcgacagcacctaccctaccaacgctacttcgacc
 D  H  N  S  N  M  L  W  L  D  S  T  Y  P  T  N  A  T  S  T
actcctggtgccaagcgtggtacttgcgatatctcctccggtgaccctgccaccattgag
 T  P  G  A  K  R  G  T  C  D  I  S  S  G  D  P  A  T  I  E
tccaccaacgccaacgcctatgtcatttactcgaacatcaagaccggtcctttcaactcg
 S  T  N  A  N  A  Y  V  I  Y  S  N  I  K  T  G  P  F  N  S
accttcaccggcggcagctcaggctcttcgtcttcttcttctatcactagcactgctacc
 T  F  T  G  G  S  S  G  S  S  S  S  S  S  I  T  S  T  A  T
agcaccaagaccacttctaccagctctacctccactaccacctcttctggctcaggaagc
 S  T  K  T  T  S  T  S  S  T  S  T  T  T  S  S  G  S  G  S
actggcgccgctgctcactggggtcagtgcggtggaaatggctggaccggtcccaccacc
 T  G  A  A  A  H  W  G  Q  C  G  G  N  G  W  T  G  P  T  T
tgtgtcagcccttacgtctgcaccaagtccaacgactggtactcccagtgcctgtaa
C  V  S  P  Y  V  C  T  K  S  N  D  W  Y  S  Q  C  L  -
```

Fig. 9. Coding sequence of Penicillium pulvillorum (TUB F-2220) cbh1a. Sequence matching the de novo peptide sequences are shaded dark grey.

```
GUX1_PENJA      MKGSISYQIYKGALLLSALLNSVSAQQVGTLTAETHPALTWSKCTAGX-CSQVSGSVVID 59
PpCBH1          MKGSISYQIYKGALLLSALLGSVQAQQAGTLATETHPSLTWSKCTAGGSCTAVSGSVVID 60
F6L7A1_PENOX    MKGSISYQIYKGALLLSSLLASVSAQGAGTLTAESHPALTWQKCSAGGSCTPVSGSVVID 60
C9EI49_9EURO    MKGSISYQIYKGALLLSSLLASVSAQGAGTLTAESHPALTWQKCSAGGSCTPVSGSVVID 60
G0RVK1_HYPJQ    --------MYRKLAVISAFLATARAQSACTLQSETHPPLTWQKCSSGGTCTQQTGSVVID 52
                        :*:   ::*::* :. ** . ** :*:**.***.**::*  *:  :******

GUX1_PENJA      ANWPXVHSTSGSTNCYTGNTWDATLCPDDVTCAANCAVDGARRQHLR-VTTSGNSLRINF 118
PpCBH1          ANWRWVHSTSGSTNCYTGNTWDATLCPDDKTCASNCAVDGASYASTYGVTTTGNSLRINF 120
F6L7A1_PENOX    ANWRWVHDKNG-KNCYTGNTWDATLCPDDKTCAANCAVDGASYASTYGVTTSGNSLRINF 119
C9EI49_9EURO    ANWRWVHDKNG-KNCYTGNTWDATLCPDDKTCAANCAVDGASYASTYGVTTSGNSLRINF 119
G0RVK1_HYPJQ    ANWRWTHATNSSTNCYDGNTWSSTLCPDNETCAKNCCLDGAAYASTYGVTTSGNSLSIGF 112
                ***  .* ... .*** ****.:*****: *** **.:***       ***:**** *.*

GUX1_PENJA      VTTASQKNIGSRLYLLENDTTYQKFNLLNQEFTFDVDVSNLPCGLNGALYFVDMDADGGM 178
PpCBH1          VTTASQKNIGSRLYLLENDTTYQKFNLLNQEFTFDVDVSNLPCGLNGALYFVDMDADGGM 180
F6L7A1_PENOX    VTQASQKNIGSRLYLLENDTTYQKFNLLNQEFTFDVDVSNLPCGLNGALYFVDMDADGGM 179
C9EI49_9EURO    VTQASQKNIGSRLYLLENDTTYQKFNLLNQEFTFDVDVSNLPCGLNGALYFVDMDADGGM 179
G0RVK1_HYPJQ    VTQSAQKNVGARLYLMASDTTYQEFTLLGNEFSFDVDVSQLPCGLNGALYFVSMDADGGV 172
                ** ::***:*:****: .*****:*.**.:**:******:************.******:

GUX1_PENJA      AKYPTNKAGAKYGTGYCDSQCPRDLKFINGQANVDGWTPSKNDVNSGIGNHGSCCAEMDI 238
PpCBH1          TKYPTNKAGAKYGTGYCDSQCPRDLKFINGQANVEGWTPSTTDANSGIGNHGSCCAEMDI 240
F6L7A1_PENOX    AKYPTNKAGAKYGTGYCDSQCPRDLKFINGIANVEGWTPSSNDPNSGVGGHGTCCAEMDI 239
C9EI49_9EURO    AKYPTNKAGAKYGTGYCDSQCPRDLKFINGIANVEGWTPSSNDPNSGVGGHGTCCAEMDI 239
G0RVK1_HYPJQ    SKYPTNTAGAKYGTGYCDSQCPRDLKFINGQANVEGWEPSSNNANTGIGGHGSCCSEMDI 232
                :*****.*********************** ***:** **..: *:*:*.**:**:****

GUX1_PENJA      WEANSISNAVTPHPCDTPSQTMCTGQRCGGTYSTDRYGGTCDPDGCDFNPYRMGVTNFYG 298
PpCBH1          WEANSVSEALTPHPCDTPGQTMCTGNTCGGTYSTDRYGGTCDPDGCDFNPYRQGVTNFYG 300
F6L7A1_PENOX    WEANSISEALTPHPCDTPGQTMCEGNACGGTYSNDRYAGTCDPDGCDFNPYRQGVTNFYG 299
```

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C9EI49_9EURO      WEANSISEALTPHPCDTPGQTMCEGNACGGTYSNDRYAGTCDPDGCDFNPYRQGVTNFYG 299
G0RVK1_HYPJQ      WEANSISEALTPHPCTTVGQEICEGDGCGGTYSDNRYGGTCDPDGCDWNPYRLGNTSFYG 292
                  *****:*:*:***** * .* :* *: ****** :**.*********:* ** * *.***

GUX1_PENJA        PG--ETIDTKSPFTVVTQFLTNDGTSTGTLSEIKRFYVQGGKVIGNPQSTIVGVSGNSIT 356
PpCBH1            SG--LTVDTKSPFTVVTQFLTDDGTSTGTLSEIKRFYVQNGKVIGQPQSTIVGNSGNSIT 358
F6L7A1_PENOX      PG--MTVDTKSPFTVVTQFLTDDGTSTGTLSEIKRFYVQNGKVIGQPQSTVAGVSGNSIT 357
C9EI49_9EURO      PG--MTVDTKSPFTVVTQFLTDDGTSTGTLSEIKRFYVQNGKVIGQPQSTVAGVSGNSIT 357
G0RVK1_HYPJQ      PGSSFTLDTTKKLTVVTQFETSG--------AINRYYVQNGVTFQQPNAELGSYSGNELN 344
                  .*   *:**.. :****** *..         *:*:***.* .: :*:: : . ***.:.

GUX1_PENJA        DSWCNAQKSAFGDTNEFSKHGGMAGMGAGLADGMVLVMSLWDDHASDMLWLDSTYPTNAT 416
PpCBH1            DAFCTAQKTAFGDIDDFTKHGALAGMGAAFAEGMVLVMSLWDDHNSNMLWLDSTYPTNAT 418
F6L7A1_PENOX      DSFCKAQKAAFGDTDDFTKHGALAGMGAAFEEGMVLVMSLWDDHNSNMLWLDSTYPTTAS 417
C9EI49_9EURO      DSFCKAQKAAFGDTDDFTKHGALAGMGAAFEEGMVLVMSLWDDHNSNMLWLDSTYPTTAS 417
G0RVK1_HYPJQ      DDYCTAEEAEFGGS-SFSDKGGLTQFKKATSGGMVLVMSLWDDYYANMLWLDSTYPTNET 403
                  * :*.*::: **.  .*:.:*.:: :  .   ***********: ::**********. :

GUX1_PENJA        STTPGAKRGTCDISRR-PNTVESTYPNAYVIYSNIKTGPLNSTFT--GGTTSS---SSTT 470
PpCBH1            STTPGAKRGTCDISSGDPATIESTNANAYVIYSNIKTGPFNSTFT--GGSSGS---SSSS 473
F6L7A1_PENOX      STTLGAKRGSCDISSGAPNDVESQNANSYVVFSNIKAGPIGSTFN--SGSTGGGNGSGST 475
C9EI49_9EURO      STTLGAKRGSCDISSGAPNDVESQNANSYVVFSNIKAGPIGSTFN--SGSTGGGNGSGST 475
G0RVK1_HYPJQ      SSTPGAVRGSCSTSSGVPAQVESQSPNAKVTFSNIKFGPIGSTGNPSGGNPPGGNPPGTT 463
                  *:* ** **:*. *   *  :**  .*: * :**** **:.** .  .*.. .   ..::

GUX1_PENJA        TTTSKSTSTSSSSKTTTTVT----TTTTSSGSSGTGARDWAQCGGNGWTGPTTCVSPYTC 526
PpCBH1            SITSTATSTKTTS-TSSTST----TTSSGSGSTG-AAAHWGQCGGNGWTGPTTCVSPYVC 527
F6L7A1_PENOX      TTTKGSTTTTKAPTTTTTTTSKATTTTAASGGNGGGAAHWAQCGGVGYTGPTTCASPYTC 535
C9EI49_9EURO      TTTKGSTTTTKAPTTTTTTTSKATTTTAASGGNGGGAAHWAQCGGVGYTGPTTCASPYTC 535
G0RVK1_HYPJQ      TTRRPATTTGSSP--------------------GPTQSHYGQCGGIGYSGPTVCASGTTC 503
                  :    :*:* .:.                   *    .:.**** *::***.*.*  .*
```

```
GUX1_PENJA        TKQNDWYSQCL 537

PpCBH1            TKSNDWYSQCL 538

F6L7A1_PENOX      TKQNEYYSQCL 546

C9EI49_9EURO      TKQNEYYSQCL 546

G0RVK1_HYPJQ      QVLNPYYSQCL 514

                     * :*****
```

Figure 10. ClustalW aligments of Penicillium pulvillorum PpCBH1 with closest BLAST neighbours (GUX1-PENJA, Penicillium janthinellum; F6L7A1 PENOX, Penicillium oxalicum; C0EI49 9EURO, Penicillium decumbens) and TrCel7a (G0RVK1-HYPQ, Trichoderma i (Hypocrea jecorina)). Signal peptide predicted with SignalP software is in italics, conserved catalytic amino acids shaded in dark grey, conserved four tryptophan residues involved in glucosyl binding in active-site are boxed, cysteine residues involved in disulphide bridge formation are shaded in light grey and carbohydrate binding domain underlined.

STATEMENT

Hereby I declare that this PhD thesis was written by me and I only used the given sources. All parts of the thesis, which were cited from other references, are indicated unambiguously.

Karolina Toth

Budapest, February 10, 2014

NYILATKOZAT

Alulírott Tóth Karolina kijelentem, hogy ezt a doktori értekezést magam készítettem és abban csak a megadott forrásokat használtam fel. Minden olyan részt, amelyet szó szerint, vagy azonos tartalomban, de átfogalmazva más forrásból átvettem, egyértelműen, a forrás megadásával megjelöltem.

Tóth Karolina

Budapest, 2014. február 10.