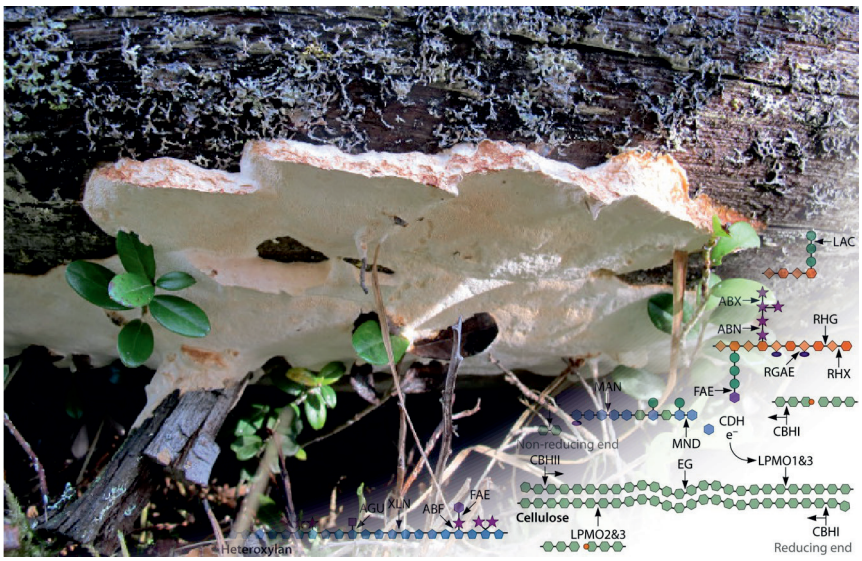
DISSERTATIONES SCHOLA DOCTORALIS SCIENTIAE CIRCUMIECTALIS,
ALIMENTARIAE, BIOLOGICAE. UNIVERSITATIS HELSINKIENSIS

15/2016

JOHANNA RYTIOJA

Enzymatic Plant Cell Wall Degradation by the White Rot Fungus *Dichomitus squalens*



DIVISION OF MICROBIOLOGY AND BIOTECHNOLOGY
DEPARTMENT OF FOOD AND ENVIRONMENTAL SCIENCES
FACULTY OF AGRICULTURE AND FORESTRY
DOCTORAL PROGRAMME IN MICROBIOLOGY AND BIOTECHNOLOGY
UNIVERSITY OF HELSINKI

Department of Food and Environmental Sciences
University of Helsinki
Finland

ENZYMATIC PLANT CELL WALL DEGRADATION BY THE WHITE ROT FUNGUS *DICHOMITUS SQUALENS*

Johanna Rytioja

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki, for public examination in Auditorium II, Infocenter Korona (Viikinkaari 11), on August 26th 2016 at 12 o'clock noon.

Helsinki, Finland 2016

Supervisor: Dr. Miia R. Mäkelä
Department of Food and Environmental Sciences
University of Helsinki, Finland

Co-supervisors: Professor Annele Hatakka
Department of Food and Environmental Sciences
University of Helsinki, Finland

Docent Kristiina Hildén
Department of Food and Environmental Sciences
University of Helsinki, Finland

Pre-examiners: Professor Christiane Liers
Department of Bio- and Environmental Science
Technische Universität Dresden, Germany

Associate Professor Emma R. Master
Department of Chemical Engineering and Applied Chemistry
University of Toronto, Canada

Department of Biotechnology and Chemical Technology
Aalto University School of Chemical Technology, Finland

Opponent: Associate Professor Kiyohiko Igarashi
Department of Biomaterial Sciences
University of Tokyo, Japan

VTT Technical Research Center of Finland, Finland

Custos: Professor Maija Tenkanen
Department of Food and Environmental Sciences
University of Helsinki, Finland

Dissertationes Schola Doctoralis Scientiae Circumiectalis, Alimentariae, Biologicae

ISSN 2342-5423 (print)
ISSN 2342-5431 (online)
ISBN 978-951-51-2331-2 (paperback)
ISBN 978-951-51-2332-9 (PDF)

Hansaprint
Helsinki 2016

Front cover: *Dichomitus squalens* growing on wood (photo: Matti J. Koivula) and schematic representation of enzymatic polysaccharide degradation (modified from Publication I).

Abstract

Basidiomycete white rot fungi are wood-rotting species and their impact to the global carbon cycle is significant. White rot fungi are capable of degrading all the polymeric cell wall components of the plant biomass from polysaccharides, cellulose, hemicelluloses and pectin, to the aromatic heteropolymer lignin. This is due to their ability to produce diverse set of extracellular enzymes that degrade or modify the plant cell wall concomitantly releasing carbon.

Research on plant-biomass-degrading fungi has concentrated on isolation and characterization of enzymes especially from the ascomycete fungi for biotechnological applications, such as bioenergy, food processing and waste treatment. More recently genomic studies have opened the reservoir of the plant-biomass-degrading potential of basidiomycete fungi including wood-rotting, litter-decomposing, plant-pathogenic and ectomycorrhizal species.

Dichomitus squalens is a white-rot fungus, which colonises softwood and is able to efficiently degrade lignin and cellulose. Previously, intensive studies on white rot fungi have been focused on lignin degradation by oxidative enzymes. The aim of this study was to analyse the potential of the plant-cell-wall-modifying enzymes of *D. squalens*. Plant biomass degradation by *D. squalens* was studied at different levels from gene expression to enzyme production. The focus was to dissect the overall degradation of plant biomass polymers, especially cellulose degrading enzymes of *D. squalens*.

The cellulose degradation by *D. squalens* was studied at the transcript level during growth on spruce wood sticks and in microcrystalline cellulose-containing liquid medium. Selected cellulases and oxidoreductases, which putatively act on cellulose were expressed simultaneously on spruce, the natural substrate of the fungus, and microcrystalline cellulose in time- and substrate-dependent manner.

To clarify the adaptation of *D. squalens* to different plant biomass, the transcriptome and secretome of the fungus were studied in different wood and non-woody substrates. The study confirmed that lignin degradation occurs at the initial stage of growth and *D. squalens* has retained the diverse enzyme set both for the degradation of wood and non-woody plant biomass. The cellobiohydrolases (CBHs) and cellobiose dehydrogenase of *D. squalens* were biochemically characterized. In hydrolysis of different plant-derived biomasses, CBHs released reducing sugars alone and in combination with oxidative laccase enzyme.

The study shows that *D. squalens* encodes a complete enzymatic repertoire for plant biomass degradation. In addition, the data emphasise the role of

oxidoreductases in the white rot fungal degradation of cellulose and other plant cell wall polymers. Results suggest that white rot fungal plant cell wall converting enzymes are promising candidates in the biotechnological applications using plant biomass.

Tiivistelmä

Valkolahosienet ovat puuta lahottavia kantasieniä, joilla on huomattava merkitys maapallon hiilen kierrossa. Ne hajottavat tehokkaasti kasvisoluseinää, joka koostuu pääosin hiilihydraattipolymeereistä eli selluloosasta, hemiselluloosista ja pektiinistä sekä aromaattisesta ligniinipolymeeristä. Tuottamiensa solunulkoisten entsyymien avulla valkolahosienet muokkaavat ja hajottavat näitä yhdisteitä ja siten vapauttavat hiiltä.

Kasvibiomassaa hajottavilla entsyymeillä on lukuisia sovelluskohteita esimerkiksi polttoaine-, elintarvike- ja jätteenkäsittelyteollisuudessa. Tähänastinen tutkimus on kohdistunut lähinnä kotelosienten tuottamien entsyymien eristämiseen ja karakterisointiin sekä niiden bioteknologiseen hyödyntämiseen. Viimeisen kymmenen vuoden aikana genomitieto on osoittanut, että myös puuta lahottavien valkolahosienten sekä muiden kasvibiomassaa muokkaavien kantasienten lignoselluloosaa hajottavien entsyymien kirjo on laaja. Onkin todennäköistä, että näillä entsyymeillä on teollisesti kiinnostavia uusia katalyyttisiä ominaisuuksia.

Valkolahosienten tutkimuksen pääkohteena ovat perinteisesti olleet ligniiniä hapettavat entsyymit. Tässä työssä täydennettiin kokonaiskuvaa valkolahosienten kasvibiomassan entsymaattisesta hajotuksesta selvittämällä myös kasvisoluseinän hiilihydraattipolymeerien, erityisesti selluloosan, hajotusta. Työn malliorganismiksi valittiin *Dichomitus squalens* -salokääpä, joka on havupuulla kasvava ja tehokkaasti selluloosaa ja ligniiniä hajottava valkolahosieni.

Salokäävän selluloosan hajotuskykyä selvitettiin seuraamalla valikoitujen, solunulkoisia entsyymejä koodaavien geenien ilmentymistä sienen kasvaessa luonnollisella kasvualustallaan, kuusella, ja mikrokiteisellä selluloosalla. Tutkimuksessa havaittiin, että sieni ilmensi kasvunsa aikana yhtäaikaisesti geenejä, jotka koodaavat sellulaaseja ja hiilihydraattipolymeerejä hapetus-pelkistysreaktioilla hajottavia entsyymejä. Lisäksi sienen kasvun vaihe ja kasvualustan hiilen lähde vaikuttivat näiden geenien ilmentymiseen.

D. squalens -sienen sopeutumista puu- ja ruohovartisten kasvibiomassojen hajotukseen selvitettiin tutkimalla sienen transkriptomeja ja eksoproteomeja erilaisilta lignoselluloosaa sisältäviltä kasvualustoilta. Nämä analyysit osoittivat, että salokääpä tuotti ligniinin hajottamiseen tarvittavia entsyymejä kasvun alkuvaiheessa, jonka jälkeen sieni eritti hiilihydraattipolymeerejä pilkkovia entsyymejä. Lisäksi työssä tuotettiin ja karakterisoitiin salokäävän mielenkiintoisimmat sellobiohydrolaasi- (CBH) ja sellobioosidehydrogenaasi-entsyymit. Kasviperäisten biomassojen hydrolyysikokeissa salokäävän CBH:t

hajottivat selluloosaa pelkistäviksi sokereiksi sekä yksin että yhdessä hapettavan lakkaasientsyymin kanssa.

Tässä työssä havaittiin, että *D. squalens* -sienellä on kattavat entsymaattiset mekanismit erilaisten kasvibiomassojen hajotukseen. Lisäksi tulokset osoittivat, että valkolahosienten tuottamilla hapettavilla entsyymeillä on olennainen rooli selluloosan ja muiden kasvisoluseinän polymeerien pilkkomisessa. Tutkimuksen mukaan valkolahosienten kasvisoluseinää hajottavat entsyymit ovat lupaavia katalyyttejä bioteknologisiin sovelluksiin.

Acknowledgements

This work was carried out at the Division of Microbiology and Biotechnology, Department of Food and Environmental Sciences, Faculty of Agriculture and Forestry, University of Helsinki. Funding from the Research Foundation of the University of Helsinki, the European Community's Seventh Framework Programme (FP7/2007-2013) under the project NEMO (grant agreement no. 222699), the Graduate School for Biomass Refining (BIOREGS, Academy of Finland), the Doctoral Programme in Microbiology and Biotechnology (MBDP) and the Finnish Cultural Foundation is acknowledged.

I would like to warmly thank my supervisors Dr. Miia Mäkelä, Prof. Annele Hatakka and docent Kristiina Hildén for all the advices and support during this thesis. It has been a pleasure to work with you.

I am deeply grateful for the co-authors, Prof. Ronald de Vries, Prof. Adrian Tsang, Dr. Jari Vehmaanperä, Dr. Marcos Di Falco, Dr. Miaomiao Zhou, M.Sc. Victoria Aguilar-Pontes, M.Sc. Susanna Mäkinen, M.Sc. Outi-Maaria Sietiö and M.Sc. Jennifer Yuzon, for their contribution to the articles. I want to express my gratitude for my pre-examiners Profs. Christiane Liers and Emma Master for reviewing the thesis and giving me valuable comments.

I would like to thank all the former and current colleagues of the Lignin group and the Division of Microbiology and Biotechnology. Especially, I would like to thank: Jaana, Minna, Outi, Suvi, Anu, Grit, Mila and Mari for sharing the long days in the lab and office. Thank you for all the help during this journey and making it fun.

Finally, dear friends and family, without you this thesis would have never been done. Most of all – Äiti, Isä, Matti, Jukka, Elise, Alma, Maarit, Maria and Taneli, thank you for your endless encouragement, love and support.

In Viikki, Helsinki, July 28th 2016

Johanna Rytioja

Contents

List of original publications

This thesis is based on the following publications:

I Rytioja J, Hildén K, Yuzon J, Hatakka A, de Vries RP, Mäkelä MR. (2014). Plant-polysaccharide-degrading enzymes from basidiomycetes. Microbiol. Mol. Biol. Rev. 78:614–649.

II Rytioja J, Hildén K, Hatakka A, Mäkelä MR. (2014). Transcriptional analysis of selected cellulose-acting enzymes encoding genes of the white-rot fungus *Dichomitus squalens* on spruce wood and microcrystalline cellulose. Fungal Genet. Biol. 72:91–98.

III Rytioja J, Hildén K, Di Falco M, Zhou M, Aguilar-Pontes MV, Sietiö O-M, Tsang A, de Vries RP, Mäkelä MR. The adaptation of the white-rot fungus *Dichomitus squalens* to wood and non-woody biomass as examined by transcriptome and exoproteome analyses. *Submitted manuscript*.

IV Rytioja J, Hildén K, Mäkinen S, Vehmaanperä J, Hatakka A, Mäkelä MR. (2015). Saccharification of lignocelluloses by carbohydrate-active enzymes of the white rot fungus *Dichomitus squalens*. PlosONE 10(12):e0145166.

The publications are referred to in the text by the roman numerals.

The contribution of the author to the publications:

I Johanna Rytioja had the main responsibility for the processing of the literature and wrote the review article together with the co-authors.

II Johanna Rytioja participated in the design of the study. She performed the experimental work, analysed and interpreted the results. She wrote the article together with the co-authors.

III Johanna Rytioja participated in the design of the study. She performed most of the cultivations and extractions. She had the main responsibility for interpreting the data. She wrote the manuscript together with the co-authors.

IV Johanna Rytioja participated in the design of the study. She performed the experimental work of protein purification, enzyme characterization and enzymatic hydrolysis. She analysed the data and interpreted the results. She wrote the article together with the co-authors.

Abbreviations

AA	auxiliary activity
AAO	aryl alcohol oxidase
ABF	α-arabinofuranosidase
ABN	endoarabinanase
ABX	exoarabinanase
AFC	α-L-fucosidase
AGD	α-glucosidase
AGL	α-galactosidase
AGU	α-glucuronidase
AMY	α-amylase
AOX	alcohol oxidase
AXL	α-xylosidase
BGL	β-glucosidase
BXL	β-xylosidase
CAZy	Carbohydrate-Active enZYme database
CAZyme	carbohydrate-active enzyme
CBH	cellobiohydrolase
CBM	carbohydrate-binding module
CDH	cellobiose dehydrogenase
cDNA	complementary DNA
CTAB	N-cetyl-N,N,N-trimethylammonium bromide
DNS	dinitrosalisylic acid
DTT	dithiothreitol
DyP	dye-decolourising peroxidase
CE	carbohydrate esterase
CRO	copper radical oxidase
DNA	deoxyribonucleic acid
EC	Enzyme Commission number
EG	endoglucanase
FAE	feruloyl esterase
FET	ferroxidase
FPLC	fast protein liquid cromatography
GAL	β-endogalactanase
GAO	galactose oxidase
GE	4-*O*-methyl-glucuronoyl methylesterase
GH	glycoside hydrolase

GLA	glucoamylase
GLX	glyoxal oxidase
GMC	glucose-methanol-choline oxidoreductase
GOX	glucose 1-oxidase
GT	glycosyl transferase
HTP	heme-thiolate peroxidases
kDa	kilo Dalton
LAC	β-galactosidase
LN-AS	low nitrogen-asparagine-succinate
LPMO	lytic polysaccharide monooxygenase
MAN	endomannanase
MND	mannosidase
MnP	manganese peroxidase
MOX	methanol oxidase
mRNA	messenger RNA
PCR	polymerase chain reaction
PGA	endopolygalacturonase
PGX	exopolygalacturonase
p*I*	isoelectric point
PL	polysaccharide lyase
PME	pectin methyl esterase
RG-I	rhamnogalacturonan I
RG-II	rhamnogalacturonan II
RGAE	rhamnogalacturonan acetyl esterase
RGX	exorhamnogalacturonase
RHA	α-rhamnosidase
RHG	endorhamnogalacturonase
RNA	ribonucleic acid
RPKM	reads per kilobase per million
RT-qPCR	real-time quantitative PCR
SDBE	starch debranching enzyme
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
VP	versatile peroxidase
XGA	xylogalacturonan
XG-EG	xyloglucanase
XLN	endoxylanase

1 Introduction

1.1 Plant cell wall composition

Plant cell wall provides the plant with rigid structure, support and protection against external stresses, such as microbial attack. It is built up of three main polysaccharides, cellulose, hemicelluloses and pectin, which form a three-layered functional matrix together with the aromatic heteropolymer lignin and structural proteins. The three plant cell wall layers are the middle lamella, and primary and secondary walls (Fig. 1A) (Harris and Stone, 2008; Sjöström, 1993).

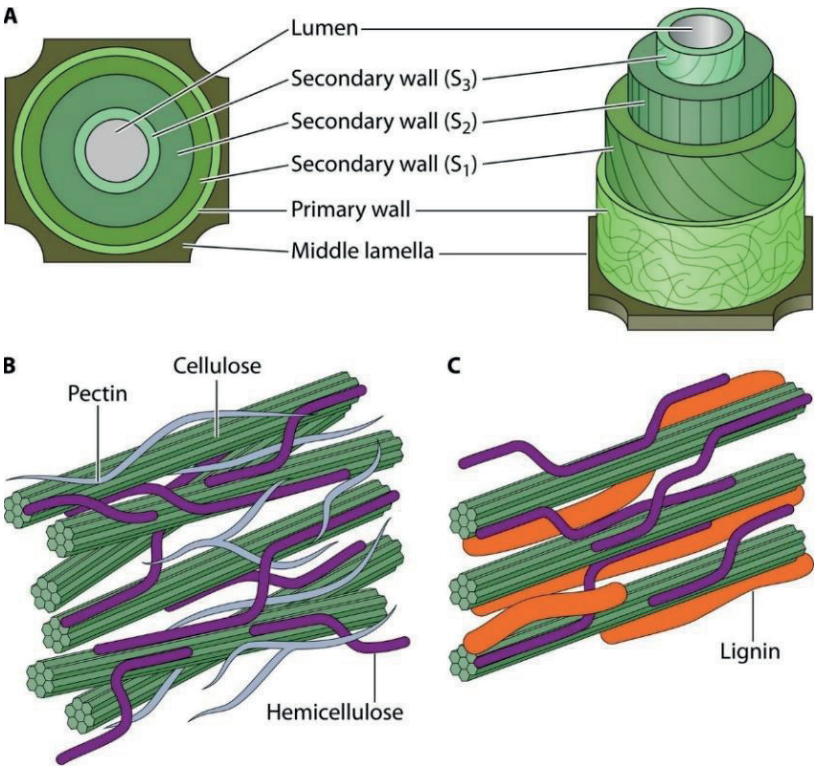


Figure 1 Simplified model of plant cell wall structure. A) The three main cell wall layers: the middle lamella, and the primary and secondary walls. The angles and directions of the polysaccharide chains differ among the primary wall and the S1-S3 layers of the secondary wall. The main polysaccharides (cellulose, hemicelluloses and pectin) and lignin in B) the primary and C) secondary walls. The lignin content in the primary cell wall (not illustrated) varies notably depending on the plant species. The illustrations are not to scale. Figure from Publication I.

The unique structure and chemical composition of each plant cell wall layer varies distinctly between plant species, tissues and the growth phases of the plant (Figs. 1B and C). The middle lamella situated between the adjacent plant cells is built up mainly of pectin, proteins and lignin (Sjöström, 1993). The primary and secondary walls differ with the content and orientation of their polysaccharides. The variation in the chemical composition of the plant-biomass derived substrates used in this study is described below (Table 1).

Table 1 Chemical composition of softwood, hardwood, monocot and dicot plant cell walls[a].

Plant material	Chemical composition (% dry wt)						
		Hemicelluloses					
	Cellulose	Mannan	Xylan	β-Glucan	Xyloglucan	Pectin	Lignin
Softwood	33-42	10-15	5-11	-	-	-	27-32
Hardwood	38-47	2-5	15-30	-	-	-	21-31
Monocots							
Primary wall	20-30	minor	20-40	10-30	1-5	5	minor
Secondary wall	35-45	minor	40-50	minor	minor	minor	20
Dicots							
Primary wall	15-30	5-10	5	nd	20-25	20-30	minor
Secondary wall	45-50	3-5	20-30	nd	minor	minor	7-10

[a]Data were obtained from Sjöström (1993) and Vogel (2008). -, not reported; nd, not detected. Table from Publication I.

Total cellulose and lignin content varies between the cell walls of softwood (e.g. pine and spruce) and hardwood (e.g. aspen, birch and oak). Hardwood has slightly higher cellulose content and lower lignin content than softwood (Table 1) (Sjöström, 1993). However, the main difference between the cell walls of softwood and hardwood is in the structure and content of hemicelluloses. Softwood hemicelluloses consist mainly of galactoglucomannans, whereas the hardwood hemicelluloses are glucuronoxylans (Sjöström, 1993).

The primary cell wall of monocots, i.e. grasses, is composed mainly of cellulose and hemicelluloses, while their secondary wall contains more cellulose, xylan as the main hemicellulose, and a significant concentration of lignin (Table 1). The primary cell wall of dicots have lower xylan and higher xyloglucan and mannan content than grasses. In addition, dicots contain notably larger amount of pectin than grasses. The secondary wall of dicots is built up of cellulose, hemicelluloses and lignin (Vogel, 2008).

1.1.1 Cellulose

Cellulose gives the plant cell wall its rigid structure and it is found in the primary and secondary cell walls (40-45% of plant dry weight, Table 1) (Sjöström, 1993). Linear cellulose chains are built up from the repeating β-1,4-linked D-glucose units, which are joined together by intermolecular hydrogen bonds forming linear crystalline microfibril structures and less crystalline amorphous regions (Kolpak and Blackwell, 1976). The ratio of crystalline and amorphous regions varies between the primary and secondary walls and plant species. The regularity of the cellulose microfibrils increases from the outer layer of the primary cell wall to the vertical microfibrils in the inner layer (Fig. 1). Also, the angles and directions of the cellulose microfibrils differ among the three sublayers (S1-S3) of the secondary plant cell wall (Harris and Stone, 2008; Sjöström, 1993).

1.1.2 Other plant cell wall polysaccharides

Hemicelluloses (20-30% of plant dry weight, Table 1), which are linked to cellulose microfibrils by hydrogen bonds, support the structure of the primary and secondary plant cell walls (Harris and Stone, 2008; Sjöström, 1993). Four main types of amorphous hemicellulose structures are xyloglucan, β-glucan, xylan and heteromannan with different main monosaccharides in their backbone. Of these, xyloglucan is a polymer of β-1,4-linked D-glucose units with 1,6-linked D-xylose sidechains, which can also contain D-galactose and L-fucose residues. Xyloglucan is present mainly in the primary walls. D-glucose units linked together with β-1,3- and β-1,4-bond form β-glucan.

The most common hemicellulose polymer is xylan. It has a β-1,4-linked D-xylose backbone, which can be decorated with branched monomers and short oligomers that comprise of L-arabinose, D-glucuronic acid, acetate and ferulic acid (Harris and Stone, 2008). Glucuronoxylans, named by the most abundant side group, (4-*O*-methyl)-D-glucuronic acid, of D-xylose backbone, are common xylans in hardwoods (Eriksson et al., 1990). Arabinoxylans, with L-arabinose linked to the D-xylose backbone, are the most common in cereals and grasses. Galactoglucomannan backbones (β-1,4-linked D-mannose and D-glucose) are decorated with branched monomers of D-galactose (Harris and Stone, 2008). It is the main hemicellulose in softwood (Eriksson et al., 1990).

Pectin is a galacturonic-acid-containing polysaccharide that is found mainly in the primary cell wall and middle lamella where it provides additional cross-links between the cellulose and hemicellulose polymers (Caffall and Mohnen, 2009). The

concentration of pectin is high in the middle lamella at an early stage of plant growth, but its concentration decreases during lignification (Sjöström, 1993).

Homogalacturonan is the simplest pectin structure. It is a linear polymer of α-1,4-linked D-galacturonic acid residues that can be methylated at the C-6 carboxyl group and acetylated at the O-2 or O-3 position. Xylogalacturonan (XGA) and rhamnogalacturonan II (RG-II) are substituted galacturonans. XGA has β-1,3-linked D-xylose residues attached to the galacturonic acid backbone, while the structure of RG-II is more complex. In total, 12 different glycosyl residues can be attached to the galacturonic acid backbone of RG-II (Caffall and Mohnen, 2009). The most complex pectin structure is rhamnogalacturonan I (RG-I), having a backbone that consists of alternating D-galacturonic acid and L-rhamnose residues, with branched structures of D-galactose and L-arabinose chains that are attached to the L-rhamnose residues.

1.1.3 Lignin

The aromatic, heterogenic biopolymer lignin (27-32% and 21-31 % of plant dry weight in softwood and hardwood, respectively, Table 1) provides resistance against microbial attack and is the key structural element that facilitates water transportation between the plant cells. Lignin and hemicelluloses are covalently linked to each other in wood plant cell walls (Harris and Stone, 2008). Lignin is synthesised in plants from phenylpropanoid precursors, i.e. *p*-coumaryl, coniferyl- and sinapyl alcohol. These monolignols are enzymatically polymerised to *p*-hydroxyphenol, guaiacyl and syringyl monomers and joined together with carbon-carbon and ether bonds (Sjöström, 1993). The composition of lignin monomers differ between plant species. In softwood, lignin is mainly composed of guaiacyl subunits, whereas hardwood lignin contains both guaiacyl and syringyl subunits (Sjöström, 1993). Lignin content in herbaceous plants is lower than in wood and it is mainly composed of *p*-hydroxyphenol units (Eriksson et al., 1990).

1.2 Wood-rotting basidiomycete fungi

Wood is a substantial carbon reservoir on earth. Wood-rotting fungi are able to degrade all the plant cell wall polymers of the complex wood structure making them important organisms in the global carbon cycle (Eriksson et al., 1990). Traditionally, the wood-rotting fungi have been divided into white rot and brown rot fungi based on the type of modification they cause in wood residue during decay.

More than 90% of all known wood-rotting basidiomycetes cause white rot by degrading both lignin and polysaccharides (Gilbertson, 1980). The remaining wood is typically white or yellowish, moist, soft and fiber-like. White rot fungi are found more commonly on angiosperm (including flowering species), than on gymnosperm (including seed-producing, conifer species) wood species, and their wood decay strategy is presented in more detail in this thesis. Brown rot fungi are found mainly on gymnosperm wood (Ryvarden, 1991) and they represent less than 10% of all known wood-decaying basidiomycetes. Brown rot fungi depolymerise cellulose efficiently, but they only modify lignin yielding characteristically brown and fragile cubical residues. However, the mechanisms of brown rot fungi for cellulose degradation combine both chemical and enzymatic strategies (reviewed in Mäkelä et al., 2014a).

The increasing genome data have revealed the genetic repertoire of different type of wood-inhabiting basidiomycetes (Fernández-Fueyo et al., 2012; Floudas et al., 2012; Hori et al., 2014b; Martinez et al., 2009, 2004; Ohm et al., 2010; Riley et al., 2014). Recent study suggests that the current dichotomous grouping of rot types may be too restricted and needs more nuanced categories (Riley et al., 2014). More research is still needed for the detailed classification of the different rot types.

1.3 White rot fungi

White rot fungi are unique in their ability to degrade all the plant cell wall polymers. They possess lignin-modifying peroxidases that efficiently depolymerise the aromatic polymer lignin and enzymes that attack crystalline cellulose (Hatakka and Hammel, 2011; Riley et al., 2014). White rot fungi have also the most extensive repertoire of putative carbohydrate-active enzymes (CAZymes (Lombard et al., 2014), described in chapter 1.4) among the wood-decaying basidiomycetes that allows them to inhabit diverse plant species, e.g. hardwood and softwood (Floudas et al., 2012, Publication I). Commonly, the most intensively studied white rot fungal species have been isolated from hardwoods (Hatakka and Hammel, 2011).

1.3.1 *Dichomitus squalens*

Dichomitus squalens is a white rot basidiomycete mainly colonising softwood, e.g. western yellow pine (*Pinus ponderosa*), and old coniferous trunks (Fig. 2) (Niemelä, 2006). It is capable of efficient cellulose and lignin degradation of pine and spruce wood (Fackler et al., 2006; Hakala et al., 2004; Venugopal et al., 2016). Under

laboratory conditions, it is able to grow on cellulose-, pectin- and lignin-containing minimal media (Floudas et al., 2012). Possibly related to its natural habitat, softwood, it grows better on galactomannan than on xylan. *D. squalens* possesses diverse set of genes encoding lignocellulose-modifying enzymes and produces corresponding extracellular enzymes (described more in detail in chapters 1.4 and 1.5), thus making it an excellent model fungus for comprehensive study on lignocellulose degradation.

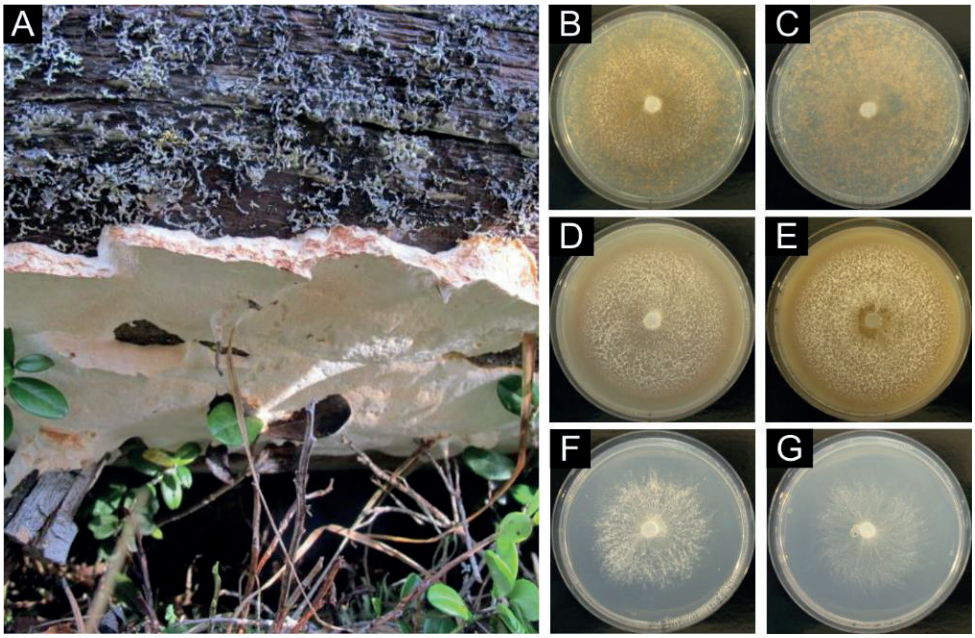


Figure 2 *D. squalens in vivo* and *in vitro*. A) Fruiting body of *D. squalens* (photo: Matti J. Koivula) and *D. squalens* growing on agar-plates containing B) spruce wood sawdust, C) aspen wood sawdust, D) powdered wheat bran, E) powdered cotton seed hulls, F) glycerol and G) no additional carbon source.

Only a few extracellular enzymes encoded by *D. squalens* that target plant cell wall polymers have been characterized at the protein level. These include two laccases (Périé et al., 1998; Šušla et al., 2007), two manganese peroxidases (MnPs) (Li et al., 1999; Périé et al., 1996), three β-1,4-endoglucanases (EGs) (Rouau and Foglietti, 1985), two cellobiohydrolases (CBHs) with xylanase activity (Rouau and Odier, 1986a, 1986b) and one α-arabinofuranosidase (ABF) (Brillouet et al., 1985). The oxalic acid metabolism of *D. squalens* in relation to wood degradation has also been studied (Mäkelä et al., 2002, 2014b, 2009).

The genome of *D. squalens* includes multiple lignin-modifying enzymes, such as 12 laccases, nine MnPs and three versatile peroxidases (VPs) (Table 3), which

are classified in the auxiliary activies (AA) families AA1_1, AA2 and AA2, respectively, in the Carbohydrate-Active enZYmes database (CAZy, http://www.cazy.org, (Lombard et al., 2014), described in chapter 1.4). *D. squalens* also possesses several cellulose and hemicellulose-acting enzymes belonging to the glycoside hydrolase (GH) families, which degrade the corresponding plant polysaccharides to oligo- and monosaccharides. It has four CBHs with signal peptides (one GH6 and three GH7 CBHs), three putative GH5 EGs and six putative GH3 β-1,4-glucosidases (BGLs) (Floudas et al., 2012). Also, a full set of mannan and xylan degradation related enzyme-encoding genes is present in the genome of *D. squalens*, e.g. three putative β-1,4-endomannanases (MANs, two GH5_7 and one GH5_31), four putative GH2 β-1,4-mannosidases (MNDs), five putative GH10 β-1,4-endoxylanases (XLNs) and five β-1,4-xylosidases (BXLs) from GH-families GH3, GH5_22 and GH43. In addition, *D. squalens* possesses one AA8-AA3_1 cellobiose dehydrogenase (CDH) and 15 putative AA9 lytic polysaccharide monooxygenase (LPMO) -encoding genes (Floudas et al., 2012). Its genome lacks the genes encoding putative CE1 xylan- and pectin-debranching enzymes.

Due to the ability to efficiently degrade lignocellulose, *D. squalens* has been studied in different biotechnological applications, such as degradation of different plant biomasses (Bak et al., 2010; Dong et al., 2014) and biopulping, which is pretreatment of wood prior to the pulping (Blanchette, 1984; Fackler et al., 2006; Itoh et al., 2003). Also, its suitability in bioremediation of wastewaters and soil for decomposition of dyes (Chander and Arora, 2007; Chander et al., 2004; Eichlerová et al., 2007, 2006; Novotný et al., 2012; Šušla et al., 2008), toxic compounds (Aggelis et al., 2002; Covino et al., 2010; in der Wiesche et al., 2003, 1996; Muzikář et al., 2011; Rosenbrock et al., 1997) and antibiotics (Čvančarová et al., 2014, 2013) has been studied broadly. The potential of *D. squalens* in bioremediation is especially due to its effective laccases capable of decolourising e.g. anthraquinone dye Remazol Brilliant Blue R (Šušla et al., 2007). *D. squalens* degrades and decreases the estrogenic activity of different endocrine disrupting compounds (Cajthaml et al., 2009). Another study has shown that *D. squalens* is able to grow on mature municipal landfill leachate and reduce its organic matter content and toxicity (Kalčíková et al., 2014).

1.4 Enzymatic plant cell wall degradation

Most of the plant cell wall polysaccharide-degrading enzymes and the lignin-modifying enzymes are classified in the CAZy database according to their structural

similarity (Lombard et al., 2014). In CAZy, the enzymes are organized in five catalytic classes, glycoside hydrolases (GH), glycosyl transferases (GT), polysaccharide lyases (PL), carbohydrate esterases (CE) and auxiliary activities (AA). In addition, carbohydrate-binding modules (CBMs) are classified in the group of associated modules. The selected CAZymes studied in this work and the number of the putative CAZyme-encoding genes of the white rot fungus *D. squalens* are listed in Table 2.

Table 2 Substrates, abbreviations and classification of selected CAZymes and the number of the corresponding putative genes of *D. squalens* (Floudas et al., 2012).

Substrate	Abbr.	Enzyme Function	EC Number[a]	CAZy Family	Putative CAZy Genes
Lignin		Laccase	1.10.3.2	AA1_1	12
	FET	Ferroxidase	1.10.3.-	AA1_2	1
	MnP-e	Manganese peroxidase, extra-long	1.11.1.13	AA2	4
	MnP-s	Manganese peroxidase, short	1.11.1.13	AA2	5
	VP	Versatile peroxidase	1.11.1.16	AA2	3
H_2O_2-supply	GMC	Glucose-methanol-choline oxidoreductase		AA3_2	26
	AAO	Aryl alcohol oxidase	1.1.3.7		
	GOX	Glucose 1-oxidase	1.1.3.4		
	AOX/ MOX	Alcohol-oxidase/ Mehtanol-oxidase	1.1.3.13	AA3_3	4
	CRO	Copper radical oxidase		AA5_1	9
	GAO	Galactose oxidase	1.1.3.9		
	GLX	Glyoxal oxidase	1.1.3.-		
Cellulose	BGL	β-1,4-Glucosidase	3.2.1.21	GH1, GH3	3 3
	EG	β-1,4-Endoglucanase	3.2.1.4	GH5_5, GH45, GH131	3 1 3
	CBHII	Cellobiohydrolase (non-reducing end)	3.2.1.91	GH6	1
	CBHI	Cellobiohydrolase (reducing end)	3.2.1.176	GH7	4
	CDH	Cellobiose dehydrogenase	1.1.99.18	AA8-AA3_1	1
	LPMO	Lytic polysaccharide monooxygenase	na	AA9	14

Substrate	Abbr.	Enzyme Function	EC Number[a]	CAZy Family	Putative CAZy Genes
Hetero-	MND	β-1,4-Mannosidase	3.2.1.25	GH2	4
mannan	MAN	β-1,4-Endomannanase	3.2.1.78	GH5_7, GH5_31	2 1
	AGL	α-1,4-Galactosidase	3.2.1.22	GH27	6
Xylan/ Xyloglucan	BXL	β-1,4-Xylosidase	3.2.1.37	GH3, GH5_22, GH43	1 2 2
	XLN	β-1,4-Endoxylanase	3.2.1.8	GH10	5
	AGU	α-Glucuronidase	3.2.1.139	GH115	2
	GE	4-*O*-Methyl-glucuronoyl methylesterase	3.1.1.-	CE15	2
	AE	Acetylesterase	3.1.1.6	CE16	10
	FAE	Feruloyl esterase	3.1.1.73	na	2
	XG-EG	Xyloglucanase	3.2.1.151	GH12, GH74	3 1
	AFC	α-L-Fucosidase	3.2.1.51	GH95	1
Starch	AMY	α-Amylase	3.2.1.1	GH13_1, GH13_5	2 1
	SDBE	Starch-debranching enzyme	na	GH13_8, GH13_2-GH133	1 1
	GLA	Glucoamylase	3.2.1.3	GH15	2
Starch/	AXL	α-Xylosidase	3.2.1.177	GH31	1
Xyloglucan	AGD	α-Glucosidase	3.2.1.20	GH31	5
Pectin	PGA	Endopolygalacturonase	3.2.1.15	GH28	2
	PGX	Exopolygalacturonase	3.2.1.67	GH28	2
	RGX	Exorhamnogalacturonase	3.2.1.-	GH28	2
	RHG	Endorhamnogalacturonase	3.2.1.171	GH28	1
	LAC	β-1,4-Galactosidase	3.2.1.23	GH35	3
	ABN	Endoarabinanase	3.2.1.99	GH43	5
	ABF	α-Arabinofuranosidase	3.2.1.55	GH51	2
	GAL	β-1,4-Endogalactanase	3.2.1.89	GH53	1
	RHA	α-Rhamnosidase	3.2.1.40	GH78	5
	UGH	Unsaturated glucuronyl hydrolase	3.2.1.-	GH88, GH105	1 1
	ABX	Exoarabinanase	3.2.1.-	GH93	1
	PME	Pectin methyl esterase	3.1.1.11	CE8	3
	RGAE	Rhamnogalacturonan acetyl esterase	3.1.1.-	CE12	2

[a]EC, Enzyme Commission; na, not categorized in the EC or CAZy.

1.4.1 Cellulose-degrading enzymes

EGs, CBHs and BGLs are the main hydrolytic enzymes that cleave the cellulose chain. Cellulose is hydrolysed from the middle of the chain by EGs releasing glucooligosaccharides (Fig. 3A), whereas exoglucanases or CBHs hydrolyse cellulose from the ends of the chain. Two types of cellobiohydrolases, CBHI and CBHII, degrade cellulose from either the reducing or the non-reducing end, respectively, resulting in the release of cellobiose units. CBHs have different processivities, i.e. the efficiencies of the sequential hydrolysis of the β-1,4-glycosidic bonds by the cellulase before the dissociation of the enzyme from the substrate (Igarashi et al., 2011; von Ossowski et al., 2003). Finally, BGL cleaves shorter oligosaccharides into glucose units.

In addition to the hydrolytic cleavage of the cellulose chain by cellulases, CDH and LPMOs participate in oxidative degradation of cellulose (Fig. 3A) (Harris et al., 2010; Quinlan et al., 2011). CDH is the only known secreted flavocytochrome and it catalyses oxidation of cellobiose and cellooligosaccharides to the corresponding lactones (Henriksson et al., 2000; Ludwig et al., 2010). The exact function of CDH in the degradation of lignocellulose is still not known, although the roles in lignin and cellulose degradation have been proposed (Henriksson et al., 2000; Ludwig et al., 2010). Accordingly, CDH has been shown to transfer electrons to LPMOs (Langston et al., 2011; Tan et al., 2015) and produce hydroxyl radicals through Fenton chemistry supporting its function in lignin modification (Henriksson et al., 2000).

CDHs are present both in basidiomycete and ascomycete fungi, and classified into two subgroups according to their primary amino acid sequences (Zámocký et al., 2004). The structure of class I basidiomycete CDHs consists of shorter polypeptides and more conserved linker regions than ascomycetes class II CDHs (Ludwig et al., 2010). In turn, ascomycete CDHs are complex polypeptides with a C-terminal CBMs.

LPMOs, which have been the target of intensive research during the recent years, are copper monooxygenases capable of cleaving glycosidic bonds of various polysaccharides, such as cellulose, xyloglucan, xylan, glucomannan, starch and chitin (Agger et al., 2014; Frommhagen et al., 2015; Langston et al., 2011; Lo Leggio et al., 2015; Phillips et al., 2011; Quinlan et al., 2011; Vaaje-Kolstad et al., 2010; Vu et al., 2014). The LPMO-catalysed reaction requires an electron donor that reduces copper II to copper I in the active site of LPMO, and molecular oxygen that forms copper-oxygen complex, which then oxidises glycosidic bond (Hemsworth et al., 2013). More recently, fungal aromatic acid metabolite 3-hydroxyanthranilic acid and insoluble high molecular weight lignins (hydrolysis lignin, organosolv lignin and Klason lignin) together with soluble low molecular weight lignin have been

proven to act as electron donors for LPMOs (Westereng et al., 2015). In addition, several compounds, e.g. ascorbic acid, have been shown to act as reductants in LPMO reactions *in vitro* (Isaksen et al., 2014; Quinlan et al., 2011).

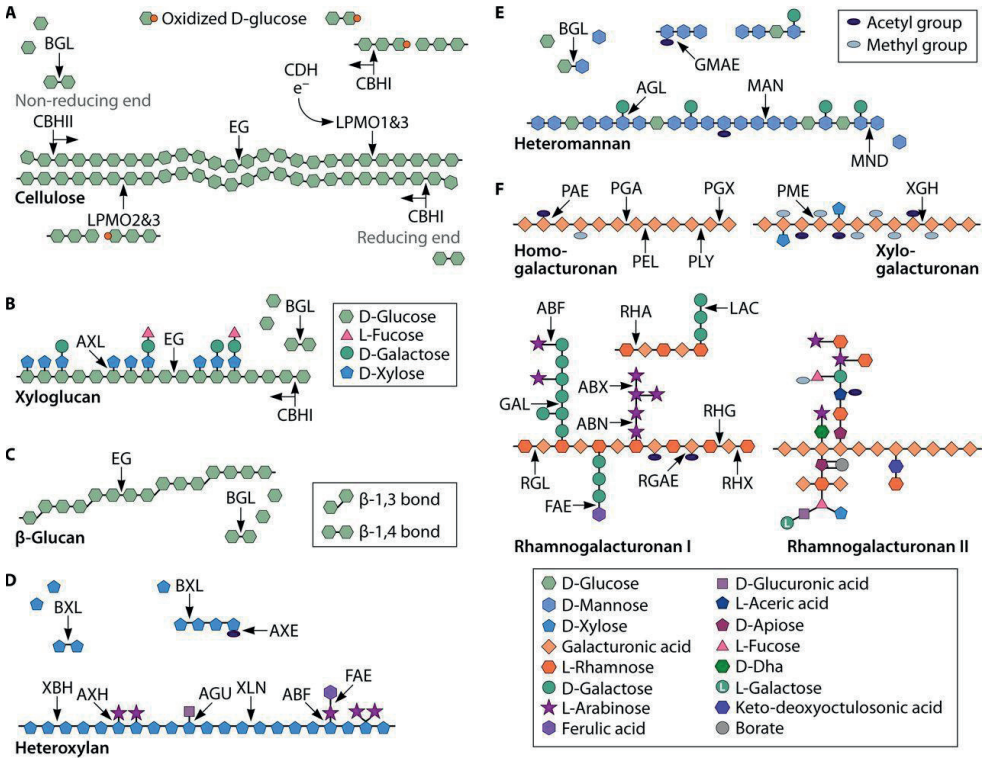


Figure 3 Schematic presentation of the plant cell wall polysaccharides and selected polysaccharide-depolymerising enzymes acting on A) cellulose, B) xyloglucan, C) β-glucan, D) heteroxylan, E) heteromannan and F) four different pectin polymers. Enzyme abbreviations are presented in Table 2. Polysaccharide structures were drawn by using data reported previously by Doblin et al. (2010) and Mohnen (2008). Figure from Publication I.

At least three types of fungal LPMOs, which act on cellulose, exist based on their sequence similarity and specific activity towards cellulose chain (Li et al., 2012). Type 1 and type 2 LPMOs oxidise the glucose unit of the cellulose chain at the C-1 position and C-4 position, respectively. Reactions of type 1 LPMOs result in aldonic acids at the reducing end of the cellulose chain while reactions of type 2 enzymes generate ketosugars at the non-reducing end of the cellulose chain (Isaksen et al., 2014; Phillips et al., 2011; Quinlan et al., 2011). Type 3 LPMOs are able to oxidise at both positions (Phillips et al., 2011). Other positions, e.g. C-6, have also been suggested to be oxidised by LPMOs (Quinlan et al., 2011).

So far five ascomycete and two basidiomycete LPMO crystal structures, i.e. Cel61B of *Trichoderma reesei* (*Hypocrea jecorina*) (PDB accession number 2VTC), GH61E of *Thielavia terrestris* (PDB accession numbers 3EII and 3EJA), GH61A of *Thermoascus aurantiacus* (PDB accession number 2YET), PMO-2 and PMO-3 of *Neurospora crassa* (PDB accession numbers 4EIR and 4EIS), PcGH61D of *Phanerochaete chrysosporium* (PDB accession number 4B5Q) and *Ls*(AA9)A of *Lentinus similis* (PDB accession number 5ACH), have been solved (Frandsen et al., 2016; Harris et al., 2010; Karkehabadi et al., 2008; Li et al., 2012; Quinlan et al., 2011; Wu et al., 2013). More detailed classification of LPMOs will be most probably obtained after biochemical characterization of more enzymes.

1.4.2 Other polysaccharide-degrading enzymes

Several different enzymes are needed to degrade hemicelluloses and pectin. The xyloglucan backbone is similar to that of cellulose, and is thus hydrolysed by similar enzyme set of EGs, CBHs and BGLs (Fig. 3B). The terminal xylose residues from the side branches are cleaved by α-1,4-xylosidase (AXL) (van den Brink and de Vries, 2011). EGs are also able to degrade the glucose chain of β-glucan into oligosaccharides, which are further cleaved by BGLs (Fig. 3C).

Depolymerisation of xylan backbone requires more specific enzymes. It is hydrolysed into shorter oligomers by XLN (Fig. 3D) and further to D-xylose monomers by BXL. In addition, BXL is able to release D-xylose from the non-reducing terminus of the larger xylooligosaccharide backbones (Polizeli et al., 2005; van den Brink and de Vries, 2011). Monomers and short oligomers, i.e. L-arabinose, D-glucuronic acid, ferulic acid and acetyl group, in the side branches of the xylan backbone are cleaved by arabinoxylan arabinofuranohydrolase (AXH), α-L-arabinofuranosidase (ABF), α-glucuronidase (AGU), feruloyl esterase (FAE) and acetyl xylan esterase (AXE) (de Vries and Visser, 2001).

Common to the hydrolysis of polysaccharides, MAN cleaves the β-1,4-linked D-mannose backbone of heteromannan to mannooligosaccharides and MND releases D-mannose from the terminal ends of the backbone (Fig. 3E). In addition, the glucose units in the galactoglucomannan backbones are cleaved by BGLs (van den Brink and de Vries, 2011). D-galactose side branches are cleaved by α-1,4-galactosidase (AGL), while galactomannan acetyl esterase (GMAE) releases acetyl groups from D-mannose.

Recently, LPMOs from the ascomycete fungus *N. crassa* have also been shown to oxidatively cleave xyloglucan, β-glucan and to some extent glucomannan (Agger et al., 2014; Frommhagen et al., 2015). In addition, LPMO from ascomycete species

Myceliophthora thermophila simultaneously cleaves xylan and cellulose (Frommhagen et al., 2015). Most probably hemicellulose acting LPMOs will be characterized also from white rot fungi.

Endopolygalacturonases (PGAs) hydrolyse the homogalacturonan backbone of pectin from the middle of the chain, whereas exopolygalacturonases (PGXs) hydrolyse at the terminal end releasing D-galacturonic acid (Fig. 3F). Xylogalacturonan hydrolases (XGHs) specifically cleave xylogalacturonan. Instead, the backbone of rhamnogalacturonan I is cleaved by several hydrolases and lyases, such as exorhamnogalacturonase (RHX), endorhamnogalacturonase (RHG), rhamnogalacturonan rhamnohydrolase (RGXB), α-rhamnosidase (RHA), pectin lyase (PEL), pectate lyase (PLY) and rhamnogalacturonan lyase (RGL) (de Vries and Visser, 2001; van den Brink and de Vries, 2011).

Side branches of pectin are cleaved by several debranching enzymes such as endoarabinase (ABN), exoarabinase (ABX), β-1,4-galactosidase (LAC), β-1,4-endogalactanase (GAL), *p*-coumaroyl esterase (pCAE), rhamnogalacturonan acetyl esterase (RGAE), pectin acetyl esterase (PAE) and pectin methyl esterase (PME) (van den Brink and de Vries, 2011).

1.4.3 Lignin-modifying enzymes

Enzymatic degradation of complex lignin structures, which contain no hydrolysable linkages, requires a diverse set of oxidative enzymes (Martínez et al., 2009). Of these, MnP, lignin peroxidase (LiP) and VP belong to class II heme peroxidases and they use H_2O_2 as an electron acceptor in their reaction cycles (Martínez et al., 2009). MnP oxidises Mn^{2+} to Mn^{3+}, which in a chelated form is able to further oxidise a number of phenolic substrates, carboxylic acids and unsaturated lipids (Glenn et al., 1986; Gold et al., 2000; Hofrichter et al., 2010; Wariishi et al., 1992).

LiP is able to catalyse the one-electron oxidation of phenolic and non-phenolic substrates, e.g. the substructures of lignin. The LiP-catalysed oxidation of the lignin model compounds, such as the non-phenolic β-*O*-4 linkage-type arylglycerol-aryl ethers, leads to C_α-C_β cleavage forming veratraldehyde and other aromatic derivatives and ring fission products (Hammel et al., 1993; Hofrichter et al., 2010; Lundell et al., 1993). VP shares the catalytic properties of both MnP and LiP. It contains tryptophan residue similarly with LiP and two glutamatic acid and one aspartic acid residue, which are involved in Mn^{2+}-oxidation, similarly with MnP (Hofrichter et al., 2010).

In addition to the class II heme peroxidases, novel peroxidases, dye-decolourising peroxidases (DyPs; EC 1.11.1.19) and heme-thiolate peroxidases

(HTPs, i.e. chloroperoxidases EC 1.11.1.10 and peroxygenases EC 1.11.2.1), have been characterized (Hofrichter and Ullrich, 2014, 2006; Linde et al., 2015). Fungal DyPs have been shown to degrade anthraquinone dyes, substituted phenols (e.g. guaiacol and 2,6-dimethoxyphenol) and non-phenolic lignin model compounds (Liers et al., 2014, 2013, 2010). However, the exact role in plant cell wall or lignin degradation and the natural substrates of DyPs and HTPs still needs to be clarified (Hofrichter and Ullrich, 2014, 2006; Linde et al., 2015).

Laccases are multicopper oxidases that are able to oxidise variety of substrates (e.g. diphenol hydroxyls and hydroxyl groups of monophenols and related compounds) using O_2 as an electron acceptor (Munk et al., 2015). Although white rot fungal laccases have the highest reported redox potential compared to laccases from other organisms (Munk et al., 2015), they are not able to oxidise the non-phenolic units of lignin without small molecular weight mediator compounds (Bourbonnais and Paice, 1990).

White rot fungi possess several oxidases related to H_2O_2-production, which is essential for the peroxidase reactions (Kirk and Farrell, 1987; Mäkelä et al., 2014a). Aryl alcohol oxidase (AAO), alcohol/methanol oxidase (AOX/MOX) and glucose 1-oxidase (GOX) are examples of flavoproteins from the glucose-methanol-choline (GMC) family. AAO catalyses the oxidation of lignin-derived compounds, phenolic aromatic aldehydes and acids, and other aromatic fungal metabolites, to their corresponding aldehydes concurrently reducing O_2 to H_2O_2 (Hernández-Ortega et al., 2012). MOX oxidises methanol forming formaldehyde and H_2O_2, while GOX oxidises glucose to corresponding lactone producing H_2O_2 in the reaction (Ozimek et al., 2005). Copper radical oxidases (CROs) include galactose oxidases (GAOs) and glyoxal oxidases (GLXs). Similar to AAO, GLX has a broad substrate specificity and is able to oxidise various simple aldehydes to their corresponding carboxylic acids (Whittaker et al., 1996).

1.5 White rot fungal repertoire of genes and enzymes related to plant cell wall degradation

To date over 85 basidiomycete genomes, including genomes of at least 15 white rot fungal species, have been sequenced and annotated (The MycoCosm, http://genome.jgi.doe.gov/, Grigoriev et al., 2014). The vast amount of genome information can be applied in understanding fungal physiology and searching for novel enzymes with a potential use in biotechnological applications.

White rot fungi harbour an extensive set of cellulase-encoding genes (Table 3), whereas some of the brown rot, plant-pathogenic and ectomycorrhizal fungi as well as basidiomycetous yeasts lack CBH-encoding genes. White rot fungi possess typically one to seven copies of genes encoding GH6 and GH7 enzymes, which are mainly CBHs (Publication I). As an exception, *Pleurotus ostreatus* harbours 16 putative genes encoding GH7 enzymes (Riley et al., 2014). Several GH3 and GH5 genes encoding putative BGLs and EGs (6 to 17 and 16 to 43 genes, respectively) are represented in all the white rot genomes (Publication I). GH1, GH12 and GH45 genes are also present in the genomes and BGL from the family GH1, and EGs from families GH12 and GH45 have been characterized from *P. chrysosporium* (Henriksson et al., 1999; Igarashi et al., 2008; Vanden Wymelenberg et al., 2005).

The three-dimensional crystal structure of GH7 Cel7D from *P. chrysosporium* (PDB accession number 1GPI) was the first white rot fungal CBHI structure to be solved (Muñoz et al., 2001). The catalytic domain of *P. chrysosporium* Cel7D shares similar β-sandwich structure with the ascomycete *T. reesei* Cel7A (PDB accession number 1CEL) (Divne et al., 1994), but its substrate binding tunnel is more open, which affects to the accessibility of the substrate to the active site. Homology modelling of six *P. chrysosporium* CBH isoenzymes suggests that they differ in their three-dimensional structures, thus supporting the presence of multiple isoenzymes with different biochemical properties in white rot fungi (Muñoz et al., 2001).

Genes from diverse CAZy families encoding hemicellulose- and pectin-active enzymes are also broadly present in the white rot fungal genomes (Table 3). Compared to the other wood-rotting and litter-decomposing basidiomycetes, white rot fungi have more copies of genes related to xylan, pectin and xyloglucan degradation in the families GH10, GH11, GH28, GH43, GH74, CE1 and CE12 (Publication I). Generally white rot fungi possess more XLN-encoding genes in the family GH10 than in GH11, and some of the species do not have any GH11 gene copies (Publication I). Both GH10 and GH11 XLNs have been biochemically characterized from the white rot fungi. Also, GH43 BXLs, ABFs (GH family not identified), AGUs (GH family not identified), CE1 AXEs and FAEs (GH family not identified) have been characterized only from one to three white rot fungal species (Publication I). So far, no enzyme with AXH activity has been characterized from white rot fungi, and they rarely even possess the putative genes from the related GH62 family (Publication I). In addition to xylanases, white rot fungi have several genes related to galactomannan degradation in the families GH2, GH5 and GH27 (Table 3). GH5 MANs, MNDs (GH family not identified) and GH36 and GH27 LACs have been biochemically characterized from white rot fungi (Publication I).

Table 3 Distribution of CAZy genes related to plant cell wall polysaccharide degradation in white rot fungi[a]. Table modified from the Publication I.

CAZy Family	Species																		
	As	*Ba*	*Cs*	*Ds*	*Fm*	*Gl*	*Gsp*	*Hi*	*Pca*	*Pch*	*Pb*	*Pg*	*Po*	*Ps*	*Sh*	*Tv*	*Sc*[b]	*Bb*[c]	*Ja*[c]
GH1	1	2	3	4	5	3	3	2	2	2	2	2	3	1	3	2	3	4	3
GH2	7	3	4	4	2	3	3	3	2	2	2	3	3	4	3	5	4	2	3
GH3	12[d]/ 14	9	6	7[d]/8	7[d]/8	12	13	11[d]/ 12	11	9[d]/ 11	8	9	13	12[d]/ 14	15[d]/ 17	11[d]/ 13	12	7	10
GH5	8[e]/ 43	19	18	5[e]/ 19	6[e]/ 20	19	18	7[e]/ 16	24	5[e]/ 19	23	6[e]	21	6[e]/ 18	6[e]/ 20	5[e]/ 22	16	29	36
GH6	2	1	1	1	2	1	1	1	1	1	1	1	3	1	1	1	1	3	3
GH7	8	5	3	4	2	3	3	1	5	9	4	5	16	5	3	4	2	7	5
GH9	0	1	0	0	1	2	1	1	1	1	1	1	1	1	1	1	1	3	1
GH10	4	4	6	5	4	7	9	2	5	6	8	4	3	5	6	6	5	11	5
GH11	3	0	1	0	0	0	0	0	1	1	0	2	2	1	1	0	1	0	0
GH12	1	2	2	3	3	3	3	4	3	2	2	3	2	2	5	5	1	0	8
GH25	1	1	1	2	1	2	2	0	0	1	1	1	2	1	0	1	1	2	1
GH26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0
GH28	14	6	6	7	17	13	10	8	4	4	5	10	6	13	17	11	3	2	7
GH30	1	1	1	2	2	2	3	2	3	2	2	2	4	3	5	4	5	3	1
GH35	6	4	1	3	2	10	7	4	4	3	4	2	4	4	7	2	4	1	2
GH36	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GH37	3	3	2	3	3	2	2	2	2	2	3	2	2	2	2	2	2	2	2
GH43	28	6	2	7	7	11	11	4	4	4	2	7	8	7	12	3	19	1	3
GH45	2	3	1	2	0	2	3	2	1	2	4	1	2	1	1	2	1	1	2
GH51	3	2	2	2	1	2	2	1	2	2	1	2	3	3	3	2	2	1	3
GH53	1	1	6	1	1	1	1	1	1	1	1	1	1	2	2	1	1	1	2
GH54	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0
GH62	0	-	0	0	0	0	0	0	0	0	-	-	1	0	0	0	1	0	0
GH67	-	-	0	0	0	0	0	0	-	0	-	-	-	-	-	-	0	-	-
GH74	1	2	1	1	4	1	1	1	2	4	1	2	3	2	2	1	1	0	1
GH78	4	2	1	5	2	5	3	2	1	1	1	1	2	7	3	3	3	0	1
GH93	1	0	0	1	0	2	1	0	0	0	0	0	0	1	1	0	2	0	0
GH115	2	2	2	2	3	4	4	1	1	1	2	1	1	1	2	2	2	1	2
CE1	4	2	2	0	0	3	2	1	2	4	2	2	2	2	1	3	11	2	2
CE5	3	0	0	0	0	0	1	0	0	0	1	0	0	1	1	0	1	0	0
CE8	2	2	2	3	3	3	3	3	2	2	3	8	2	6	5	2	2	2	2
CE12	2	1	0	2	2	1	1	2	0	0	0	1	2	1	3	0	2	1	0
CE15	6	2	2	2	1	2	2	1	3	3	2	1	1	2	1	2	2	3	0
CE16	12	15	5	13	6	17	17	6	5	1	8	6	9	12	14	8	11	7	8
PL1	2	1	0	0	2	0	0	2	0	0	0	0	10	4	4	0	5	4	0
PL3	1	2	0	0	0	0	0	0	0	0	0	0	3	0	0	0	4	2	0
PL4	1	1	0	1	0	0	0	1	0	0	1	0	2	3	3	1	3	1	0
PL9	0	-	0	0	0	0	0	0	0	0	-	-	0	0	0	0	1	0	0
PL11	-	-	0	0	0	0	0	0	-	0	-	-	-	-	-	-	0	-	-
AA3_1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3	1
AA9	20	28	9	15	13	15	16	10	11	15	12	15	29	14	16	18	22	32	15
AA1_1	7	0	7	12	10	-	16	16	0	0	8	0	11	12	15	7	2	0	1
AA1_2	1	1	1	1	1	-	-	1	1	1	-	-	1	1	2	2	0	1	1
AA2[f]	0	12	0	0	0	-	0	0	4	10	5	3		0	0	10	0	0	0
AA2[g]	5	6	13	9	16	-	6	8	7	5	10	7	9[fgh]	10	5	13	0	0	0
AA2[h]	0	1	2	3	0	-	2	0	0	0	0	0		0	0	2	0	0	0
AA3_3	6	-	3	4	3	-	-	3	4	3	-	3	4	4	7	4	4	3	2
AA5_1	9	7	4	9	4	-	9	4	6	7	8	6	16	9	8	9	4	5	4

[a]Gene numbers are based on published data (updated according to Riley et al. (2014) and Hori et al. (2014)) for the following organisms: *As, Auricularia subglabra* (Floudas et al., 2012), *Ba, Bjerkandera adusta* (Binder et al., 2013), *Bb, Botryobasidium botryosum* (Riley et al., 2014), *Cs, Ceriporiopsis (Gelatoporia) subvermispora* (Fernández-Fueyo et al., 2012), *Ds, Dichomitus squalens*, *Fm, Fomitiporia mediterranea* (Floudas et al., 2012), *Gl, Ganoderma lucidum* (Chen et al., 2012), *Gsp, Ganoderma* sp. (Binder et al., 2013), *Hi, Heterobasidion irregulare* (Olson et al., 2012), *Ja, Jaapia argillacea* (Riley et al., 2014), *Pca, Phanerochaete carnosa* (Suzuki et al., 2012), *Pch, Phanerochaete chrysosporium* (Martinez et al., 2004), *Pb, Phlebia brevispora* (Binder et al., 2013), *Pg, Phlebiopsis gigantea* (Hori et al., 2014b), *Po, Pleurotus ostreatus* (Riley et al., 2014), *Ps, Punctularia strigosozonata* (Floudas et al., 2012), *Sc, Schizophyllum commune* (Ohm et al., 2010), *Tv, Trametes versicolor*, (Floudas et al., 2012). [b]White rot-like; [c]ecological classification uncertain; [d]No β-N-acetylhexosaminidase was included. [e]β-1,4-Endoglucanase and β-1,4-endomannanase are included. [f]LiP, [g]MnP, [h]VP. -, not annotated in publications.

Some of the white rot fungi have few pectinolytic genes from the families PL1 and PL4, but genes from PL3, PL9 and PL11 are almost absent in all white rot fungi. *P. ostreatus* is an exception with ten copies of PL1 genes. *P. chrysosporium* has a limited number of pectinolytic genes. For example no PL1, PL3 or PL9 pectin/pectate lyase-, GH93 ABX-, or GH28 rhamnogalacturonan hydrolase-encoding genes are present in its genome (Martinez et al., 2004). It grows also poorly on rhamnogalacturonan and polygalacturonic acid (Benoit et al., 2012). However, *P. chrysosporium* is able to grow on solid cultures of soy, apple or lemon pectins, which contain a high degree of methyl esterification, possibly by utilizing GH28 PGAs, GH35 LACs, GH53 galactan- and arabinan-hydrolysing GAL and GH51 ABF (Benoit et al., 2012).

White rot fungi possess several AA9 LPMO-encoding genes (up to 29) and typically one CDH-encoding gene (Publication I). As an exception, *B. botryosum*, which ecological classification and rot type is uncertain, has the highest number of genes encoding LPMOs and CDHs (32 and 3, respectively) (Riley et al., 2014). White rot fungal CDHs are able to oxidise variable substrates including cellobiose, higher cellodextrins, lactose, mannobiose, galactosylmannose (Henriksson et al., 2000), maltose (Harreither et al., 2009; Nakagame et al., 2006) and xylobiose (Ludwig et al., 2004). However, the substrate specifity of different basidiomycete CDHs varies substantially (Hai et al., 2000).

P. chrysosporium PcGH61D is so far the only biochemically characterized basidiomycete LPMO that acts on cellulose (Westereng et al., 2011). In the presence of ascorbic acid, PcGH61D is able to oxidise phosphoric acid-swollen cellulose and release lactone, which is spontaneously converted to aldonic acid. PcGH61D does not oxidise soluble cellooligosaccharides (Westereng et al., 2011). Accordingly to the three-dimensional crystal structure, PcGH61D (PDB accession number 4B5Q) possess the copper-bound active site that is common to LPMOs (Wu et al., 2013). Nevertheless, compared to the structurally characterized LPMOs from ascomycete

species, *P. chrysosporium* PcGH61D has notable differences in the loop structures near the binding face emphasising the diversity of the LPMOs.

The main difference compared to other basidiomycetes and ascomycetes is that white rot basidiomycetes exclusively harbour variable sets of genes encoding ligninolytic enzymes (Table 3) (Fernández-Fueyo et al., 2012; Floudas et al., 2012; Hori et al., 2014b; Martinez et al., 2004; Olson et al., 2012). While all the white rot fungi harbour five to 16 copies of the genes encoding AA2 MnPs, the number of genes encoding AA2 LiPs and AA2 VPs is highly variable between different white rot fungal species (Hori et al., 2014b; Mäkelä et al., 2014a). All white rot fungi with genome sequence available, except *P. chrysosporium*, *Phanerochaete carnosa* and *Phlebiopsis gigantea*, have several AA1_1 laccase-encoding genes (Hori et al., 2014b; Mäkelä et al., 2014a; Suzuki et al., 2012). In general, the characterization of white rot fungal CAZymes has been concentrated on ligninolytic enzymes (Baldrian, 2008; Hatakka, 2005).

Genes encoding H_2O_2-producing oxidoreductases from AA3 GMC superfamily and AA5_1 CRO group are widely present in white rot fungi (Hori et al., 2014b; Mäkelä et al., 2014a). All the currently sequenced white rot fungi harbour at least one AA3_3 AOX/MOX, and one to nine genes encoding AA5_1 CROs (Hori et al., 2014b; Mäkelä et al., 2014a). The highest number of genes encoding AA3_3 AOX/MOXs (14 and 7, respectively) is present in the genome of *Stereum hirsutum* (Floudas et al., 2012).

1.6 Expression and secretion of the CAZyme-encoding genes and enzymes of white rot fungi in plant biomass derived substrates

The expression and secretion of the CAZyme-encoding genes and enzymes have been studied in the several basidiomycetes under different culture conditions (Alfaro et al., 2014; Mäkelä et al., 2014a). In this chapter, the studies covering those aspects on the model white rot fungal species are presented.

P. chrysosporium is probably the most studied white rot fungus and it is able to completely degrade cellulose and hardwood hemicelluloses into their building blocks (Martinez et al., 2004; Vanden Wymelenberg et al., 2006). In contrast to the ligninolytic (carbon-limited) culture conditions, several cellulose-degradation related CAZymes have been upregulated and secreted in microcrystalline cellulose (Avicel) cultures of *P. chrysosporium* (Vanden Wymelenberg et al., 2009, 2006, 2005). This suggests that the fungus does not produce cellulolytic CAZymes constitutively. In addition, *P. chrysosporium* expresses LPMO and CDH-encoding

genes during growth on Avicel and secretes the corresponding enzymes in cellulose- and xylan-containing cultures together with the classical cellulases, i.e. CBHs, EGs and BGLs (Hori et al., 2011; Vanden Wymelenberg et al., 2009).

In contrast to cellulases, *P. chrysosporium* has been suggested to constitutively express the genes encoding hemicellulolytic and pectinolytic enzymes, including GH10 XLN, a putative GH28 PXG and a putative CE1 AXE-encoding genes, which the fungus expresses in both Avicel and ligninolytic cultures. Also, the corresponding enzymes have been secreted in those cultures (Vanden Wymelenberg et al., 2009, 2006, 2005). *P. chrysosporium* expresses ligninolytic genes in cultures containing aspen, red oak, pine or Avicel (Sato et al., 2009; Vanden Wymelenberg et al., 2011, 2010, 2009). In aspen-containing cultures of *P. chrysosporium*, modest upregulation of two LiP genes and high expression of MOX-encoding genes have been detected (Vanden Wymelenberg et al., 2010). The active production of H_2O_2 by *P. chrysosporium* in lignocellulosic substrates have also been shown by the high expression of MOX and CRO-encoding genes and secretion of the corresponding enzymes in pine-containing cultures (Vanden Wymelenberg et al., 2011). Similarly to *P. chrysosporium*, high expression of AOXs have been detected during the growth of the white rot fungi *Pycnoporus coccineus* and *P. gigantea* in aspen and/or pine cultures (Couturier et al., 2015; Hori et al., 2014b; Vanden Wymelenberg et al., 2011).

In several studies, the plant-polysaccharide-degrading ability of *P. chrysosporium* has been compared to other basidiomycetes, e.g. *Ceriporiopsis (Gelatoporia) subvermispora* and *P. carnosa*. *C. subvermispora* is a selective white rot fungus that removes mainly lignin and hemicelluloses while leaving cellulose almost intact. Although the distribution of its GH families is similar to *P. chrysosporium*, the selective degradation caused by *C. subvermispora* has been suggested to be due to reduced expression of cellulolytic GH5, GH6, GH7 and GH12 genes (Fernández-Fueyo et al., 2012). *C. subvermispora* has been proposed to compensate the low cellulase expression by a higher dependence on oxidoreductases, which is in accordance with its preference for lignin depolymerisation (Fernández-Fueyo et al., 2012).

P. carnosa, another representative of the genus *Phanerochaete*, and *P. gigantea* are white rot fungi found on softwoods, while most of the other widely studied species including *P. chrysosporium* are typically present on hardwoods (Hori et al., 2014b; Suzuki et al., 2012). Similar pattern of GH3, GH6 and GH7 cellulases, GH10 and GH11 XLNs, GH43 debranching hydrolases and CE1 glucuronoyl esterases together with putative AA9 LPMOs have been detected in the secretomes of *P. carnosa* and *P. chrysosporium* (Mahajan and Master, 2010). In contrast to

P. chrysosporium, hemicellulases specifically related to softwood degradation have been observed in cellulose-containing cultures of *P. carnosa* and in pine-containing cultures of *P. gigantea* supporting the adaptation of these species for conversion of softwood (Hori et al., 2014b; Mahajan and Master, 2010; Vanden Wymelenberg et al., 2009, 2006).

High expression of MnP and LiP-encoding genes were detected at early stages of growth of *P. carnosa* on fir, pine, spruce and maple, followed by the prolonged expression of CAZymes at later stages of growth (MacDonald and Master, 2012; MacDonald et al., 2011). Corresponding ligninolytic enzymes have also been observed in the spruce cultures of *P. carnosa* (Mahajan and Master, 2010). In line with the high expression of H_2O_2-producing enzymes encoding genes by *P. chrysosporium* during growth on plant biomass, majority of the ligninolytic enzymes detected in the *P. carnosa* secretomes resulting from growth on Avicel or spruce were putative H_2O_2-producing oxidoreductases (Mahajan and Master, 2010). Selective white rot fungus *P. ostreatus*, which is the second common edible mushroom, has also been shown to overproduce four laccases, one VP and one MnP in poplar and wheat straw cultures, thus corroborating their essential role in white rot fungal lignocellulose degradation (Fernández-Fueyo et al., 2016).

1.7 Plant-biomass-modifying enzymes in biotechnological applications

The most abundant renewable material, plant biomass, and the extensive repertoire of the enzymes degrading its complex structure opens up various prospects in biotechnological applications. The products of the enzymatic catalysis of plant biomass can be used as precursors to produce bio-based fuels, chemicals, food, feed or materials (Himmel et al., 2007). Main drivers towards the bio-based economy include slowing the pace of the global climate change and reducing the use of non-renewable feedstocks through replacement with renewable raw materials (Sheldon, 2014).

1.7.1 Biorefinery concept

Biorefineries utilize renewable raw materials aiming to reduce the CO_2-emissions, minimize the waste by using all the formed side products and avoid the use of toxic or hazardous substances (Sheldon, 2014). Currently, the main target of biorefineries is to convert lignocellulosic non-food biomass to products, heat and power.

Complex structure of lignocellulose makes it a versatile raw material, but also hampers the conversion processes.

Prior the production of fuels or chemicals, the lignocellulosic material needs to be depolymerised (Sheldon, 2014). First the biomass is usually pre-treated with physical and/or chemical means, e.g. with steam and pressure or with acids in high temperatures (Mosier et al., 2005). The main aim of the pretreatment is to open up the lignin and hemicellulose structure, which then enables the hydrolysis of the carbohydrates. The harsh pretreatment conditions create inhibitory compounds, which can hinder the following hydrolysis or fermentation steps (Palmqvist and Hahn-Hägerdal, 2000). Fungal laccases have been studied in the detoxification of the lignin-derived phenolic inhibitory compounds after pretreatment and in the hydrolysis of plant biomass (Kudanga and Le Roes-Hill, 2014).

After pretreatment, the carbohydrates of the biomass are hydrolysed to C5 and/or C6 sugars, depending on the raw material and the (hemi-)cellulases used for the hydrolysis. The released sugars can be used as precurors in biotechnological or chemical processes. In biotechnological approaches sugars can be directly fermented to lower alcohols, diols and mono- and di-carboxylic acids (Sheldon, 2014). In addition, alcohols and diols can be further processed to commodity hydrocarbon chemicals, which are likely to increase their proportion as the end-products of biomass refineries (Rass-Hansen et al., 2007).

The hydrolysis step is a demanding enzymatic process; thus efficient cellulases, CBHs, EGs and BGLs that tolerate the inhibiting compounds and are able to overcome other hindrances, such as crystallinity of cellulose and end-product inhibition, are still needed. LPMOs can be used in hydrolysis to open up new chain ends to CBHs thereby enhancing the overall hydrolysis (Horn et al., 2012).

Currently, the ascomycete *T. reesei* is the most common source of the commercial lignocellulose-degrading enzyme cocktails, which contain mostly its CBHs (Horn et al., 2012). Since *T. reesei* possesses only two LPMO-encoding genes, the commercial cellulose-degrading enzyme cocktails such as Cellic CTec2 and CTec3 sold by Novozymes A/S are supplemented with LPMOs to improve the hydrolysis (Cannella et al., 2012; Harris et al., 2014). Cellic CTec3 has also EG, BGL and xylanase acitivities (Mahajan et al., 2016). While processivity of *T. reesei* enzymes is possibly necessary for effective degradation of crystalline cellulose, it decreases the speed of the overall hydrolysis (Horn et al., 2012, 2006; Igarashi et al., 2011). To solve this, the use of more synertistically acting enzymes or less processive enzyme cocktails have been suggested (Horn et al., 2006; Igarashi et al., 2011). However, this would also require improvement of the biomass pretreatment step in order to increase the amount of amorphous regions in cellulose. So far the

application of basidiomycete enzymes in the commercial enzyme cocktails has been scarce, which is largely due to the challenges in the efficient production of basidiomycete enzymes in ascomycete hosts (Casado López et al. 2016). However, the genome sequences show that basidiomycete fungi hold a huge potential with respect to enzymes that could be used for lignocellulose conversion in industrial applications.

2 Aims of the study

The main objective of this study was to explore the biotechnological potential of the white rot fungus *D. squalens*. The aim was to study plant biomass degradation caused by *D. squalens* at different levels from mRNA to the protein production and enzyme activities. This fungus was selected as a model organism based on its selectivity towards lignin and its capacity to degrade spruce wood, which is economically important raw material in mechanical pulping in Northern Europe and North America. While intensive studies on white rot fungi have been concentrated on their ability to degrade lignin, the research of fungal plant cell wall polysaccharide degradation is mainly focused on the studies of ascomycete fungi. Therefore the goal of this study was to elucidate this less explored aspect of plant biomass degradation of white rot fungi.

Since gene expression of classical cellulases has not been investigated together with oxidative polysaccharide acting enzymes including the more recently found LPMOs in white rot fungi, this study aimed to determine expression of cellulose degradation related genes of *D. squalens* on different plant biomass substrates. In order to evaluate how *D. squalens* has adapted to its natural substrate at the molecular level, the transcriptome and exoproteome of the fungus were studied in wood and non-woody substrates. CBH and CDH enzymes of *D. squalens* were selected for detailed investigation. They were produced, biochemically characterized and their contribution to saccharification of different plant biomass was studied.

The hypothesis of this study was that white rot fungi have a full repertoire of plant cell wall degrading enzyme activities. As a basis for this work, a comprehensive literature review of the current knowledge on degradation of plant polysaccharides by basidiomycetes was performed (Publication I). The specific aims of the work were:

1. To determine the time-dependent expression and production of hydrolases and oxidative (hemi-)cellulose acting enzymes in microcrystalline cellulose and spruce wood cultures of *D. squalens*. (Publication II)
2. To clarify the adaptation of *D. squalens* to wood and non-woody substrates by transcriptome and exoproteome analysis. (Manuscript III)
3. To characterize CBH and CDH enzymes of *D. squalens* and test their contribution to the saccharification of plant biomass. (Publication IV)

3 Materials and methods

The key materials and methods used in this work are summarized in this chapter and all the methods are listed in Table 4. The materials and methods are described in detail in the original publications and manuscript (II-IV).

Table 4 Methods used in this work.

Method	Short description	Publication
Cultivation of fungi	Liquid cultures	II, III, IV
	Submerged cultures	II, III, IV
	Solid-state spruce wood cultures	II
Enzyme activity measurements	CBHI (4-methylumbelliferyl-β-D-lactoside)	II, IV
	EG (hydroxyethyl cellulose)	II, IV
	XYL (birch/beech xylan)	II, IV
	BGL (4-nitrophenyl β-D-glucopyranoside)	II, IV
	Laccase (2,6-dimethoxyphenol)	II, IV
	MnP (Mn^{2+}-$Mn^{3+)}$	IV
	CDH (2,6-dichlorophenol-indophenol)	IV
Enzymatic hydrolysis	Released sugars (DNS)	IV
RNA extraction	CTAB-based method	II
	TRI Reagent (Sigma-Aldrich)	IV
	CTAB and RNeasy Plant Mini kit (Qiagen)	III
cDNA synthesis	Smart RACE cDNA amplification kit (Clontech), Superscript III reverse transcriptase (Invitrogen)	II, III, IV
Real time quantitive PCR	Maxima SYBR Green (Fermentas)	II, III
Cloning	pJET 1.2/blunt vector	IV
Recombinant protein production	*Trichoderma reesei*	IV
Protein purification	FPLC (ÄKTA, GE Healthcare)	IV
Statistical analyses	Normality test	II, IV
	Repeated measures ANOVA	II
	t-test	II
	ONE-way ANOVA	IV

DNS, dinitrosalisylic acid; CTAB, N-cetyl-N,N,N-trimethylammonium bromide; FPLC, fast protein liquid chromatography

3.1 Cultivation of *D. squalens*

D. squalens FBCC312 was obtained from the Fungal Biotechnology Culture Collection (FBCC), Department of Food and Environmental Sciences, University of Helsinki, Finland (email: fbcc@helsinki.fi) and maintained on on 2% malt agar plates (2% (wt/vol) malt extract (Biokar, France), 2% (wt/vol) agar agar (Biokar, France)). For the inoculumn *D. squalens* was cultivated stationary in 250 ml Erlenmeyer flasks containing 75 ml liquid 2% (wt/vol) malt extract medium (Publications II and IV) or in 75 ml liquid low-nitrogen-asparagine-succinate (LN-AS) medium supplemented with 6.8 mM glycerol (LN-AS-glycerol) (pH 4.5) (Manuscript III).

Solid-state spruce wood cultures (Publication II) contained 2 g (dry weight) of Norway spruce (*Picea abies*) wood sticks on top of the 1% (wt/vol) water agar. The agitated (120 rpm) microcrystalline cellulose (Avicel) cultures (Publications II and IV) were performed in 250 ml Erlenmeyer flasks containing 100 ml liquid medium (pH 6.0), which consisted of 2.5 g/l meat peptone (Lab MLimited, UK), 1 g/l yeast extract (LabMLimited, UK), 1 g/l potassium dihydrogen phosphate (Sigma–Aldrich, Japan), 0.5 g/l magnesium sulphate (Merck, Germany) and 1% (wt/vol) Avicel® PH-101 cellulose (Fluka, Ireland).

Semi-solid cultures (Manuscript III) contained 100 ml LN-AS culture medium supplemented with 1 g of fresh and dried spruce sapwood (*Picea abies*) sawdust (1-2 mm in diameter), fresh and dried aspen sapwood (*Populus tremula*) sawdust (1-2 mm in diameter), powdered wheat bran (WB; Windkorenmolen De Vlijt, Wageningen, The Netherlands) or powdered cotton seed hulls (CSH; a gift from Garold Gresham at the Interfacial Chemistry Idaho National Laboratory, Idaho Falls, USA). All the cultures were performed at 28 ºC.

3.2 RNA extraction and transcript profiling with RT-qPCR and RNA-seq

The *D. squalens* total RNA was extracted from the fungal-colonised spruce sticks and the fungal mycelia from the submerged cultures (Publication II and Manuscript III). Mycelia was milled with grinder (IKA, Germany) or ground in mortar under liquid N_2. RNA was extracted by N-cetyl-N,N,N-trimethylammonium bromide (CTAB, Sigma, Germany) -based method (Chang et al., 1993). Concentration of DNaseI (RNase free, Fermentas) -treated RNA was determined spectrophotometrically at 260 nm using NanoDrop ND-1000 (NanoDrop

Technologies Inc., USA) (Publication II). In the Manuscript III RNA was further purified with RNeasy Plant Mini kit (Qiagen, Germany), and quantity and integrity of RNA were determined with NanoDrop ND-1000 and RNA6000 Nano Assay (Agilent 2100 Bioanalyzer, Agilent Technologies, USA).

Prior to RT-qPCR (Publication II and Manuscript III), cDNA was synthesised according to the instructions of the manufacturers (Table 4). Gene specific primers were designed to overlap one intron according to the genome sequence of *D. squalens* LYAD-421 SS1 or the *cel7b* sequence of *D. squalens* FBCC312. The primer pairs were validated by determining the amplification efficiency which varied from 91.9% to 108.7% (R^2 >0.98). Dissociation curves resulted in single peak thus confirming the specificity of the primers. The RT-qPCR amplification products were further verified by running them on 1% agarose gels and sequencing (Macrogen Corp., The Netherlands).

For RNA-seq (Manuscript III), purification of mRNA, synthesis of cDNA library and sequencing reactions were conducted in the BGI Tech Solutions Co., Ltd. (Hong Kong, China), and the data analysis and statistical treatment of data were performed as described previously (Patyshakuliyeva et al., 2015). On average, ~10 million reads of 51 bp per sample were obtained. The sequence reads were mapped to the genome sequence of *D. squalens* LYAD-421 SS1 (v1.0 annotation, the Joint Genome Institute). Results were quantified by using RPKM method (Mortazavi et al., 2008). Genes with RPKM value from 20-100 were considered as lowly, 100-300 as moderately and over 300 as highly expressed. Genes with RPKM value lower than 20 under all conditions were considered as not significantly expressed and filtered out of the cluster analysis.

The clustering of the RNA-seq data of CAZyme-encoding genes was performed using Genesis (Sturn et al., 2002) with the Euclidean distance and complete linkage. A cut-off of fold change of >1.5 and *P*-value of <0.05 were used to identify differentially expressed genes between the time points and the statistical methods were conducted according to Patyshakuliyeva et al. (2015). The RNA-seq data were deposited to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (Edgar et al., 2002) with accession number: GSE79674. The overall workflow of transcriptome and exoproteome analyses is presented in Fig. 4.

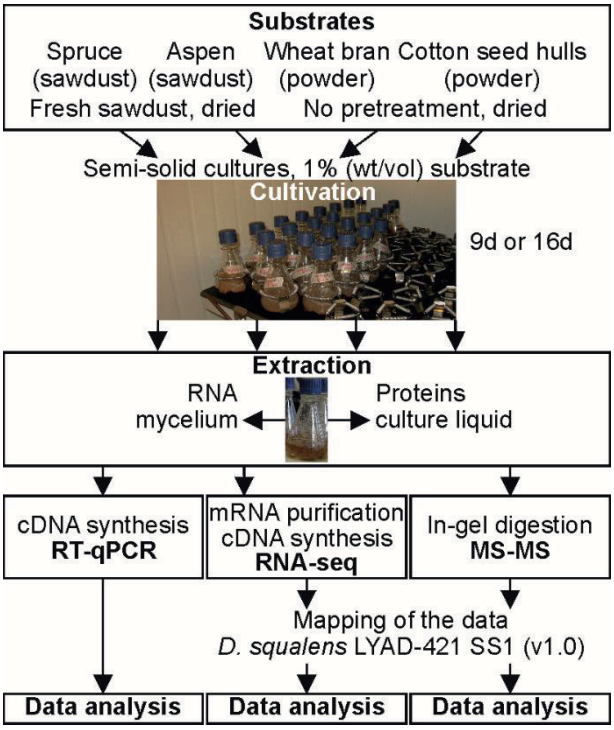


Figure 4 Workflow of the transcriptome and exoproteome experiments.

3.3 Exoproteome analysis, protein purification and biochemical characterization

For the secretome analysis (Manuscript III) proteins were extracted from *D. squalens* culture liquids. First, fungal mycelium was separated from culture liquid by filtration and centrifugation. Proteins were precipitated with 20% tricloroacetic acid, 20 mM dithiothreitol (DTT) and 80% acetone solution on ice for 1 h, and resuspended and reprecipitated with 20 mM DTT and 80% acetone solution overnight at -20°C.

In-gel digested peptide extracts were analysed on LTQ-Velos-Orbitrap mass spectrometer (Thermo-Scientific, San Jose, CA) and the acquired MS-MS data were searched against the *D. squalens* LYAD-421 SS1 (v1.0 annotation, the Joint Genome Institute) database containing 12290 protein sequences for peptide/protein identification as previously reported (Ozturkoglu Budak et al., 2014). Total normalized ion profiles from the top three identified peptides for each protein as determined in Scaffold (Proteome Software, Portland, OR) was used to quantify relative levels of secreted proteins. The amount of protein was defined as

percentages of total identified secreted proteins within individual samples. For analysis, secreted proteins representing higher than 0.02% of the total exoproteome were considered as significantly produced and lower than that were removed. Proteins accumulated to 0.02-1% level of total exoproteome were considered low and 1-5% moderate while those accumulated >5% were deemed high.

For the purification of *D. squalens* CBHI fraction and CDH (Publication IV), the frozen Avicel-culture liquid was melted, filtered and concentrated. CBHI and CDH activities were fractionated with anion exhange and size exclusion chromatography with Äkta Explorer apparatus (GE Healthcare, Sweden).

Protein fractions separated by SDS-PAGE in MiniProtean TGX gels (Bio-Rad, USA) and analysed by isoelectric focusing (IEF) were visualised with EZBlue staining reagent (Sigma-Aldrich, USA). Their protein concentrations were determined by using bicinchoninic acid method (Pierce BCA Protein Assay kit, Thermo Scientific, USA). LC-MS/MS sequencing was used to obtain the internal peptides of the purified CBHI fraction and CDH (Proteomics Unit, Institute of Biotechnology, University of Helsinki, Finland).

Extracellular enzyme activity measurements were performed in 96-well plates as triplicates using Tecan Infinite M200 plate reader (Tecan, Austria). The only exception was CDH activity, which was determined by using Shimadzu PharmaSpec UV-Vis-1700 spectrophotometer.

3.4 Enzymatic hydrolysis of plant-derived biomass

Small scale enzymatic hydrolysis of Avicel, sugar beet pulp (powdered, SBP; Danisco Ingredients, Denmark) and wheat bran (powdered, WB; Windkorenmolen De Vlijt, Wageningen, The Netherlands) was conducted in 250 µl reactions with 1% (w/v) solid concentration of substrates in 50 mM Na-citrate buffer (pH 4.0) for 4 h at 50°C under agitation (1400 rpm). The hydrolysis reactions contained purified CBHI fraction and CDH of *D. squalens*, recombinant Cel6A of *D. squalens* (rCel6A), or a commercial laccase from *M. thermophila* (Novozym 51003, Novozymes, Denmark) in different combinations. After hydrolysis, the supernatants were collected and the amount of reducing sugars was determined by using dinitrosalisylicacid (DNS) method (Miller, 1959).

4 Results and discussion

4.1 Transcript levels of selected cellulolytic enzyme-encoding genes on spruce and in microcrystalline cellulose (II)

The expression patterns of genes encoding four CBHs, five LPMOs and one CDH were determined by RT-qPCR during the growth of *D. squalens* on solid-state spruce wood and submerged microcrytalline cellulose (Avicel) cultures. Selected genes showed variable expression patterns in both cultures in the course of cultivation (Fig. 5). The most highly expressed CBH-encoding gene was *cel7c* in both cultures and it showed constitutive expression on spruce during the four-week cultivation. The other CBH-encoding genes were expressed at low levels in both cultures. Transcript levels of *cel6a* and *cel7a* decreased, whereas the levels of *cel7b* increased significantly ($p<0.05$) from day seven to day 28 on spruce (Fig. 5A). In Avicel cultures, the transcript amount of *cel7b* and *cel6a* decreased significantly ($p<0.01$) and *cel7a* was expressed at same level during the cultivation (Fig. 5B).

Corresponding to the results of this study, Avicel has been shown to upregulate the expression of *cbh* genes in *P. chrysosporium* (Vanden Wymelenberg et al., 2009) and divergence in the expression levels of *cbh* genes have been detected in e.g. semi-solid ball-milled aspen cultures of *P. chrysosporium* and *C. subvermispora* (Fernández-Fueyo et al., 2012; Vanden Wymelenberg et al., 2006). In addition, *cel7* and *cel6* genes of *P. chrysosporium* have shown time-dependent regulation on solid-state aspen wood chip (Vallim et al., 1998) and in submerged Avicel and ball-milled straw cultures (Broda et al., 1995), respectively. This is also supported by the time-dependent expression of the *cel6* of *P. carnosa* in cultures containing ground wood (fir, pine, spruce or maple) as a carbon source (MacDonald and Master, 2012). Together these findings suggest that white rot fungal CBH-encoding genes are regulated differently and indicate possible temporal roles for the individual enzymes during the degradation of wood cellulose.

D. squalens expressed the *lpmo* genes carrying CBM (*lpmo3-5*) at significantly higher level ($p<0.01$) than those without CBM (*lpmo1-2*) on spruce (Fig. 5A). On contrary, in Avicel cultures similar transcript levels were observed for *lpmo2* and the *lpmo*s with CBMs (*lpmo3-5*) after 14 days of cultivation ($p>0.05$). On spruce, *lpmo2* and the CBM-containing *lpmo*s (*lpmo3-5*) were expressed constitutively, whereas the expression of *lpmo1* increased significantly ($p<0.01$) towards the end

of the cultivation. In addition to the expression of LPMO-encoding genes, *D. squalens* constitutively expressed low levels of *cdh* in Avicel cultures and on spruce during the first three weeks of cultivation. Corresponding to previous studies, several putative *lpmo*s have been upregulated in the aspen cultures of *P. chrysosporium* and *C. subvermispora* (Fernández-Fueyo et al., 2012) and in Avicel-containing liquid cultures of *P. chrysosporium* (Vanden Wymelenberg et al., 2009). However, more studies on the *lpmo* expression in white rot fungi are still needed in order to complete the overall role of the corresponding enzymes in plant biomass degradation.

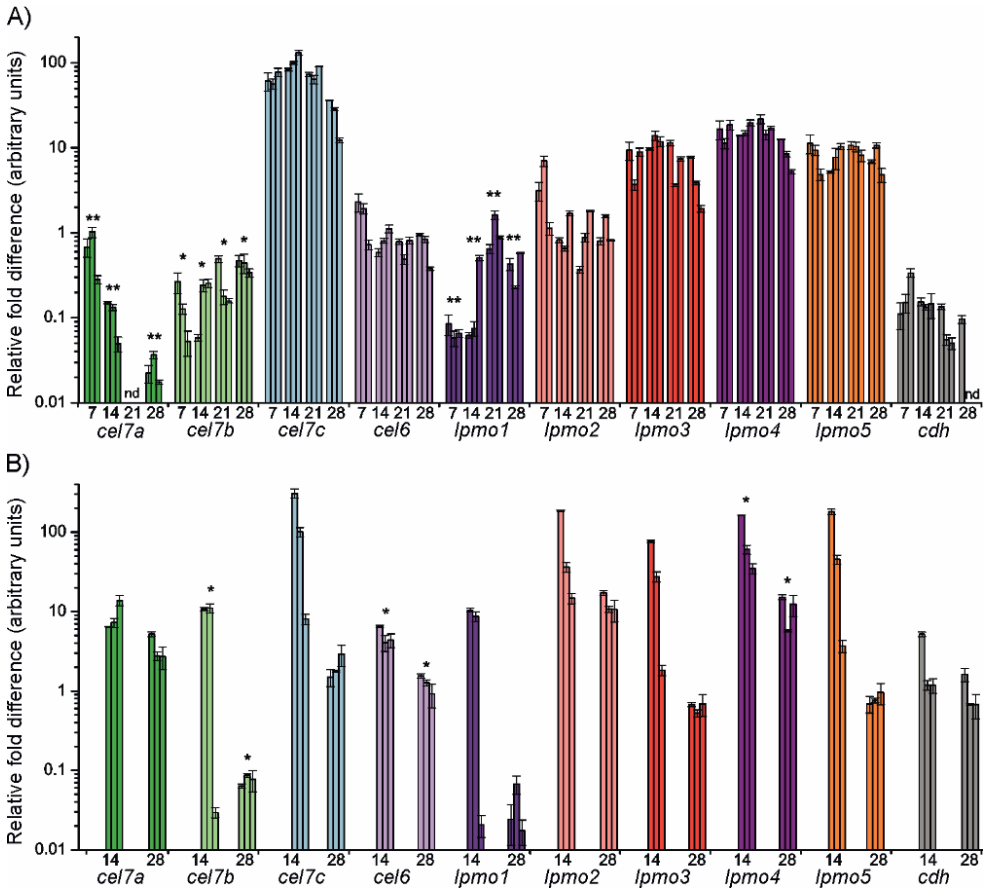


Figure 5 Expression of the selected genes encoding CBHs, LPMOs and CDH A) on spruce and B) in Avicel cultures of *D. squalens* determined by RT-qPCR. Three columns at the indicated time points represent the biological replicates and the standard deviation of the RT-qPCR reactions is shown as error bars. * $p<0.05$ and ** $p<0.01$, significant difference between the time points by repeated measures test. nd = not detected.

4.2 Adaptation of *D. squalens* to wood and non-woody substrates (III)

4.2.1 Selection of plant biomass substrates

The molecular response of *D. squalens* to its natural carbon sources (wood) and non-woody plant biomass was analysed to clarify the adaptation of the fungus to its natural substrates. An initial experiment was performed in order to select appropriate substrates and time points for transcriptomic and exoproteomic analyses. The fungus was cultivated on seven different plant-derived biomass substrates (Avicel, spruce, wheat bran, cotton seed hulls, apple pectin, beech xylan and guar gum) and two control carbon sources (glucose and cellobiose) for 21 days. After nine days of cultivation extracellular proteins were observed in all culture liquids by SDS-PAGE analysis (Fig. 6), and thus this time point was chosen as an early phase of substrate degradation. Clear differences were observed in protein profiles between day 9 and day 16, and therefore day 16 was selected as the time point for later phase of substrate degradation.

D. squalens produced the most variable set of extracellular proteins in the cultures amended with the lignocellulosic substrates such as softwood (spruce sawdust) and monocot (powdered wheat bran) and dicot (powdered cotton seed hulls) plants. In addition to these substrates, a representative of hardwood (aspen sawdust) was selected as carbon sources for cultivations for RNA-seq and exoproteome analyses. The selected substrates varied in their chemical composition (Table 5).

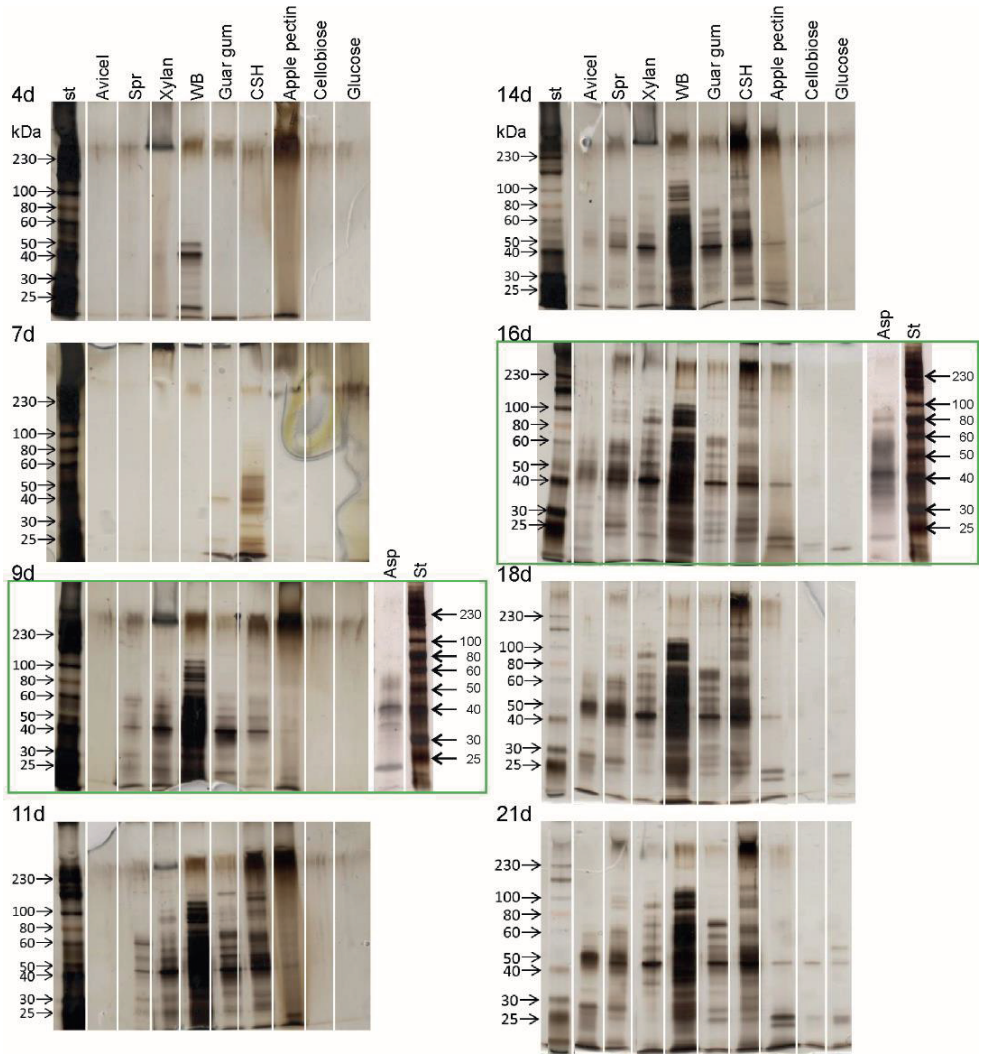


Figure 6 Extracellular protein profiles of *D. squalens* grown in liquid cultures with different plant-derived substrates as sole carbon sources. Crude culture liquid samples after 4 to 21 days of cultivation were analysed on SDS-PAGE gels. St, molecular weight standard; kDa, kilodalton; Spr, spruce; WB, wheat bran; CSH, cotton seed hulls; Asp, aspen. Figure from Manuscript III.

Table 5 Monosaccharide (mol%) and polysaccharide composition of spruce, aspen, wheat bran and cotton seed hulls representing softwood, hardwood, monocots and dicots, respectively.

Plant material	Rha	Ara	Xyl	Man	Gal	Glu	Ura	Polysaccharides[a]
Spruce	0	3	12	17	3	58	7	Cellulose, galactomannan, glucuronoxylan
Aspen	1	2	29	5	1	53	8	Cellulose, glucuronoxylan, galactomannan
Wheat bran[b]	0	17	35	1	2	42	3	Cellulose, (arabino)xylan
Cotton seed hulls	1	16	26	3	7	40	8	Cellulose, xylan, xyloglucan, pectin

Rha, rhamnose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glu, glucose, Ura, uronic acid. [a]Main polysaccharide composition is predicted from the monomer values. [b](Ozturkoglu Budak et al., 2014). Table modified from Manuscript III.

4.2.2 Transcriptomic and exoproteomic response of *D. squalens* to wood and non-woody plant biomasses

During growth on the selected plant biomasses, *D. squalens* notably expressed (RPKM>20) 297 putative CAZyme-encoding genes, 135 of these were related to plant cell wall degradation (Fig. 7A). In addition, 12 expansin-like and two FAE-encoding genes were notably expressed and included in the analyses. At the protein level, 175 notably secreted (%-value>0.02) CAZymes were detected, of which 120 were related to plant cell wall degradation (Fig. 7B).

When the expression of the selected genes were analysed on all of the substrates, the time point representing early degradation of spruce and aspen were the most similar to each other, while the later time point of aspen clustered with the non-woody substrates (Fig. 8). In cluster analysis, gene expression levels at the later time point from spruce cultivations differed the most from the other substrates, especially in the clusters G, K and R (Fig. 8). In general, the repertoire of the CAZymes expressed and secreted was similar in softwood and hardwood, and corresponded to a typical set of CAZymes described for white rot fungal species such as *P. coccineus* (Couturier et al., 2015; Riley et al., 2014).

Overall, the CAZyme-encoding transcripts and secreted enzymes correlated well (Fig. 9-12). However, several expansin-like genes were highly expressed, for which no corresponding proteins were detected. Most of the expansin-like genes (8-12 out of 13) were expressed in all the studied cultivation conditions but only two expansin-like proteins were observed in aspen and one in wheat bran cultures of

D. squalens. Similarly, high expression of expansin-like genes has also been detected in *P. carnosa* grown on aspen and spruce (Suzuki et al., 2014). Fungal expansin-like proteins possibly participate in development of fungal cell wall or modification of plant cell wall structure (Cosgrove, 2015; Quiroz-Castañeda et al., 2011; Tovar-Herrera et al., 2015; Veneault-Fourrey et al., 2014). The differences in transcriptome and exoproteome can be due to various reasons. One explanation is that the proteins are bound to fungal cell wall or biomass, which hinders their detection in exoproteome analysis (Hori et al., 2014a). This was likely in the case of *D. squalens* expansin-like proteins, which scarcely identified in the exoproteomes. In addition, corresponding genes of three GH27 AGLs and four AA5_1 CROs detected in all cultures lacked notable expression, which can be due to the different stability of the mRNA and protein, or different rates of transcription, translation and secretion (Patyshakuliyeva et al., 2015; Vogel and Marcotte, 2012).

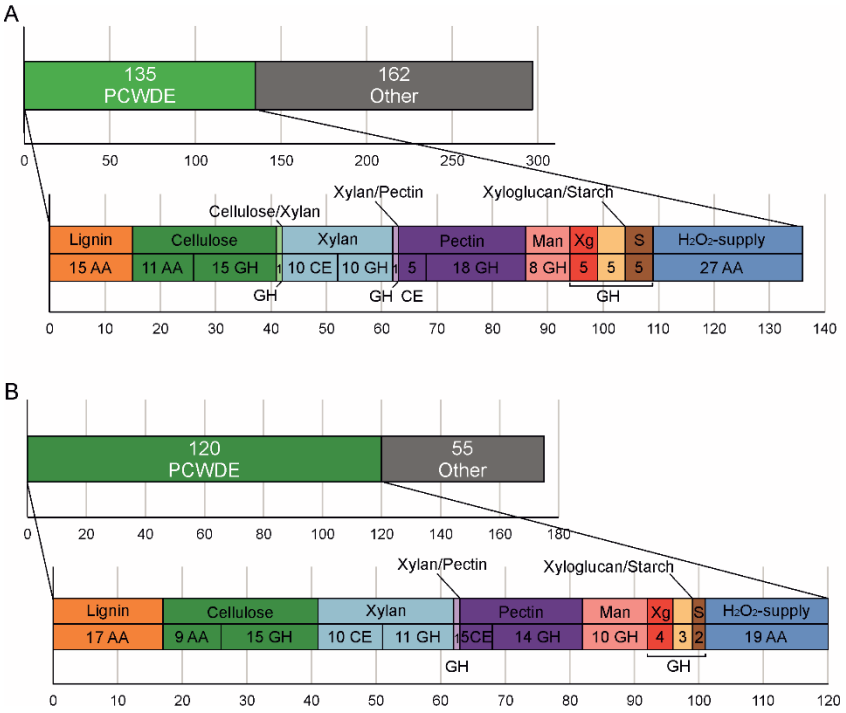


Figure 7 Total number of CAZy A) transcripts (RPKM>20) and B) extracellular proteins (%-value>0.02) detected from *D. squalens* cultivations on spruce, aspen, wheat bran and cotton seed hulls. CAZy transcripts and proteins related to plant cell wall degradation are divided by their corresponding substrates. PCWDE, plant cell wall-degrading enzymes; Xylan, heteroxylan; Man, heteromannan; Xg, xyloglucan S, starch; AA, auxiliary activities; GH, glycoside hydrolases; CE, carbohydrate esterases. Figure from Manuscript III.

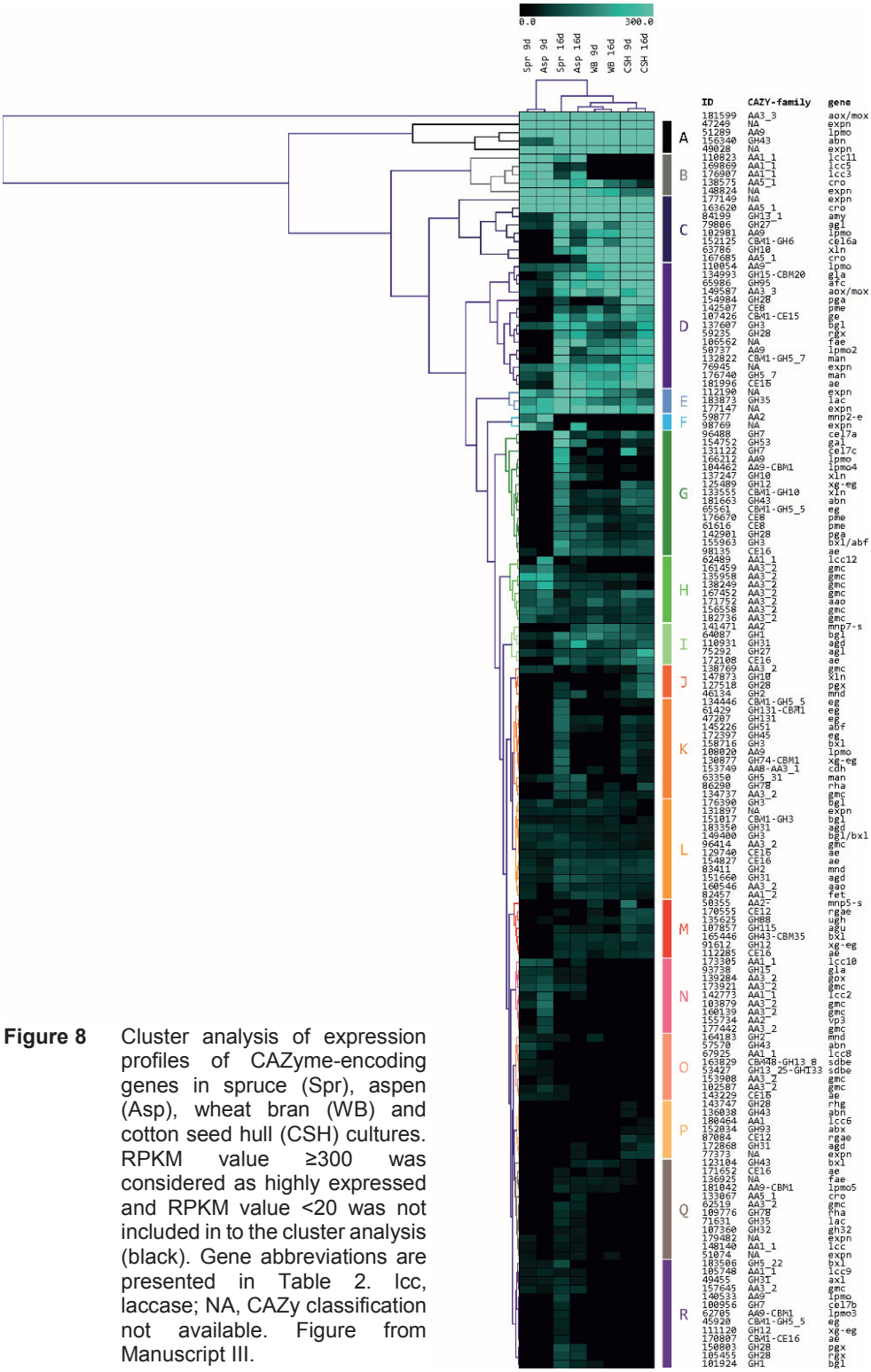


Figure 8 Cluster analysis of expression profiles of CAZyme-encoding genes in spruce (Spr), aspen (Asp), wheat bran (WB) and cotton seed hull (CSH) cultures. RPKM value ≥300 was considered as highly expressed and RPKM value <20 was not included in to the cluster analysis (black). Gene abbreviations are presented in Table 2. lcc, laccase; NA, CAZy classification not available. Figure from Manuscript III.

4.2.2.1 *Lignin degradation*

D. squalens expressed more AA1_1 and AA2 lignolytic genes at the early phase (10) than in the later phase (6) of the degradation in spruce-containing medium (Fig. 9A). In contrast, in aspen cultures the number of ligninolytic genes was similar (9-10) at both time points. The most notable difference between wood and non-woody growth substrates was that *D. squalens* expressed only two to three ligninolytic genes in the non-woody substrates. Also, the number of corresponding extracellular enzymes increased during the growth of *D. squalens* in wood cultures from five to 11 in spruce and from ten to 15 in aspen. This is in accordance with the high lignin content of spruce and aspen (25-33% and 20-25%, respectively, Sjöström, 1993).

Despite the low expression levels, the number of ligninolytic enzymes was at similar level in the non-woody (5-11) than in wood cultures. However, the number of the enzymes decreased (from 11 to 7) in time in wheat bran, whereas in cotton seed hulls it remained at the same level (5-6) during the whole cultivation. The lower number of detected ligninolytic genes and enzymes during growth of *D. squalens* on the non-woody than on the wood substrates indicated that the presence of lignin specifically induced the expression of ligninolytic genes and is not part of a general response of the fungus to the presence of lignocellulosic biomass.

In *D. squalens* wood cultures, transcripts of genes encoding extra-long MnPs and their corresponding enzymes were more abundant than short MnPs or VPs. Extra-long MnPs have been only found in a few other white-rot genomes such as *C. subvermispora* and *Punctularia strigosozonata* (Fernández-Fueyo et al., 2012; Floudas et al., 2012). Catalytically, the long and extra-long MnPs of *C. subvermispora* are similar. However, their substrate specificity and pH stability differ significantly from the short MnPs of *C. subvermispora* (Fernández-Fueyo et al., 2014). In spruce cultures, *D. squalens* produced the highest level of extra-long MnPs (Fig. 9A), which were detected at the later time point. Due to the polar tail structure of the extra-long MnPs, which has been reported to give extra stability for the proteins (Li et al., 2001, 1999), they may be advantageous especially in the degradation of recalcitrant softwood lignin. In addition, two short MnP and one VP isoenzymes were notably secreted in aspen cultures although the corresponding transcripts were not detected. *D. squalens* highly expressed (RPKM>300) two to three laccase-encoding genes in wood. Laccases were also the most abundant proteins in wood at the later time point.

In non-woody substrates, the expression profiles of CAZyme-encoding genes were highly similar during early and later time points (Fig. 9-12). In contrast to the wood cultures, the most highly expressed ligninolytic genes were those encoding short MnPs, and the corresponding enzymes were detected at low level in non-

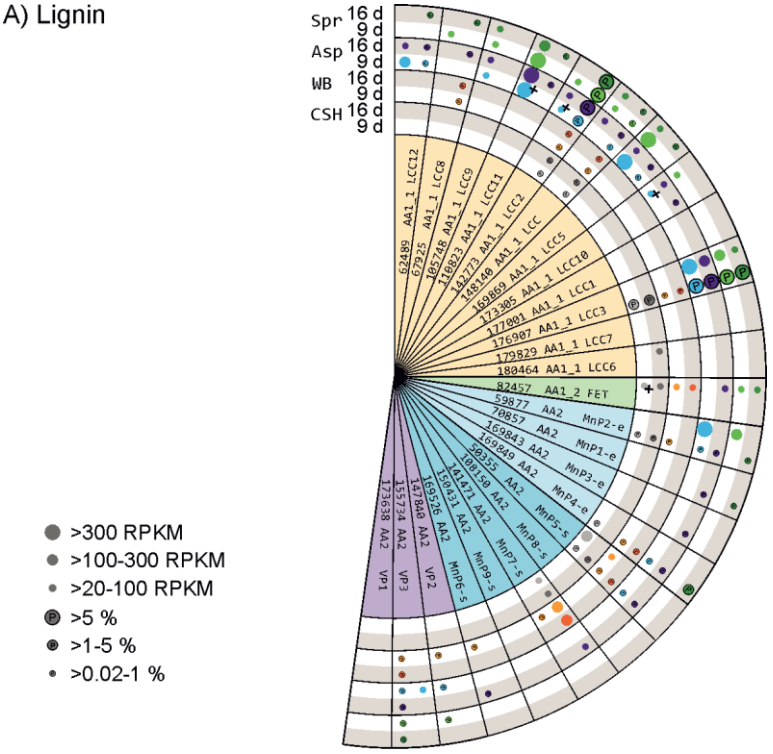
woody cultures (Fig. 9A). Extra-long MnPs and VPs were also detected at low protein levels in the non-woody cultures. Notable expression was detected only for one laccase-encoding gene in cotton seed hulls and only low amounts of up to four laccase isoenzymes were observed in both non-woody substrates.

The high expression of ligninolytic genes and several corresponding enzymes observed on both wood substrates in the early phase of degradation by *D. squalens* supports the white rot decay mechanism in which lignin depolymerisation precedes cellulose degradation. In addition, decreased expression of ligninolytic genes at the later time point suggests that the need for lignin degradation reduces after sufficient access to the plant cell wall polymers is achieved. These observations are in line with earlier studies (Fernández-Fueyo et al., 2016; MacDonald and Master, 2012). The similar pattern of abundant ligninolytic response has been detected for instance with the white-rot fungus *P. coccineus* that produces laccases during the early growth phase on wood (Couturier et al., 2015) and with the litter-decomposing fungus *Agaricus bisporus* that secretes ligninolytic enzymes in compost at early stages of growth (Patyshakuliyeva et al., 2015). In addition, similar to this study, laccases were found to be the main proteins in the secretomes of *P. ostreatus* grown on poplar (Fernández-Fueyo et al., 2016).

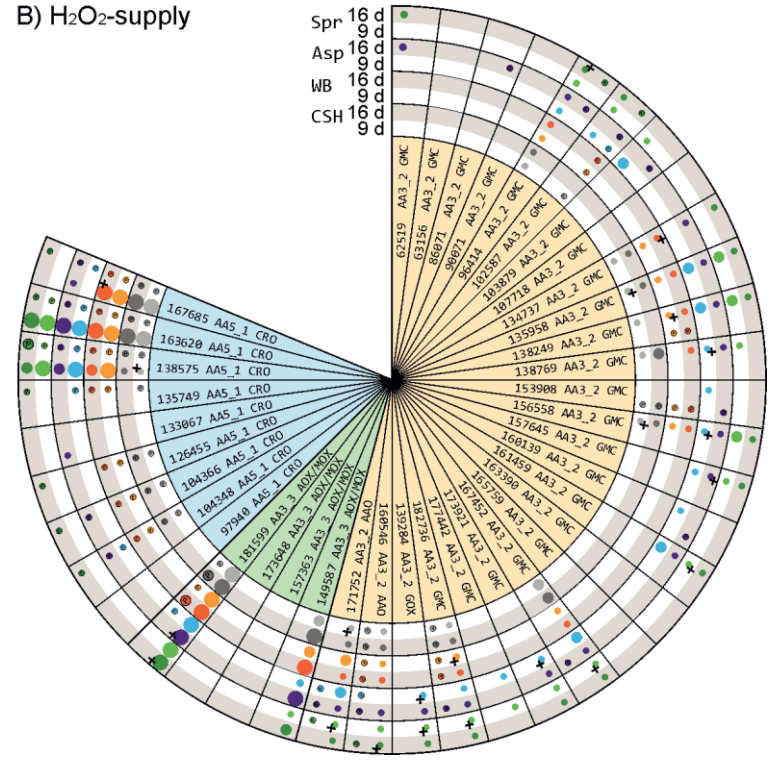
During the early phase of degradation, fewer extracellular proteins were detected in the *D. squalens* spruce cultures than in the other studied cultures (Manuscript III). This can possibly be due to slow growth of the fungus that is most probably caused by the guaiacyl subunit structure of softwood lignin, which has been suggested to be more resistant to enzymatic degradation than the guaiacyl-syringyl lignin in hardwoods (Mooney et al., 1998; Ramos et al., 1992; Sjöström, 1993). However, the overall response of *D. squalens* to wood was similar in spruce and aspen cultures.

The importance of the extracellular H_2O_2-production for the ligninolytic peroxidase activity, and therefore for the efficient lignin degradation has been shown previously (Guillén et al., 1994; Kersten, 1990). Our results are in line with the functional studies. *D. squalens* expressed higher number of putative H_2O_2-producing enzyme-encoding genes (20-21) during the growth in wood than in non-woody substrates (14-15) (Fig. 9B). In addition, the number of H_2O_2-production related enzymes increased in the course of the cultivation in wood but stayed at the same level in non-woody substrates.

A) Lignin



B) H_2O_2-supply



(See figure on previous page.)

Figure 9 A) Lignin-degradation and B) H_2O_2-supply related transcripts and extracellular proteins from spruce wood (Spr, green circles), aspen wood (Asp, blue circles), wheat bran (WB, orange circles) and cotton seed hull (CSH, grey circles) cultures of *D. squalens* after 9 and 16 days of cultivation. Difference in gene expression is significant (fold change>1.5, $p<0.05$) if the size of the circles corresponding to one gene varies; otherwise significant upregulation is marked with + or no change with =. ID numbers for the gene and predicted protein model are according to the Protein ID numbers of JGI. LCC, laccase. Other enzyme abbreviations are presented in Table 2. Circles were drawn with iTol (Letunic and Bork, 2011). Figure modified from Manuscript III.

The genes encoding AA5_1 CROs were highly expressed and several corresponding enzymes were detected in softwood and hardwood. In contrast, the genes encoding AA3_3 AOX/MOXs were highly expressed in both wood cultures, but only one of these was secreted in aspen. The high expression levels of AA5_1 CRO and AA3_3 AOX/MOX-encoding genes is in line with the studies of *P. gigantea*, *P. chrysosporium* and *P. coccineus* in wood cultures (Couturier et al., 2015; Hori et al., 2014b; Vanden Wymelenberg et al., 2010).

Low or moderate expression was detected for AA3_2 GMC oxidoreductase-encoding genes and most of them (58%) showed reduced expression at the later time point in wood. Low level secretion was detected for ten out of 24 putative GMC oxidoreductases. In addition, in the non-woody substrates low level expression and production of corresponding enzymes was detected for most of the AA3_2 GMC oxidoreductases (Fig. 9B). Transcriptome and exoproteome patterns of other putative H_2O_2-producing enzymes (from families AA5_1 and AA3_3) were similar in wood and non-woody substrates.

4.2.2.2 *Cellulose degradation*

In contrast to the ligninolytic genes, the number of expressed cellulolytic genes increased during the cultivation of *D. squalens* in spruce and aspen (from 8 to 27 and 7 to 17, respectively). In non-woody substrates, the number of expressed cellulolytic genes decreased slightly (from 18 to 13) or stayed at the same level (17-18). The number of cellulolytic enzymes increased in spruce (from 10 to 23), but stayed similar in aspen (20) cultures. Expression of at least half of the GH cellulase-encoding genes reduced over time in non-woody substrates, which was opposite to wood substrates (Fig. 10). GH7 CBHIs were the most abundant proteins that *D. squalens* secreted in both non-woody substrates (up to 15% and 16% in wheat bran and cotton seed hulls, respectively).

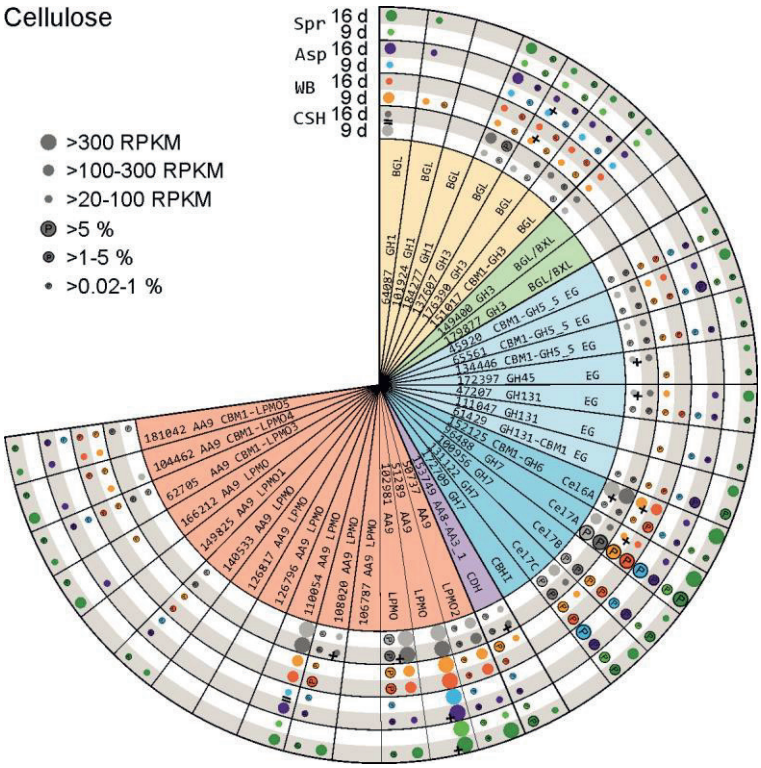


Figure 10 Cellulose-degradation related transcripts and extracellular proteins from spruce wood (Spr, green circles), aspen wood (Asp, blue circles), wheat bran (WB, orange circles) and cotton seed hull (CSH, grey circles) cultures of *D. squalens* after 9 and 16 days of cultivation. Details of the figure are presented in the caption of Fig. 9. Enzyme abbreviations are presented in Table 2. Circles were drawn with iTol (Letunic and Bork, 2011). Figure modified from Manuscript III.

D. squalens expressed a complete set of cellulolytic enzyme-encoding genes and produced the corresponding enzymes during the growth in wood and non-woody substrates. A whole repertoire of cellulolytic enzyme-encoding genes or corresponding enzymes has also been detected from other white rot fungi grown in wood-containing medium (Couturier et al., 2015; Hori et al., 2014a). All the three genes encoding CBHI isoenzymes and a gene encoding CBHII, which were also previously detected on solid spruce wood and Avicel-containing cultures (Publication II) were expressed and produced in spruce, wheat bran and cotton seed hull cultures. The corresponding enzymes were also present in the aspen cultures.

The barrier that lignin forms to hinder the utilization of cellulose at the onset of wood degradation was evident. The main difference between the transcription of ligninolytic and cellulolytic genes in wood was that transcripts of most cellulases were significantly higher (fold change >1.5, p<0.05) at day 16 compared to day 9

(Fig. 10), which is inline with the earlier studies (MacDonald and Master, 2012). However, the fungus secreted GH7 CBHIs already at the early time point and they were the second most abundant proteins detected in the wood cultures (6-10% in spruce and 8-16% in aspen). Production of CBHs and EGs have been induced already at the early stage of growth in aspen cultures of *C. subvermispora* (Hori et al., 2014a). In this study, EGs and BGLs represented a smaller proportion of the total proteins than CBHs. This is different from the secretome of *P. ostreatus* grown on poplar, where the BGLs were the most abundant cellulases (Fernández-Fueyo et al., 2016).

Expression of AA9 LPMO-encoding genes was higher (fold change >1.5, $p<0.05$) at day 16 compared to day 9 in wood cultures and low level production of eight and six out of 15 LPMO isoenzymes were detected in spruce and aspen cultures, respectively. Whereas, the expression of LPMO-encoding genes either stayed at the same level or reduced over time in non-woody cultures. LPMOs were also the second most abundant extracellular proteins (6%) at the early phase wheat bran degradation, whereas they were present in lower amounts in cotton seed hull cultures. The several expressed LPMOs and their clustering with different sets of CAZymes (Figs. 8 and 10), supports their multifunctional nature in the degradation of various lignocellulose polymers (Westereng et al., 2015).

4.2.2.3 *Hemicellulose degradation*

In *D. squalens* cultures, similar expression pattern was detected for xylanolytic and cellulolytic genes. The number of expressed xylanolytic genes increased in wood (1 up to 9), whereas in non-woody substrates their number stayed at same level (6-9). In contrast, the number of expressed mannanolytic genes (6-8) was similar among all the substrates and time points.

In wood cultures, expression of the majority of the hemicellulolytic CAZyme-encoding genes of *D. squalens* was higher (fold change >1.5, $p<0.05$) at day 16 than at day 9 (Fig. 11A-B). Despite the structural differences in the hemicellulose content of spruce and aspen (Table 5), not clear molecular level adaptation to the spruce or aspen was observed in the *D. squalens* transcriptome or exoproteome. Complete set of both xylanases and mannanases was expressed and secreted in wood which is in line with the detected CAZymes from the pine and aspen cultures of *P. coccineus* (Couturier et al., 2015).

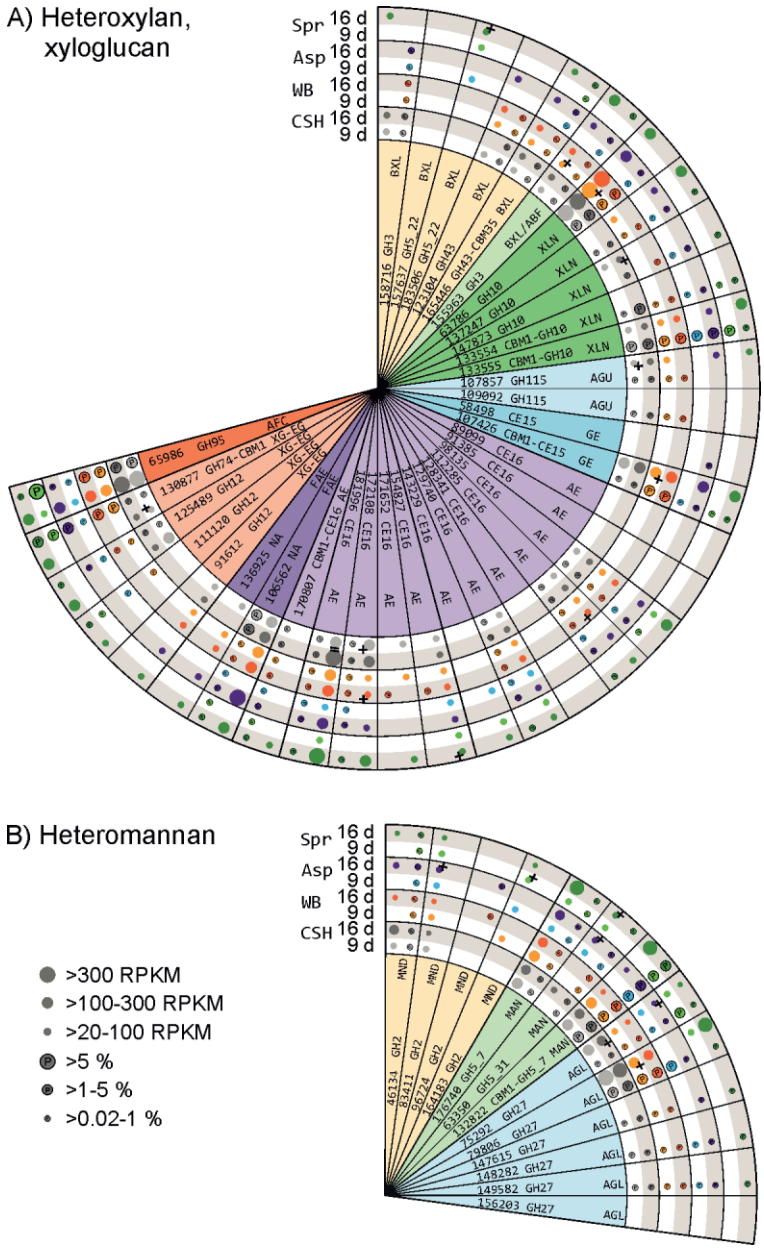


Figure 11 A) Heteroxylan, xyloglucan- and B) heteromannan-degradation related transcripts and extracellular proteins from spruce wood (Spr, green circles), aspen wood (Asp, blue circles), wheat bran (WB, orange circles) and cotton seed hull (CSH, grey circles) cultures of *D. squalens* after 9 and 16 days of cultivation. Details of the figure are presented in the caption of Fig. 9. Enzyme abbreviations are presented in Table 2. Circles were drawn with iTol (Letunic and Bork, 2011). Figure modified from Manuscript III.

The higher expression of three mannanase genes in spruce cultures compared to aspen cultures was constant with the higher mannan content of spruce. In line with that, the MANs and AGLs have been highly expressed in pine cultures of the white-rot fungus *P. gigantea* (Hori et al., 2014b). In addition, the transcriptomes from the wood cultures of *D. squalens* were all notably different from the ones from the non-woody cultures (Fig. 8). However, the transcriptome of 16-day aspen culture was more similar to the non-woody transcriptomes than to the other wood transcriptomes. This is probably due to the higher xylan content of aspen compared to spruce. Similarly, the difference in xylan content has been suggested to cause an increased expression of three XLN-encoding genes in the aspen cultures of *P. coccineus* compared to the pine cultures (Couturier et al., 2015).

Both the studied non-woody substrates, wheat bran and cotton seed hulls, contain significant amounts of xylan, which resulted in the expression of a full set of xylanolytic genes (Fig. 11A). The highest transcript level of these genes was detected for a GH10 XLN that also represented 2.8% and 2.4% of total proteins in wheat bran and cotton seed hull cultures, respectively, in the early phase of degradation. In contrast to xylan, wheat bran and cotton seed hulls contain low amounts of mannan (Table 5). However, *D. squalens* expressed several mannanolytic genes in non-woody substrates, and high transcript levels were detected for one of the six AGL-encoding genes in cotton seed hulls at both time points (Fig. 11B). In addition, *D. squalens* produced relatively high amount (3-7%) of mannanolytic proteins in non-woody substrates.

Good correspondence between the transcriptome and exoproteome profiles of non-woody substrates and their chemical composition suggests that *D. squalens* has ability to respond sufficiently to these plant biomasses, which are not its substrates in nature. Furthermore, the results indicate that *D. squalens* has a less specific adaptation to its substrates in the natural substrates than has previously been observed for several ascomycete fungal species, such as *Podospora anserina* and *Magnaporthe oryzae* (Battaglia et al., 2011; Espagne et al., 2008). In addition, the low content of lignin in non-woody substrates resulted in an early time point expression of (hemi-)cellulolytic genes, which indicates that lignin prevents their induction. Altogether, this indicates that *D. squalens* initially concentrates most of its energy to degrade lignin in order to obtain access to the polysaccharides. The energy required for this initial stage of wood degradation is likely produced by the release of sugars from hemicelluloses by the limited number of enzymes that are targeted to these polymers (Hori et al., 2014b).

4.2.2.4 *Degradation of other polysaccharides*

D. squalens expressed and produced pectin- and starch-degradation related genes and enzymes in wood and non-woody cultures (Fig. 12A-B). The number of pectinolytic genes expressed by *D. squalens* increased in wood from early to later phase of degradation (from 4 to 17). In contrast, in non-woody substrates similar number of genes was expressed at both time points (10-11 in wheat bran and 16-17 in cotton seed hulls).

While pectin is a minor component in wood, the expression of most pectinolytic genes was higher (fold change>1.5, $p<0.05$) at day 16 than at day 9 in *D. squalens* wood cultures (Fig. 12A) similarly to cellulolytic and xylanolytic genes. Genes encoding GH43 ABN and GH35 LAC were expressed at high level at the later time point. LACs, acting on the side chains of pectin, were also the most abundantly secreted pectinases. In contrast to these results, GH28 PGX has been among the highly expressed genes and secreted enzymes in *P. chrysosporium* aspen-containing cultures and expression of GH28 polygalacturonases has been induced in *P. coccineus* pine and aspen cultures (Couturier et al., 2015; Vanden Wymelenberg et al., 2011, 2010).

In non-woody substrates, which pectin content is higher compared to wood, pectinolytic genes showed variable expression patterns (Fig. 12A). Similar to wood cultures the high amount of GH43 ABN transcripts was detected in both non-woody substrates at day 16. Furthermore, high transcript levels of genes encoding GH28 PGA and CE8 PME were detected in the cotton seed hull cultures. The overall repertoire of extracellular pectinases was similar in both non-woody substrates. These results show that *D. squalens* produces a wide set of pectinases for the degradation of both wood and non-woody biomass.

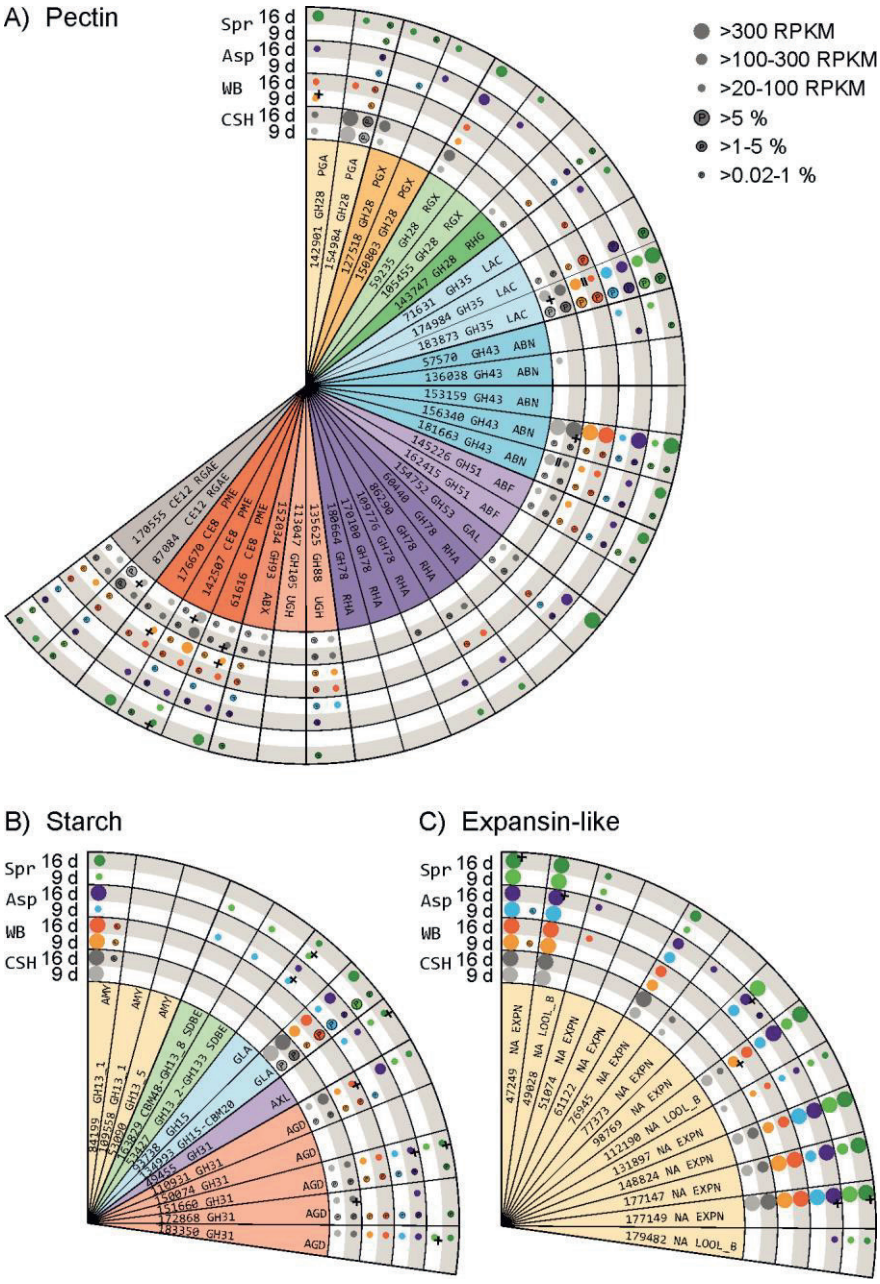


Figure 12 A) Pectin- and B) starch-degradation related and C) expansin-like transcripts and extracellular proteins from spruce wood (Spr, green circles), aspen wood (Asp, blue circles), wheat bran (WB, orange circles) and cotton seed hull (CSH, grey circles) cultures of *D. squalens* after 9 and 16 days of cultivation. Details of the figure are presented in the caption of Fig. 9. Enzyme abbreviations are presented in Table 2. Circles were drawn with iTol (Letunic and Bork, 2011). Figure modified from Manuscript III.

4.3 Enzyme activities in microcrystalline cellulose cultures (II, IV)

D. squalens produced several extracellular lignocellulose-modifying enzyme activities during the cultivation in microcrystalline cellulose (Avicel) -containing medium (Fig. 13). Avicel induced the production of cellulases and hemicellulase of *D. squalens* as the activity of CBHI, EG and xylanase increased up to 21 days of cultivation. The highest CBHI and EG activities (7.7 nkat/ml and 53 nkat/ml, respectively) were observed after 21 days, which is also in line with the exoproteome results from wood cultures with increasing amount of cellulases from day 9 to day 16 (Manuscript III). BGL activity reached the highest level (3.6 nkat/ml) already at the day seven and remained at similar level (3.0±0.8 nkat/ml) during the whole *D. squalens* cultivation. The highest xylanase activity (108 nkat/ml) was detected after 28 days of cultivation of *D. squalens* (Fig. 13).

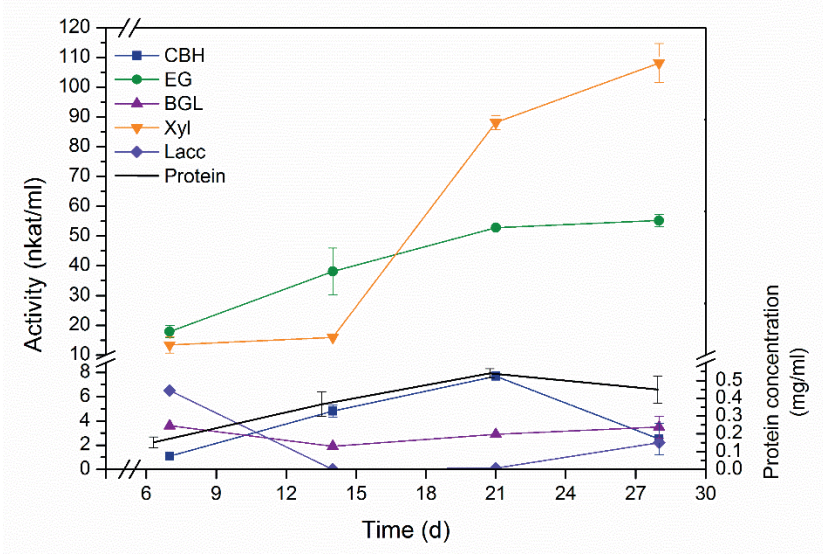


Figure 13 Activity profile and protein concentration from the submerged Avicel cultures of *D. squalens*. CBH, cellobiohydrolase I; EG, endoglucanase; BGL, β-glucosidase; Xyl, xylanase; Lacc, laccase; Protein, protein concentration. Error bars represent the standard deviation between three parallel cultivations. Figure from Publication II.

Significant decrease in the amount of *cel7b* transcripts after 28 days of cultivation in Avicel was supported by simultaneous decline of CBHI activity (Figs. 5B and 13). Increased cellulase activities have also been observed in the course of cultivation of *P. gigantea* in Avicel- and carboxymethylcellulose (CMC) -

containing media (Niranjane et al., 2007), and Avicel has been used as a carbon source for the production of cellulases with *P. chrysosporium* (Szabó et al., 1996).

The activity of CDH was not detected from the Avicel medium without addition of the surfactant Tween20 that is known to enhance secretion of the enzymes (Reese and Maguire, 1969). In Tween20-amended cultures, CDH activity was observed after ten days of growth and it accumulated until the end of the cultivation. However, the increase of EG and CBHI activities was slower in Tween20-amended cultures than in the cultures without the surfactant.

In addition to (hemi-)cellulases, a low laccase activity (2.2-6.5 nkat/ml) was detected after seven and 28 days of cultivation (Fig. 13). Similarly, low laccase activities have been reported from Avicel- and CMC-containing cultures of white-rot fungi *Cerrena maxima*, *Fomes fomentarius* and *Trametes* (*Pseudotrametes*) *gibbosa* (Elisashvili and Kachlishvili, 2009). No MnP activity was detected from the Avicel cultures of *D. squalens* in Publication II, but minor activity (from 0.1 to 0.2 nkat/ml at days 12 and 22) was detected in the similar cultivation in Publication IV. This suggests that MnP activity is sporadic during the growth of *D. squalens* in Avicel. *D. squalens* has been reported to produce laccase and MnP activities in the low-nitrogen and complex malt extract liquid media (Arora and Gill, 2000; Mäkelä et al., 2002; Périé and Gold, 1991) and in the solid spruce wood substrate (Fackler et al., 2006; Mäkelä et al., 2002). These results support the different regulation patterns for cellulases and ligninolytic enzymes in *D. squalens*.

4.4 *D. squalens* CBHs and CDH (IV)

*D. s*qualens cellulases were produced in the 1% Avicel cultures in order to characterize the CBH and CDH enzymes of the fungus. After chromatographical purification, CBHI fraction was detected as single protein band in SDS-PAGE gel electrophoresis with molecular mass of 45 kDa (Fig. 14A), which was slightly lower than the theoretical molecular masses (http://web.expasy.org/compute_pi) of the three translated CBHI-encoding genes (mature parts) of *D. squalens* (protein IDs #96488, #100956 and #131122, http://genome.jgi-psf.org/Dicsq1/Dicsq1.home.html). Peptide mapping of the CBHI fraction resulted with one internal peptide sequence corresponding with all the three translated CBHI-encoding genes, two with *cel7a* and *cel7b*, one with *cel7a* and *cel7b* and one that was unique to the *cel7a* of *D. squalens* (Floudas et al., 2012; Publication II). This suggests that *D. squalens* produced three different CBHI isoenzymes simultaneously in the Avicel cultures. In addition, analytical IEF showed several

isoforms or possibly also different glycoforms of CBHI fraction from p*I* 3.8 to 4.1 (Fig. 14B), which is in line with the theoretical p*I* values for *D. squalens* CBHIs (from 4.24 to 4.93) (http://web.expasy.org/compute_pi). To confirm this, more peptide sequences are needed to gain unique peptides for each isoenzyme.

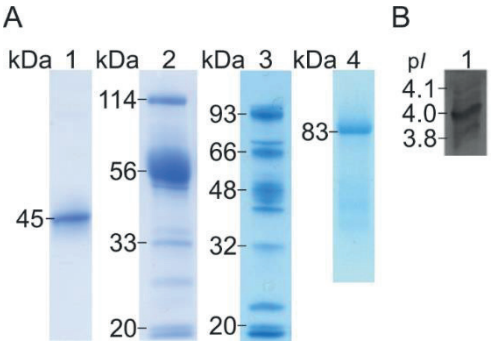


Figure 14 Gel electrophoretic separation of *D. squalens* cellulolytic enzymes. A) SDS-PAGE of chromatographically purified CBHI fraction, heterologously produced rCel6A and purified CDH of *D. squalens*. Lane 1, CBHI fraction; lane 2, rCel6A; lane 3, *T. reesei* culture filtrate without *cel6a* insert; lane 4 CDH. B) IEF analysis of CBHI fraction of *D. squalens*. Figure from Publication IV.

The simultaneous expression of the genes encoding CBHIs (Publication II) and secretion of the corresponding enzymes by *D. squalens* in the Avicel cultures is in agreement with the reports from white rot fungal biomass-containing cultures (Fernandez-Fueyo et al., 2012; Hori et al., 2013; Publication I).

The pH and temperature ranges of the purified CBHI fraction of *D. squalens* corresponded with those previously reported for white rot fungal CBHI enzymes (Publication I and IV). *D. squalens* CBHI fraction had more acidic pH optimum, pH 4.0, and was less thermotolerant with the temperature optimum of 50°C than that of *T. reesei* Cel7A, which has a pH and temperature optima of 5.0 and 60°C, respectively (Boer et al., 2000). However, the *D. squalens* CBHI fraction maintained its activity for 60 min at 50°C, and was stable in 50 mM Na-citrate buffer from pH 3.0 to pH 6.0.

In the mid 1980's, Rouau and Odier (1986b) purified two enzymes (Ex1 and Ex2) from another strain of *D. squalens* (CBS432-34) with both CBHI and xylanase activity. Ex1 and Ex2 had lower molecular masses (39.0 and 36.0 kDa, respectively) and higher isoelectric points (4.6 and 4.5) compared to the CBHI fraction characterized in this work (Publication IV). In addition, the CBHI fraction did not have any activity towards xylan and its temperature optimum was lower than that

of Ex1 and Ex2, confirming that the CBHI fraction is different from Ex1 and Ex2 enzymes.

The CBHII-encoding *cel6a* gene of *D. squalens* was amplified from cDNA originating from the mycelium grown in Avicel cultures. After codon optimization, a synthetic *cel6a* gene sequence was transformed into *T. reesei* and CBHII (Cel6A) enzyme was heterologously produced in *T. reesei* host strain that lacks the four major cellulases. Basidiomycete CBHIIs from *Irpex lacteus* and *Coprinopsis cinerea* have been produced heterologously in *Pichia pastoris* and *Escherichia coli* (Liu et al., 2009; Toda et al., 2008; Yoshida et al., 2009), respectively, but this was the first time when white rot fungal CBHII was produced in *T. reesei*.

The molecular mass of the recombinant protein rCel6A, 56 kDa (Fig. 14A) was higher than the computed theoretical molecular mass (45.3 kDa) of the mature *D. squalens* Cel6A (http://web.expasy.org/compute_pi/), in line with that of the typical basidiomycete CBHIIs, varying between 50 and 60 kDa (Publication I). *T. reesei* control strain, which is similar to *T. reesei host* strain, but lacks the *cel6a* expression cassette, did not produce major protein bands with molecular mass of 50-60 kDa (Fig. 14A). In addition, no CBHI activity was detected with 4-methylumbelliferyl-β-D-lactoside as a substrate from the host and control strain culture liquids. However, the *T. reesei* rCel6A culture filtrate contained side activities of XLN (52.7 nkat/mg), EG (4.1 nkat/mg) and BGL (3.0 nkat/mg), and therefore the culture liquid of the *T. reesei* control strain was used as a background control in hydrolysis experiments.

In addition, the native *D. squalens* CDH was chromatographically purified from the Avicel culture medium. The molecular mass of the CDH was 83 kDa (Fig. 14A), corresponding with the other characterized white rot fungal CDHs with molecular masses ranging from 81 to 113 kDa (Publication I). Three internal peptides obtained from the purified *D. squalens* CDH (KVLLLER, VILSAGSFGTPR and SGVFAGASPK) were identical with the translated (mature part) *cdh* gene of *D. squalens* FBCC312 (Publication II). Previously Avicel and α-cellulose have been found to be good substrates for CDH production in the basidiomycete plant pathogen *Sclerotium* (*Athelia*) *rolfsii* (Baminger et al., 2001). In addition, expression of CDH-encoding genes in Avicel and secretion of corresponding enzymes in cellulose- and xylan-containing cultures of *P. chrysosporium* have been detected together with the classical cellulases (Hori et al., 2011; Vanden Wymelenberg et al., 2009). Together these results support the role of CDH in cellulose depolymerisation.

4.5 Plant biomass saccharification by *D. squalens* CBHI and rCBHII (IV)

Different combinations of *D. squalens* rCel6A, CBHI fraction and CDH, and *M. thermophila* laccase were tested for the hydrolysis of crude plant biomass substrates sugar beet pulp and wheat bran, and microcrystalline cellulose Avicel. Substrates were selected based on their different chemical compositions and lignin concentrations. Sugar beet pulp, a major by-product of the sugar refining industry, is rich in glucose, arabinose and uronic acids (Ozturkoglu Budak et al., 2014). It contains 1-2% (as dry weight) lignin (Micard et al., 1996), whereas wheat bran contains approximately 10% (as dry weight) lignin (Bergmans et al., 1996). The major monosaccharides in wheat bran are glucose, xylose and arabinose (Ozturkoglu Budak et al., 2014). In addition to cellulose, Avicel contains only low amount of other polymers like xylan or lignin (Várnai et al., 2011).

Since no specific activity measurement method for CBHII is available, the activity of *D. squalens* rCel6A was verified by saccharification assays. *D. squalens* rCel6A and CBHI fraction hydrolysed all the tested substrates, sugar beet pulp, wheat bran and Avicel (Fig. 15). The highest sugar yields were achieved from the hydrolysis of Avicel by the CBHs. The lower sugar yields from the hydrolysis of sugar beet pulp and wheat bran were most probably due to the complexity of these feedstocks (Fig. 15).

Higher amount of reducing sugars was released from all of the substrates by *D. squalens* rCel6A, which contains a cellulose binding domain 1 (CBM1), than CBHI fraction, which lacks the CBM. Ascomycetous CBHIs and CBHIIs with CBMs have also been shown to be more efficient in hydrolysis at low substrate concentration (1% w/w) than CBHs that do not possess CBMs (Várnai et al., 2013). When 5 μg of CBHI fraction per mg of substrate was used, sugar yield from Avicel, sugar beet pulp and wheat bran (2.9%, 0.4% and 1.4% of the theoretical maximum, respectively) was low, but comparable with the hydrolysis yields with *T. reesei* Cel7A from Avicel (2.5–4%) (Rahikainen et al., 2013).

Significant synergistic effect that has also been reported for basidiomycetes *I. lacteus* and *P. chrysosporium* CBHs (Toda et al., 2008; Uzcategui et al., 1991), was detected only in the hydrolysis of Avicel with the *D. squalens* rCel6A and CBHI. The released sugar yield was 1.2-fold higher in simultaneous reaction of 5 μg of rCel6A and CBHI fraction per mg of Avicel, than the theoretical maximum of released sugars obtained as a results of the hydrolysis of the individual CBHs (Fig. 15).

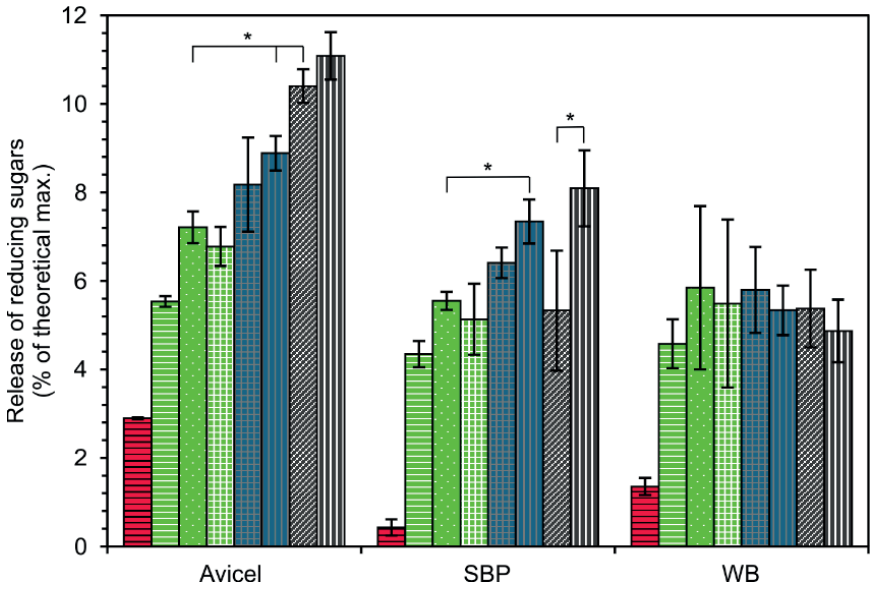


Figure 15 Hydrolysis of 1% (w/v) Avicel, sugar beet pulp (SBP) and wheat bran (WB). Reactions with CBHI fraction (5 µg/mg, red with horizontal stripes); rCel6A (5 µg/mg, green with white horizontal stripes; 10 µg/mg, green with white dots); rCel6A and CDH (10 µg/mg and 1 µg/mg, respectively, green with white grid); rCel6A, CDH and laccase (10, 0.5 and 0.5 µg/mg, respectively, blue with grey grid); rCel6A and laccase (10 and 1 µg/mg, respectively, blue with grey vertical stripes); CBHI fraction and rCel6A (5 and 5 µg/mg, respectively, grey with diagonal stripes) or CBHI fraction, rCel6A and laccase (5, 5 and 1 µg/mg, respectively, grey with white vertical stripes), for 4 h at 50°C. Significant differences (p<0.05) in the amount of released reducing sugars between the compared reactions are indicated by asterisk. Figure from Publication IV.

The addition of *D. squalens* CDH to the hydrolysis reactions with rCel6A (10 µg/mg) did not increase the amount of released sugars from any of the substrates (Fig. 15). For instance, *P. chrysosporium* CDH has been shown to improve the CBHI activity by oxidation of cellobiose to cellobionolactone and thus decreasing the inhibitory effect of cellobiose on CBHI reaction (Igarashi et al., 1998). CDH has also been hypothesized to link the cellulose and lignin depolymerising reactions (Temp and Eggert, 1999). Recently, a strong synergy was detected for CDH and LPMO (Langston et al., 2011), and thus addition of LPMO to the hydrolysis reactions should be studied.

Laccases have been suggested to loosen the lignocellulose structure and by detoxify the phenolic inhibitory compounds when added to the pretreatment and hydrolysis of plant biomass (Kudanga and Le Roes-Hill, 2014). Interestingly, adding *M. thermophila* laccase to the hydrolysis reaction with *D. squalens* CBHI fraction and rCel6A significantly improved (1.5-fold) the sugar yield from sugar

beet pulp when compared to the reaction without laccase. The enhancement was also detected when laccase was supplemented to the hydrolysis of sugar beet pulp with *D. squalens* rCel6A (Fig. 15).

Compared to sugar beet pulp, wheat bran is rich in lignin, which was most probably the reason why laccase did not significantly increase the hydrolysis of wheat bran. The commercial laccase from *M. thermophila*, which is an ascomycete fungus and possibly not involved in lignin modification in nature, may be unable to alter the lignin structure of the wheat bran. Laccase treatment has also been shown either decrease or increase the amount of bound cellulases to lignin depending on the hydrolysed substrate (Moilanen et al., 2011). However, *D. squalens* secreted low amounts of laccases in wheat bran cultures (Manuscript III) and promising results have been achieved with other basidiomycete laccases in the hydrolysis of lignin-rich plant biomass (reviewed in Kudanga and Le Roes-Hill, 2014).

Results obtained in this study suggest that laccase altered the lignin in sugar beet pulp so that better access of CBHs to cellulose fibres was obtained. Interestingly, laccase addition to *D. squalens* rCel6A improved also the amount of reduced sugars acquired from Avicel, which lignin content is very low (<1% of dry weight; Várnai et al., 2011), but did not enhance the hydrolysis of wheat bran. Thus, a more detailed understanding of the modifications to various biomasses caused by laccases from different fungal sources is needed in order to clarify the effect of laccase in the improvement of enzymatic plant biomass saccharification.

5 Conclusions

The main hypothesis that white rot fungi have a full repertoire of plant cell wall degrading enzyme activities was confirmed in this study for *D. squalens.* The fungus expressed different sets of genes and secreted a large diversity of enzymes for the degradation of softwood, hardwood and non-woody plant biomass. The gene and enzyme sets corresponded well with the high lignin content of wood substrates and confirmed that lignin degradation occurs at the initial stage of growth. The expressed genes and produced enzymes also correlated well with the overall composition of the wood and non-woody substrates. Based on its ability to use different carbohydrates, it is postulated that *D. squalens* has not specialized to the same extent to its natural habitat than has been observed in several ascomycete fungi. Restriction of *D. squalens* to grow and propagate on wood is thus likely the result of other factors, such as competition with other microbes.

Based on the results of this study and the currently available data, expression patterns of cellulose-degrading genes are consistent in the plant biomass containing cultures of the white rot fungi. The most notable difference in the sequenced white rot fungal genomes with respect to lignocellulose degradation is the presence of *sensu stricto* laccase encoding genes. However, the majority of the genome-sequenced white rot fungal species so far harbour laccase encoding genes. *D. squalens* secreted laccases at high level during growth on plant biomass similarly to other laccase possessing white rot fungi such as *P. ostreatus*. Furthermore, the results emphasised the role of oxidative enzymes in the white rot fungal degradation of the cellulose and plant biomass.

Focussing on specific enzyme classes, the biochemical properties of the *D. squalens* CBHI fraction were align with the typical white rot fungal CBHIs. Both CBHI and *D. squalens* CBHII, which was the first basidiomycetous CBHII heterologously produced in *T. reesei*, were able to saccharify microcrystalline cellulose and crude plant biomass substrates. These results and the ability of *D. squalens* to produce enzyme mixtures that correlate well with the diversity of plant biomass substrates underline the high potential of white rot fungi for biotechnology, which has so far been barely explored.

The functional data achieved in this study can help to futher distinguish the subtler differences in approaches the wood-rotting white rot species use in the degradation of plant biomass. The results also show that in order to deeper understand the plan cell wall degradation process, the roles of the uncharacterized

enzymes, such as putative LPMOs and expansin-like proteins, need to be clarified. In addition, it is possible that the putative unknown proteins encoded by the genome of *D. squalens*, which were not the focus of this study, have a role in plant cell wall degradation, and this is an interesting topic for future research. Further characterization of *D. squalens* CBHs regarding e.g. their processivity and tolerance of inhibitory compounds in the hydrolysis of plant biomass can elucidate the applicability of the white rot fungal enzymes in commercial enzyme cocktails.

6 References

Aggelis, G., Ehaliotis, C., Nerud, F., Stoychev, I., Lyberatos, G., Zervakis, G., 2002. Evaluation of white-rot fungi for detoxification and decolorization of effluents from the green olive debittering process. Appl. Microbiol. Biotechnol. 59, 353–360. doi:10.1007/s00253-002-1005-9

Agger, J.W., Isaksen, T., Várnai, A., Vidal-Melgosa, S., Willats, W.G.T., Ludwig, R., Horn, S.J., Eijsink, V.G.H., Westereng, B., 2014. Discovery of LPMO activity on hemicelluloses shows the importance of oxidative processes in plant cell wall degradation. Proc. Natl. Acad. Sci. U. S. A. 111, 6287–6292. doi:10.1073/pnas.1323629111

Alfaro, M., Oguiza, J.A., Ramírez, L., Pisabarro, A.G., 2014. Comparative analysis of secretomes in basidiomycete fungi. J. Proteomics 102, 28–43. doi:10.1016/j.jprot.2014.03.001

Arora, D.S., Gill, P.K., 2000. Laccase production by some white rot fungi under different nutritional conditions. Bioresour. Technol. 73, 283–285. doi:10.1016/S0960-8524(99)00141-8

Bak, J.S., Kim, M.D., Choi, I.-G., Kim, K.H., 2010. Biological pretreatment of rice straw by fermenting with *Dichomitus squalens*. N. Biotechnol. 27, 424–434. doi:10.1016/j.nbt.2010.02.021

Baldrian, P., 2008. Enzymes of saprotrophic basidiomycetes, in: Boddy, L., Frankland, J., van West, P. (Eds.), Ecology of Saprotrophic Basidiomycetes. Academic Press, pp. 19–41. doi:10.1016/S0275-0287(08)80004-5

Baminger, U., Subramaniam, S.S., Renganathan, V., Haltrich, D., 2001. Purification and characterization of cellobiose dehydrogenase from the plant pathogen *Sclerotium* (*Athelia*) *rolfsii*. Appl. Environ. Microbiol. 67, 1766–1774. doi:10.1128/AEM.67.4.1766-1774.2001

Battaglia, E., Benoit, I., van den Brink, J., Wiebenga, A., Coutinho, P.M., Henrissat, B., de Vries, R.P., 2011. Carbohydrate-active enzymes from the zygomycete fungus *Rhizopus oryzae*: a highly specialized approach to carbohydrate degradation depicted at genome level. BMC Genomics 12, 38. doi:10.1186/1471-2164-12-38

Benoit, I., Coutinho, P.M., Schols, H.A., Gerlach, J.P., Henrissat, B., de Vries, R.P., 2012. Degradation of different pectins by fungi: correlations and contrasts between the pectinolytic enzyme sets identified in genomes and the growth on pectins of different origin. BMC Genomics 13. doi:10.1186/1471-2164-13-321

Bergmans, M.E.F., Beldman, G., Gruppen, H., Voragen, A.G.J., 1996. Optimisation of the selective extraction of (glucurono)arabinoxylans from wheat bran: use of barium and calcium hydroxide solution at elevated temperatures. J. Cereal Sci. 23, 235–245. doi:10.1006/jcrs.1996.0024

Binder, M., Justo, A., Riley, R., Salamov, A., Lopez-Giraldez, F., Sjökvist, E., Copeland, A., Foster, B., Sun, H., Larsson, E., Larsson, K.H., Townsend, J., Grigoriev, I.V., Hibbett, D.S., 2013. Phylogenetic and phylogenomic overview of the Polyporales. Mycologia 105, 1350–1373. doi:10.3852/13-003

Blanchette, R.A., 1984. Screening wood decayed by white rot fungi for preferential lignin degradation. Appl. Environ. Microbiol. 48, 647–653.

Boer, H., Teeri, T.T., Koivula, A., 2000. Characterization of *Trichoderma reesei* cellobiohydrolase Cel7A secreted from *Pichia pastoris* using two different promoters.

Biotech. Bioeng. 69, 486–494. doi:10.1002/1097-0290(20000905)69:5<486::AID-BIT3>3.0.CO;2-N

Bourbonnais, R., Paice, M.G., 1990. Oxidation of non-phenolic substrates: An expanded role for laccase in lignin biodegradation. FEBS Lett. 267, 99–102. doi:10.1016/0014-5793(90)80298-W

Brillouet, J.-M., Moulin, J.-C., Agosin, E., 1985. Production, purification, and properties of an α-l-arabinofuranosidase from *Dichomitus squalens*. Carbohydr. Res. 144, 113–126. doi:10.1016/0008-6215(85)85012-6

Broda, P., Birch, P.R., Brooks, P.R., Sims, P.F., 1995. PCR-mediated analysis of lignocellulolytic gene transcription by *Phanerochaete chrysosporium*: substrate-dependent differential expression within gene families. Appl. Environ. Microbiol. 61, 2358–2364.

Caffall, K.H., Mohnen, D., 2009. The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. Carbohydr. Res. 344, 1879–1900. doi:10.1016/j.carres.2009.05.021

Cajthaml, T., Kresinová, Z., Svobodová, K., Möder, M., 2009. Biodegradation of endocrine-disrupting compounds and suppression of estrogenic activity by ligninolytic fungi. Chemosphere 75, 745–750. doi:10.1016/j.chemosphere.2009.01.034

Cannella, D., Hsieh, C.-W.C., Felby, C., Jørgensen, H., 2012. Production and effect of aldonic acids during enzymatic hydrolysis of lignocellulose at high dry matter content. Biotechnol. Biofuels 5, 26. doi:10.1186/1754-6834-5-26

Casado Lópes, S., Sietiö, O.-S., Hildén, K., de Vries, R.P., Mäkelä, M.R., 2016. Homologous and heterologous expression of basidiomycete genes related to plant biomass degradation, in Schmoll, M., Dattenböck, C. (Eds.), Gene expression systems in fungi: advancements and applications, Fungal Biology. Springer International Publishing, Switzerland, 119–160. doi:10.1007/978-3-319-27951-0_5

Chander, M., Arora, D.S., 2007. Evaluation of some white-rot fungi for their potential to decolourise industrial dyes. Dye. Pigment. 72, 192–198. doi:10.1016/j.dyepig.2005.08.023

Chander, M., Arora, D.S., Bath, H.K., 2004. Biodecolourisation of some industrial dyes by white-rot fungi. J. Ind. Microbiol. Biotechnol. 31, 94–97. doi:10.1007/s10295-004-0116-y

Chang, S., Puryear, J., Cairney, J., 1993. A simple and efficient method for isolating RNA from pine trees. Plant Mol. Biol. Report. 11, 113–116. doi:10.1007/BF02670468

Chen, S., Xu, J., Liu, C., Zhu, Y., Nelson, D.R., Zhou, S., Li, C., Wang, L., Guo, X., Sun, Y., Luo, H., Li, Y., Song, J., Henrissat, B., Levasseur, A., Qian, J., Li, J., Luo, X., Shi, L., He, L., Xiang, L., Xu, X., Niu, Y., Li, Q., Han, M. V, Yan, H., Zhang, J., Chen, H., Lv, A., Wang, Z., Liu, M., Schwartz, D.C., Sun, C., 2012. Genome sequence of the model medicinal mushroom *Ganoderma lucidum*. Nat. Commun. 3, 913. doi:10.1038/ncomms1923

Cosgrove, D.J., 2015. Plant expansins: diversity and interactions with plant cell walls. Curr. Opin. Plant Biol. 25, 162–172. doi:10.1016/j.pbi.2015.05.014

Couturier, M., Navarro, D., Chevret, D., Henrissat, B., Piumi, F., Ruiz-Dueñas, F.J., Martínez, A.T., Grigoriev, I.V., Riley, R., Lipzen, A., Berrin, J.-G., Master, E.R., Rosso, M.-N., 2015. Enhanced degradation of softwood versus hardwood by the white-rot fungus *Pycnoporus coccineus*. Biotechnol. Biofuels 8, 216. doi:10.1186/s13068-015-0407-8

Covino, S., Svobodová, K.K., Cvancarová, M., D'Annibale, A., Petruccioli, M., Federici, F., Kresinová, Z., Galli, E., Cajthaml, T., 2010. Inoculum carrier and contaminant bioavailability affect fungal degradation performances of PAH-contaminated solid matrices from a wood preservation plant. Chemosphere 79, 855–864.

doi:10.1016/j.chemosphere.2010.02.038

Čvančarová, M., Moeder, M., Filipová, A., Cajthaml, T., 2014. Biotransformation of fluoroquinolone antibiotics by ligninolytic fungi - metabolites, enzymes and residual antibacterial activity. Chemosphere 136, 311–320. doi:10.1016/j.chemosphere.2014.12.012

Čvančarová, M., Moeder, M., Filipová, A., Reemtsma, T., Cajthaml, T., 2013. Biotransformation of the antibiotic agent flumequine by ligninolytic fungi and residual antibacterial activity of the transformation mixtures. Environ. Sci. Technol. 47, 14128–14136. doi:10.1021/es403470s

de Vries, R.P., Visser, J., 2001. Aspergillus enzymes involved in degradation of plant cell wall polysaccharides. Microbiol. Mol. Biol. Rev. 65, 497–522. doi:10.1128/MMBR.65.4.497-522.2001

Divne, C., Ståhlberg, J., Reinikainen, T., Ruohonen, L., Pettersson, G., Knowles, J.K.C., Teeri, T.T., Jones, T.A., 1994. The three-dimensional crystal structure of the catalytic core of cellobiohydrolase I from *Trichoderma reesei*. Science. 265, 524–528. doi:10.1126/science.8036495

Doblin, M.S., Pettolino, F., Bacic, A., 2010. Plant cell walls: the skeleton of the plant world. Funct. Plant Biol. 37, 357–381 doi:10.1071/FP09279

Dong, Y.-C., Dai, Y.-N., Xu, T.-Y., Cai, J., Chen, Q.-H., 2014. Biodegradation of chestnut shell and lignin-modifying enzymes production by the white-rot fungi *Dichomitus squalens*, *Phlebia radiata*. Bioprocess Biosyst. Eng. 37, 755–764. doi:10.1007/s00449-013-1045-9

Edgar, R., Domrachev, M., Lash, A.E., 2002. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res. 30, 207–210. doi:10.1093/nar/30.1.207

Eichlerová, I., Homolka, L., Benada, O., Kofronová, O., Hubálek, T., Nerud, F., 2007. Decolorization of Orange G and Remazol Brilliant Blue R by the white rot fungus *Dichomitus squalens*: toxicological evaluation and morphological study. Chemosphere 69, 795–802. doi:10.1016/j.chemosphere.2007.04.083

Eichlerová, I., Homolka, L., Nerud, F., 2006. Synthetic dye decolorization capacity of white rot fungus *Dichomitus squalens*. Bioresour. Technol. 97, 2153–9. doi:10.1016/j.biortech.2005.09.014

Elisashvili, V., Kachlishvili, E., 2009. Physiological regulation of laccase and manganese peroxidase production by white-rot basidiomycetes. J. Biotechnol. 144, 37–42. doi:10.1016/j.jbiotec.2009.06.020

Eriksson, K.-E., Blanchette, R.A., Ander, P., 1990. Microbial and enzymatic degradation of wood and wood components. Springer-Verlag, Berlin, Germany, 333 p.

Espagne, E., Lespinet, O., Malagnac, F., Da Silva, C., Jaillon, O., Porcel, B.M., Couloux, A., Aury, J.-M., Ségurens, B., Poulain, J., Anthouard, V., Grossetete, S., Khalili, H., Coppin, E., Déquard-Chablat, M., Picard, M., Contamine, V., Arnaise, S., Bourdais, A., Berteaux-Lecellier, V., Gautheret, D., de Vries, R.P., Battaglia, E., Coutinho, P.M., Danchin, E.G., Henrissat, B., Khoury, R.E., Sainsard-Chanet, A., Boivin, A., Pinan-Lucarré, B., Sellem, C.H., Debuchy, R., Wincker, P., Weissenbach, J., Silar, P., 2008. The genome sequence of the model ascomycete fungus *Podospora anserina*. Genome Biol. 9, R77. doi:10.1186/gb-2008-9-5-r77

Fackler, K., Gradinger, C., Hinterstoisser, B., Messner, K., Schwanninger, M., 2006. Lignin degradation by white rot fungi on spruce wood shavings during short-time solid-state fermentations monitored by near infrared spectroscopy. Enzyme Microb. Technol. 39, 1476–1483. doi:10.1016/j.enzmictec.2006.03.043

Fernández-Fueyo, E., Acebes, S., Ruiz-Dueñas, F.J., Martínez, M.J., Romero, A., Medrano,

F.J., Guallar, V., Martínez, A.T., 2014. Structural implications of the C-terminal tail in the catalytic and stability properties of manganese peroxidases from ligninolytic fungi. Acta Crystallogr. D. Biol. Crystallogr. 70, 3253–3265. doi:10.1107/S1399004714022755

Fernández-Fueyo, E., Ruiz-Dueñas, F.J., Ferreira, P., Floudas, D., Hibbett, D.S., Canessa, P., Larrondo, L.F., James, T.Y., Seelenfreund, D., Lobos, S., Polanco, R., Tello, M., Honda, Y., Watanabe, T., Watanabe, T., Ryu, J.S., Kubicek, C.P., Schmoll, M., Gaskell, J., Hammel, K.E., St. John, F.J., Vanden Wymelenberg, A., Sabat, G., Splinter BonDurant, S., Syed, K., Yadav, J.S., Doddapaneni, H., Subramanian, V., Lavin, J.L., Oguiza, J.A., Perez, G., Pisabarro, A.G., Ramírez, L., Santoyo, F., Master, E., Coutinho, P.M., Henrissat, B., Lombard, V., Magnuson, J.K., Kües, U., Hori, C., Igarashi, K., Samejima, M., Held, B.W., Barry, K.W., LaButti, K.M., Lapidus, A., Lindquist, E.A., Lucas, S.M., Riley, R., Salamov, A.A., Hoffmeister, D., Schwenk, D., Hadar, Y., Yarden, O., de Vries, R.P., Wiebenga, A., Stenlid, J., Eastwood, D., Grigoriev, I.V., Berka, R.M., Blanchette, R.A., Kersten, P., Martínez, A.T., Vicuña, R., Cullen, D., 2012. Comparative genomics of *Ceriporiopsis subvermispora* and *Phanerochaete chrysosporium* provide insight into selective ligninolysis. Proc. Natl. Acad. Sci. U. S. A. 109, 5458–5463. doi:10.1073/pnas.1119912109

Fernández-Fueyo, E., Ruiz-Dueñas, F.J., López-Lucendo, M.F., Pérez-Boada, M., Rencoret, J., Gutiérrez, A., Pisabarro, A.G., Ramírez, L., Martínez, A.T., 2016. A secretomic view of woody and nonwoody lignocellulose degradation by *Pleurotus ostreatus*. Biotechnol. Biofuels 9, 49. doi:10.1186/s13068-016-0462-9

Floudas, D., Binder, M., Riley, R., Barry, K., Blanchette, R.A., Henrissat, B., Martínez, A.T., Otillar, R., Spatafora, J.W., Yadav, J.S., Aerts, A., Benoit, I., Boyd, A., Carlson, A., Copeland, A., Coutinho, P.M., de Vries, R.P., Ferreira, P., Findley, K., Foster, B., Gaskell, J., Glotzer, D., Górecki, P., Heitman, J., Hesse, C., Hori, C., Igarashi, K., Jurgens, J.A., Kallen, N., Kersten, P., Kohler, A., Kües, U., Kumar, T.K.A., Kuo, A., LaButti, K., Larrondo, L.F., Lindquist, E., Ling, A., Lombard, V., Lucas, S., Lundell, T., Martin, R., McLaughlin, D.J., Morgenstern, I., Morin, E., Murat, C., Nagy, L.G., Nolan, M., Ohm, R.A., Patyshakuliyeva, A., Rokas, A., Ruiz-Dueñas, F.J., Sabat, G., Salamov, A., Samejima, M., Schmutz, J., Slot, J.C., St John, F., Stenlid, J., Sun, H., Sun, S., Syed, K., Tsang, A., Wiebenga, A., Young, D., Pisabarro, A., Eastwood, D.C., Martin, F., Cullen, D., Grigoriev, I.V., Hibbett, D.S., 2012. The Paleozoic origin of enzymatic lignin decomposition reconstructed from 31 fungal genomes. Science. 336, 1715–1719. doi:10.1126/science.1221748

Frandsen, K.E.H., Simmons, T.J., Dupree, P., Poulsen, J.-C.N., Hemsworth, G.R., Ciano, L., Johnston, E.M., Tovborg, M., Johansen, K.S., von Freiesleben, P., Marmuse, L., Fort, S., Cottaz, S., Driguez, H., Henrissat, B., Lenfant, N., Tuna, F., Baldansuren, A., Davies, G.J., Lo Leggio, L., Walton, P.H., 2016. The molecular basis of polysaccharide cleavage by lytic polysaccharide monooxygenases. Nat. Chem. Biol. 12, 298–303. doi:10.1038/nchembio.2029

Frommhagen, M., Sforza, S., Westphal, A.H., Visser, J., Hinz, S.W.A., Koetsier, M.J., van Berkel, W.J.H., Gruppen, H., Kabel, M.A., 2015. Discovery of the combined oxidative cleavage of plant xylan and cellulose by a new fungal polysaccharide monooxygenase. Biotechnol. Biofuels 8, 101. doi:10.1186/s13068-015-0284-1

Gilbertson, R.L., 1980. Wood-rotting fungi of North America. Mycologia 72, 1–49.

Glenn, J.K., Akileswaran, L., Gold, M.H., 1986. Mn(II) oxidation is the principal function of the extracellular Mn-peroxidase from *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. 251, 688–696. doi:10.1016/0003-9861(86)90378-4

Gold, M.H., Youngs, H.L., Gelpke, M.D., 2000. Manganese peroxidase. Met. Ions Biol. Syst. 37, 559–586.

Grigoriev, I.V., Nikitin, R., Haridas, S., Kuo, A., Ohm, R., Otillar, R., Riley, R., Salamov, A., Zhao, X., Korzeniewski, F., Smirnova, T., Nordberg, H., Dubchak, I., Shabalov, I., 2014. MycoCosm portal: gearing up for 1000 fungal genomes. Nucleic Acids Res. 42, D699–704. doi:10.1093/nar/gkt1183

Guillén, F., Martínez, A.T., Martínez, M.J., Evans, C.S., 1994. Hydrogen-peroxide-producing system of *Pleurotus eryngii* involving the extracellular enzyme aryl-alcohol oxidase. Appl. Microbiol. Biotechnol. 41, 465–470. doi:10.1007/BF00939037

Hai, P.Q., Nozaki, K., Amano, Y., Kanda, T., 2000. Purification and characterization of cellobiose dehydrogenase from *Irpex lacteus* and its adsorption on cellulose. J. Appl. Glycosci. 47, 311–318. doi:10.5458/jag.47.311

Hakala, T.K., Maijala, P., Konn, J., Hatakka, A., 2004. Evaluation of novel wood-rotting polypores and corticioid fungi for the decay and biopulping of Norway spruce (*Picea abies*) wood. Enzyme Microb. Technol. 34, 255–263. doi:10.1016/j.enzmictec.2003.10.014

Hammel, K.E., Jensen, K.A., Mozuch, M.D., Landucci, L.L., Tien, M., Pease, E.A., 1993. Ligninolysis by a purified lignin peroxidase. J. Biol. Chem. 268, 12274–12281.

Harreither, W., Sygmund, C., Dünhofen, E., Vicuña, R., Haltrich, D., Ludwig, R., 2009. Cellobiose dehydrogenase from the ligninolytic basidiomycete *Ceriporiopsis subvermispora*. Appl. Environ. Microbiol. 75, 2750–2757. doi:10.1128/AEM.02320-08

Harris, P.V., Welner, D., McFarland, K.C., Re, E., Navarro Poulsen, J.-C., Brown, K., Salbo, R., Ding, H., Vlasenko, E., Merino, S., Xu, F., Cherry, J., Larsen, S., Lo Leggio, L., 2010. Stimulation of lignocellulosic biomass hydrolysis by proteins of glycoside hydrolase family 61: structure and function of a large, enigmatic family. Biochemistry 49, 3305–3316. doi:10.1021/bi100009p

Harris, P.V., Xu, F., Kreel, N.E., Kang, C., Fukuyama, S., 2014. New enzyme insights drive advances in commercial ethanol production. Curr. Opin. Chem. Biol. 19, 162–70. doi:10.1016/j.cbpa.2014.02.015

Harris, P.J., Stone, B.A., 2008. Chemistry and molecular organization of plant cell walls, in: Himmel, M.E. (Ed.), Biomass Recalcitrance. Blackwell Publishing Ltd., Oxford, UK, pp. 61–93. doi:10.1002/9781444305418.ch4

Hatakka, A., 2005. Biodegradation of lignin, in: Steinbüchel, A., Hofrichter, M. (Eds.), Biopolymers Online. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, pp. 129–145. doi:10.1002/3527600035.bpol1005

Hatakka, A., Hammel, K., 2011. Fungal biodegradation of lignocelluloses, in: Hofrichter, M. (Ed.), Industrial Applications, The Mycota. Springer Berlin Heidelberg, Germany, pp. 319–340. doi:10.1007/978-3-642-11458-8_15

Hemsworth, G.R., Davies, G.J., Walton, P.H., 2013. Recent insights into copper-containing lytic polysaccharide mono-oxygenases. Curr. Opin. Struct. Biol. 23, 660–668. doi:10.1016/j.sbi.2013.05.006

Henriksson, G., Johansson, G., Pettersson, G., 2000. A critical review of cellobiose dehydrogenases. J. Biotechnol. 78, 93–113. doi:10.1016/S0168-1656(00)00206-6

Henriksson, G., Nutt, A., Henriksson, H., Pettersson, B., Ståhlberg, J., Johansson, G., Pettersson, G., 1999. Endoglucanase 28 (Cel12A), a new *Phanerochaete chrysosporium* cellulase. Eur. J. Biochem. 259, 88–95. doi:10.1046/j.1432-1327.1999.00011.x

Hernández-Ortega, A., Ferreira, P., Martínez, A.T., 2012. Fungal aryl-alcohol oxidase: a peroxide-producing flavoenzyme involved in lignin degradation. Appl. Microbiol. Biotechnol. 93, 1395–410. doi:10.1007/s00253-011-3836-8

Himmel, M.E., Ding, S.-Y., Johnson, D.K., Adney, W.S., Nimlos, M.R., Brady, J.W., Foust,

T.D., 2007. Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science. 315, 804–807. doi:10.1126/science.1137016

Hofrichter, M., Ullrich, R., 2014. Oxidations catalyzed by fungal peroxygenases. Curr. Opin. Chem. Biol. 19, 116–125. doi:10.1016/j.cbpa.2014.01.015

Hofrichter, M., Ullrich, R., 2006. Heme-thiolate haloperoxidases: versatile biocatalysts with biotechnological and environmental significance. Appl. Microbiol. Biotechnol. 71, 276–288. doi:10.1007/s00253-006-0417-3

Hofrichter, M., Ullrich, R., Pecyna, M.J., Liers, C., Lundell, T., 2010. New and classic families of secreted fungal heme peroxidases. Appl. Microbiol. Biotechnol. 87, 871–897. doi:10.1007/s00253-010-2633-0

Hori, C., Gaskell, J., Igarashi, K., Kersten, P., Mozuch, M., Samejima, M., Cullen, D., 2014a. Temporal alterations in the secretome of the selective ligninolytic fungus *Ceriporiopsis subvermispora* during growth on aspen wood reveal this organism's strategy for degrading lignocellulose. Appl. Environ. Microbiol. 80, 2062–2070. doi:10.1128/AEM.03652-13

Hori, C., Gaskell, J., Igarashi, K., Samejima, M., Hibbett, D., Henrissat, B., Cullen, D., 2013. Genomewide analysis of polysaccharides degrading enzymes in 11 white- and brown-rot Polyporales provides insight into mechanisms of wood decay. Mycologia 105, 1412–1427. doi:10.3852/13-072

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. doi:10.1111/j.1574-6968.2011.02307.x

Hori, C., Ishida, T., Igarashi, K., Samejima, M., Suzuki, H., Master, E., Ferreira, P., Ruiz-Dueñas, F.J., Held, B., Canessa, P., Larrondo, L.F., Schmoll, M., Druzhinina, I.S., Kubicek, C.P., Gaskell, J.A., Kersten, P., St. John, F., Glasner, J., Sabat, G., Splinter BonDurant, S., Syed, K., Yadav, J., Mgbeahuruike, A.C., Kovalchuk, A., Asiegbu, F.O., Lackner, G., Hoffmeister, D., Rencoret, J., Gutiérrez, A., Sun, H., Lindquist, E., Barry, K., Riley, R., Grigoriev, I.V., Henrissat, B., Kües, U., Berka, R.M., Martínez, A.T., Covert, S.F., Blanchette, R.A., Cullen, D., 2014b. Analysis of the *Phlebiopsis gigantea* genome, transcriptome and secretome provides insight into its pioneer colonization strategies of wood. PLoS Genet. 10, e1004759. doi:10.1371/journal.pgen.1004759

Horn, S.J., Sikorski, P., Cederkvist, J.B., Vaaje-Kolstad, G., Sørlie, M., Synstad, B., Vriend, G., Vårum, K.M., Eijsink, V.G.H., 2006. Costs and benefits of processivity in enzymatic degradation of recalcitrant polysaccharides. Proc. Natl. Acad. Sci. U. S. A. 103, 18089–18094. doi:10.1073/pnas.0608909103

Horn, S.J., Vaaje-Kolstad, G., Westereng, B., Eijsink, V.G.H., 2012. Novel enzymes for the degradation of cellulose. Biotechnol. Biofuels 5. doi:10.1186/1754-6834-5-45

Igarashi, K., Ishida, T., Hori, C., Samejima, M., 2008. Characterization of an endoglucanase belonging to a new subfamily of glycoside hydrolase family 45 of the basidiomycete *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 74, 5628–5634. doi:10.1128/AEM.00812-08

Igarashi, K., Samejima, M., Eriksson, K.E., 1998. Cellobiose dehydrogenase enhances *Phanerochaete chrysosporium* cellobiohydrolase I activity by relieving product inhibition. Eur. J. Biochem. 253, 101–106. doi:10.1046/j.1432-1327.1998.2530101.x

Igarashi, K., Uchihashi, T., Koivula, A., Wada, M., Kimura, S., Okamoto, T., Penttilä, M., Ando, T., Samejima, M., 2011. Traffic jams reduce hydrolytic efficiency of cellulase on cellulose surface. Science. 333, 1279–1282. doi:10.1126/science.1208386

in der Wiesche, C., Martens, R., Zadrazil, F., 2003. The effect of interaction between white-rot fungi and indigenous microorganisms on degradation of polycyclic aromatic hydrocarbons in soil. Water, Air, Soil Pollut. Focus 3, 73–79.

doi:10.1023/A:1023944527951

in der Wiesche, C., Martens, R., Zadrazil, F., 1996. Two-step degradation of pyrene by white-rot fungi and soil microorganisms. Appl. Microbiol. Biotechnol. 46, 653–659. doi:10.1007/s002530050876

Isaksen, T., Westereng, B., Aachmann, F.L., Agger, J.W., Kracher, D., Kittl, R., Ludwig, R., Haltrich, D., Eijsink, V.G.H., Horn, S.J., 2014. A C4-oxidizing lytic polysaccharide monooxygenase cleaving both cellulose and cello-oligosaccharides. J. Biol. Chem. 289, 2632–2642. doi:10.1074/jbc.M113.530196

Itoh, H., Wada, M., Honda, Y., Kuwahara, M., Watanabe, T., 2003. Bioorganosolve pretreatments for simultaneous saccharification and fermentation of beech wood by ethanolysis and white rot fungi. J. Biotechnol. 103, 273–280. doi:10.1016/S0168-1656(03)00123-8

Kalčíková, G., Babič, J., Pavko, A., Gotvajn, A.Ž., 2014. Fungal and enzymatic treatment of mature municipal landfill leachate. Waste Manag. 34, 798–803. doi:10.1016/j.wasman.2013.12.017

Karkehabadi, S., Hansson, H., Kim, S., Piens, K., Mitchinson, C., Sandgren, M., 2008. The first structure of a glycoside hydrolase family 61 member, Cel61B from *Hypocrea jecorina*, at 1.6 Å resolution. J. Mol. Biol. 383, 144–154. doi:10.1016/j.jmb.2008.08.016

Kersten, P.J., 1990. Glyoxal oxidase of *Phanerochaete chrysosporium*: its characterization and activation by lignin peroxidase. Proc. Natl. Acad. Sci. U. S. A. 87, 2936–40. doi: 10.1073/pnas.87.8.2936

Kirk, T.K., Farrell, R.L., 1987. Enzymatic "combustion": the microbial degradation of lignin. Annu. Rev. Microbiol. 41, 465–501. doi: 10.1146/annurev.mi.41.100187.002341

Kolpak, F.J., Blackwell, J., 1976. Determination of the structure of cellulose II. Macromolecules 9, 273–278.

Kudanga, T., Le Roes-Hill, M., 2014. Laccase applications in biofuels production: current status and future prospects. Appl. Microbiol. Biotechnol. 98, 6525–6542. doi:10.1007/s00253-014-5810-8

Langston, J.A., Shaghasi, T., Abbate, E., Xu, F., Vlasenko, E., Sweeney, M.D., 2011. Oxidoreductive cellulose depolymerization by the enzymes cellobiose dehydrogenase and glycoside hydrolase 61. Appl. Environ. Microbiol. 77, 7007–7015. doi:10.1128/AEM.05815-11

Letunic, I., Bork, P., 2011. Interactive Tree Of Life v2: online annotation and display of phylogenetic trees made easy. Nucleic Acids Res. 39, W475–W478. doi:10.1093/nar/gkr201

Li, D., Li, N., Ma, B., Mayfield, M.B., Gold, M.H., 1999. Characterization of genes encoding two manganese peroxidases from the lignin-degrading fungus *Dichomitus squalens*. Biochim. Biophys. Acta - Protein Struct. Mol. Enzymol. 1434, 356–364. doi:10.1016/S0167-4838(99)00191-0

Li, D., Youngs, H.L., Gold, M.H., 2001. Heterologous expression of a thermostable manganese peroxidase from *Dichomitus squalens* in *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. 385, 348–356. doi: 10.1006/abbi.2000.2159

Li, X., Beeson IV, W.T., Phillips, C.M., Marletta, M.A., Cate, J.H.D., 2012. Structural basis for substrate targeting and catalysis by fungal polysaccharide monooxygenases. Structure 20, 1051–1061. doi:10.1016/j.str.2012.04.002

Liers, C., Aranda, E., Strittmatter, E., Piontek, K., Plattner, D.A., Zorn, H., Ullrich, R., Hofrichter, M., 2014. Phenol oxidation by DyP-type peroxidases in comparison to fungal and plant peroxidases. J. Mol. Catal. B Enzym. 103, 41–46.

doi:10.1016/j.molcatb.2013.09.025

Liers, C., Bobeth, C., Pecyna, M., Ullrich, R., Hofrichter, M., 2010. DyP-like peroxidases of the jelly fungus *Auricularia auricula-judae* oxidize nonphenolic lignin model compounds and high-redox potential dyes. Appl. Microbiol. Biotechnol. 85, 1869–1879. doi:10.1007/s00253-009-2173-7

Liers, C., Pecyna, M.J., Kellner, H., Worrich, A., Zorn, H., Steffen, K.T., Hofrichter, M., Ullrich, R., 2013. Substrate oxidation by dye-decolorizing peroxidases (DyPs) from wood- and litter-degrading agaricomycetes compared to other fungal and plant heme-peroxidases. Appl. Microbiol. Biotechnol. 97, 5839–49. doi:10.1007/s00253-012-4521-2

Linde, D., Ruiz-Dueñas, F.J., Fernández-Fueyo, E., Guallar, V., Hammel, K.E., Pogni, R., Martínez, A.T., 2015. Basidiomycete DyPs: genomic diversity, structural-functional aspects, reaction mechanism and environmental significance. Arch. Biochem. Biophys. 574, 66–74. doi:10.1016/j.abb.2015.01.018

Liu, Y., Igarashi, K., Kaneko, S., Tonozuka, T., Samejima, M., Fukuda, K., Yoshida, M., 2009. Characterization of glycoside hydrolase family 6 enzymes from *Coprinopsis cinerea*. Biosci. Biotechnol. Biochem. 73, 1432–1434. doi:10.1271/bbb.80888

Lo Leggio, L., Simmons, T.J., Poulsen, J.-C.N., Frandsen, K.E.H., Hemsworth, G.R., Stringer, M.A, von Freiesleben, P., Tovborg, M., Johansen, K.S., De Maria, L., Harris, P. V, Soong, C.-L., Dupree, P., Tryfona, T., Lenfant, N., Henrissat, B., Davies, G.J., Walton, P.H., 2015. Structure and boosting activity of a starch-degrading lytic polysaccharide monooxygenase. Nat. Commun. 6, 5961. doi:10.1038/ncomms6961

Lombard, V., Golaconda Ramulu, H., Drula, E., Coutinho, P.M., Henrissat, B., 2014. The carbohydrate-active enzymes database (CAZy) in 2013. Nucleic Acids Res. 42, D490–D495. doi:10.1093/nar/gkt1178

Ludwig, R., Harreither, W., Tasca, F., Gorton, L., 2010. Cellobiose dehydrogenase: a versatile catalyst for electrochemical applications. Chemphyschem 11, 2674–2697. doi:10.1002/cphc.201000216

Ludwig, R., Salamon, A., Varga, J., Zámocky, M., Peterbauer, C.K., Kulbe, K.D., Haltrich, D., 2004. Characterisation of cellobiose dehydrogenases from the white-rot fungi *Trametes pubescens* and *Trametes villosa*. Appl. Microbiol. Biotechnol. 64, 213–222. doi:10.1007/s00253-003-1501-6

Lundell, T., Schoemaker, H., Hatakka, A., Brunow, G., 1993. New mechanism of the Cα-Cβ cleavage in non-phenolic arylglycerol β-aryl ether lignin substructures catalyzed by lignin peroxidase. Holzforschung 47, 219–224. doi:10.1515/hfsg.1993.47.3.219

MacDonald, J., Doering, M., Canam, T., Gong, Y., Guttman, D.S., Campbell, M.M., Master, E.R., 2011. Transcriptomic responses of the softwood-degrading white-rot fungus *Phanerochaete carnosa* during growth on coniferous and deciduous wood. Appl. Environ. Microbiol. 77, 3211–3218.

MacDonald, J., Master, E.R., 2012. Time-dependent profiles of transcripts encoding lignocellulose-modifying enzymes of the white rot fungus *Phanerochaete carnosa* grown on multiple wood substrates. Appl. Environ. Microbiol. 78, 1596–1600. doi:10.1128/AEM.06511-11

Mahajan, C., Basotra, N., Singh, S., Di Falco, M., Tsang, A., Chadha, B.S., 2016. *Malbranchea cinnamomea*: A thermophilic fungal source of catalytically efficient lignocellulolytic glycosyl hydrolases and metal dependent enzymes. Bioresour. Technol. 200, 55–63. doi:10.1016/j.biortech.2015.09.113

Mahajan, S., Master, E.R., 2010. Proteomic characterization of lignocellulose-degrading enzymes secreted by *Phanerochaete carnosa* grown on spruce and microcrystalline cellulose. Appl. Microbiol. Biotechnol. 86, 1903–1914. doi:10.1007/s00253-010-2516-4

Martínez, Á.T., Ruiz-Dueñas, F.J., Martínez, M.J., del Río, J.C., Gutiérrez, A., 2009. Enzymatic delignification of plant cell wall: from nature to mill. Curr. Opin. Biotechnol. 20, 348–357. doi:10.1016/j.copbio.2009.05.002

Martinez, D., Challacombe, J., Morgenstern, I., Hibbett, D., Schmoll, M., Kubicek, C.P., Ferreira, P., Ruiz-Dueñas, F.J., Martínez, A.T., Kersten, P., Hammel, K.E., Vanden Wymelenberg, A., Gaskell, J., Lindquist, E., Sabat, G., BonDurant, S.S., Larrondo, L.F., Canessa, P., Vicuña, R., Yadav, J., Doddapaneni, H., Subramanian, V., Pisabarro, A.G., Lavín, J.L., Oguiza, J.A., Master, E., Henrissat, B., Coutinho, P.M., Harris, P., Magnuson, J.K., Baker, S.E., Bruno, K., Kenealy, W., Hoegger, P.J., Kües, U., Ramaiya, P., Lucas, S., Salamov, A., Shapiro, H., Tu, H., Chee, C.L., Misra, M., Xie, G., Teter, S., Yaver, D., James, T., Mokrejs, M., Pospisek, M., Grigoriev, I.V., Brettin, T., Rokhsar, D., Berka, R., Cullen, D., 2009. Genome, transcriptome, and secretome analysis of wood decay fungus *Postia placenta* supports unique mechanisms of lignocellulose conversion. Proc. Natl. Acad. Sci. U. S. A. 106, 1954–1959. doi:10.1073/pnas.0809575106

Martinez, D., Larrondo, L.F., Putnam, N., Sollewijn Gelpke, M.D., Huang, K., Chapman, J., Helfenbein, K.G., Ramaiya, P., Detter, J.C., Larimer, F., Coutinho, P.M., Henrissat, B., Berka, R., Cullen, D., Rokhsar, D., 2004. Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium* strain RP78. Nat. Biotechnol. 22, 695–700. doi:10.1038/nbt967

Micard, V., Renard, C.M.G.C., Thibault, J.-F., 1996. Enzymatic saccharification of sugar-beet pulp. Enzyme Microb. Technol. 19, 162–170. doi:10.1016/0141-0229(95)00224-3

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31, 426–428. doi:10.1021/ac60147a030

Mohnen, D., 2008. Pectin structure and biosynthesis. Curr. Opin. Plant Biol. 11, 266-277. doi:10.1016/j.pbi.2008.03.006

Moilanen, U., Kellock, M., Galkin, S., Viikari, L., 2011. The laccase-catalyzed modification of lignin for enzymatic hydrolysis. Enzyme Microb. Technol. 49, 492–498. doi:10.1016/j.enzmictec.2011.09.012

Mooney, C.A., Mansfield, S.D., Touhy, M.G., Saddler, J.N., 1998. The effect of initial pore volume and lignin content on the enzymatic hydrolysis of softwoods. Bioresour. Technol. 64, 113–119. doi:10.1016/S0960-8524(97)00181-8

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., Ladisch, M., 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour. Technol. 96, 673–686. doi:10.1016/j.biortech.2004.06.025

Munk, L., Sitarz, A.K., Kalyani, D.C., Mikkelsen, J.D., Meyer, A.S., 2015. Can laccases catalyze bond cleavage in lignin? Biotechnol. Adv. 33, 13–24. doi:10.1016/j.biotechadv.2014.12.008

Muñoz, I.G., Ubhayasekera, W., Henriksson, H., Szabó, I., Pettersson, G., Johansson, G., Mowbray, S.L., Ståhlberg, J., 2001. Family 7 cellobiohydrolases from *Phanerochaete chrysosporium*: crystal structure of the catalytic module of Cel7D (CBH58) at 1.32 Å resolution and homology models of the isozymes. J. Mol. Biol. 314, 1097–1111. doi:10.1006/jmbi.2000.5180

Muzikář, M., Křesinová, Z., Svobodová, K., Filipová, A., Čvančarová, M., Cajthamlová, K., Cajthaml, T., 2011. Biodegradation of chlorobenzoic acids by ligninolytic fungi. J. Hazard. Mater. 196, 386–394. doi:10.1016/j.jhazmat.2011.09.041

Mäkelä, M., Galkin, S., Hatakka, A., Lundell, T., 2002. Production of organic acids and oxalate decarboxylase in lignin-degrading white rot fungi. Enzyme Microb. Technol. 30, 542–549. doi:10.1016/S0141-0229(02)00012-1

Mäkelä, M.R., Hildén, K., Hatakka, A., Lundell, T.K., 2009. Oxalate decarboxylase of the white-rot fungus *Dichomitus squalens* demonstrates a novel enzyme primary structure and non-induced expression on wood and in liquid cultures. Microbiology 155, 2726–2738. doi:10.1099/mic.0.028860-0

Mäkelä, M.R., Hildén, K.S., de Vries, R.P., 2014a. Degradation and modification of plant biomass by fungi, in: Nowrousian, M. (Ed.), Fungal Genomics, The Mycota. Springer, Berlin Heidelberg, Germany, pp. 175–208. doi:10.1007/978-3-642-45218-5_8

Mäkelä, M.R., Sietiö, O.-M., de Vries, R.P., Timonen, S., Hildén, K., 2014b. Oxalate-metabolising genes of the white-rot fungus *Dichomitus squalens* are differentially induced on wood and at high proton concentration. PLoS One 9, e87959. doi:10.1371/journal.pone.0087959

Nakagame, S., Furujyo, A., Sugiura, J., 2006. Purification and characterization of cellobiose dehydrogenase from white-rot basidiomycete *Trametes hirsuta*. Biosci. Biotechnol. Biochem. 70, 1629–1635. doi:10.1271/bbb.50692

Niemelä, T., 2006. Käävät, puiden sienet, Norrlinia, 13. Luonnontieteellinen keskusmuseo, Helsinki.

Niranjane, A.P., Madhou, P., Stevenson, T.W., 2007. The effect of carbohydrate carbon sources on the production of cellulase by *Phlebia gigantea*. Enzyme Microb. Technol. 40, 1464–1468. doi:10.1016/j.enzmictec.2006.10.041

Novotný, C., Trošt, N., Šlušla, M., Svobodová, K., Mikesková, H., Válková, H., Malachová, K., Pavko, A., 2012. The use of the fungus *Dichomitus squalens* for degradation in rotating biological contactor conditions. Bioresour. Technol. 114, 241–246. doi:10.1016/j.biortech.2012.03.080

Ohm, R.A., De Jong, J.F., Lugones, L.G., Aerts, A., Kothe, E., Stajich, J.E., de Vries, R.P., Record, E., Levasseur, A., Baker, S.E., Bartholomew, K.A., Coutinho, P.M., Erdmann, S., Fowler, T.J., Gathman, A.C., Lombard, V., Henrissat, B., Knabe, N., Kües, U., Lilly, W.W., Lindquist, E., Lucas, S., Magnuson, J.K., Piumi, F., Raudaskoski, M., Salamov, A., Schmutz, J., Schwarze, F.W.M.R., van Kuyk, P.A., Horton, J.S., Grigoriev, I.V., Wösten, H.A.B., 2010. Genome sequence of the model mushroom *Schizophyllum commune*. Nat. Biotechnol. 28, 957–963. doi:10.1038/nbt.1643

Olson, Å., Aerts, A., Asiegbu, F., Belbahri, L., Bouzid, O., Broberg, A., Canbäck, B., Coutinho, P.M., Cullen, D., Dalman, K., Deflorio, G., van Diepen, L.T.A., Dunand, C., Duplessis, S., Durling, M., Gonthier, P., Grimwood, J., Fossdal, C.G., Hansson, D., Henrissat, B., Hietala, A., Himmelstrand, K., Hoffmeister, D., Högberg, N., James, T.Y., Karlsson, M., Kohler, A., Kües, U., Lee, Y.-H., Lin, Y.-C., Lind, M., Lindquist, E., Lombard, V., Lucas, S., Lundén, K., Morin, E., Murat, C., Park, J., Raffaello, T., Rouzé, P., Salamov, A., Schmutz, J., Solheim, H., Ståhlberg, J., Vélëz, H., de Vries, R.P., Wiebenga, A., Woodward, S., Yakovlev, I., Garbelotto, M., Martin, F., Grigoriev, I.V., Stenlid, J., 2012. Insight into trade-off between wood decay and parasitism from the genome of a fungal forest pathogen. New Phytol. 194, 1001–1013. doi:10.1111/j.1469-8137.2012.04128.x

Ozimek, P., Veenhuis, M., Van Der Klei, I.J., 2005. Alcohol oxidase: a complex peroxisomal, oligomeric flavoprotein. FEMS Yeast Res. 5, 975–983. doi:10.1016/j.femsyr.2005.06.005

Ozturkoglu Budak, S., Zhou, M., Brouwer, C., Wiebenga, A., Benoit, I., Di Falco, M., Tsang, A., de Vries, R.P., 2014. A genomic survey of proteases in Aspergilli. BMC Genomics 15, 523. doi:10.1186/1471-2164-15-523

Palmqvist, E., Hahn-Hägerdal, B., 2000. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Bioresour. Technol. 74, 25–33. doi:10.1016/S0960-8524(99)00161-3

Patyshakuliyeva, A., Post, H., Zhou, M., Jurak, E., Heck, A.J.R., Hildén, K.S., Kabel, M.A.,

Mäkelä, M.R., Altelaar, M.A., de Vries, R.P., 2015. Uncovering the abilities of *Agaricus bisporus* to degrade plant biomass throughout its life cycle. Environ. Microbiol. 17, 3098–3109. doi:10.1111/1462-2920.12967

Périé, F.H., Gold, M.H., 1991. Manganese regulation of manganese peroxidase expression and lignin degradation by the white rot fungus *Dichomitus squalens*. Appl. Environ. Microbiol. 57, 2240–2245.

Périé, F.H., Reddy, G.V.B., Blackburn, N.J., Gold, M.H., 1998. Purification and characterization of laccases from the white-rot basidiomycete *Dichomitus squalens*. Arch. Biochem. Biophys. 353, 349–355. doi:10.1006/abbi.1998.0625

Périé, F.H., Sheng, D., Gold, M.H., 1996. Purification and characterization of two manganese peroxidase isozymes from the white-rot basidiomycete *Dichomitus squalens*. Biochim. Biophys. Acta - Protein Struct. Mol. Enzymol. 1297, 139–148. doi:10.1016/S0167-4838(96)00096-9

Phillips, C.M., Beeson, W.T., Cate, J.H., Marletta, M.A., 2011. Cellobiose dehydrogenase and a copper-dependent polysaccharide monooxygenase potentiate cellulose degradation by *Neurospora crassa*. ACS Chem. Biol. 6, 1399–1406. doi:10.1021/cb200351y

Polizeli, M.L.T.M., Rizzatti, A.C.S., Monti, R., Terenzi, H.F., Jorge, J.A., Amorim, D.S., 2005. Xylanases from fungi: properties and industrial applications. Appl. Microbiol. Biotechnol. 67, 577–591. doi:10.1007/s00253-005-1904-7

Quinlan, R.J., Sweeney, M.D., Lo Leggio, L., Otten, H., N Poulsen, J.-C., Johansen, K.S., Krogh, K.B.R.M., Jørgensen, C.I., Tovborg, M., Anthonsen, A., Tryfona, T., Walter, C.P., Dupree, P., Xu, F., Davies, G.J., Walton, P.H., 2011. Insights into the oxidative degradation of cellulose by a copper metalloenzyme that exploits biomass components. Proc. Natl. Acad. Sci. U. S. A. 108, 15079–15084. doi:10.1073/pnas.1105776108

Quiroz-Castañeda, R.E., Martínez-Anaya, C., Cuervo-Soto, L.I., Segovia, L., Folch-Mallol, J.L., 2011. Loosenin, a novel protein with cellulose-disrupting activity from *Bjerkandera adusta*. Microb. Cell Fact. 10, 8. doi:10.1186/1475-2859-10-8

Rahikainen, J.L., Moilanen, U., Nurmi-Rantala, S., Lappas, A., Koivula, A., Viikari, L., Kruus, K., 2013. Effect of temperature on lignin-derived inhibition studied with three structurally different cellobiohydrolases. Bioresour. Technol. 146, 118–25. doi:10.1016/j.biortech.2013.07.069

Ramos, L.P., Breuil, C., Saddler, J.N., 1992. Comparison of steam pretreatment of eucalyptus, aspen, and spruce wood chips and their enzymatic hydrolysis. Appl. Biochem. Biotechnol. 34-35, 37–48. doi:10.1007/BF02920532

Rass-Hansen, J., Falsig, H., Jørgensen, B., Christensen, C.H., 2007. Bioethanol: fuel or feedstock? J. Chem. Technol. Biotechnol. 82, 329–333. doi:10.1002/jctb.1665

Reese, E.T., Maguire, A., 1969. Surfactants as stimulants of enzyme production by microorganisms. Appl. Microbiol. 17, 242–245.

Riley, R., Salamov, A.A., Brown, D.W., Nagy, L.G., Floudas, D., Held, B.W., Levasseur, A., Lombard, V., Morin, E., Otillar, R., Lindquist, E.A., Sun, H., LaButti, K.M., Schmutz, J., Jabbour, D., Luo, H., Baker, S.E., Pisabarro, A.G., Walton, J.D., Blanchette, R.A., Henrissat, B., Martin, F., Cullen, D., Hibbett, D.S., Grigoriev, I.V., 2014. Extensive sampling of basidiomycete genomes demonstrates inadequacy of the white-rot/brown-rot paradigm for wood decay fungi. Proc. Natl. Acad. Sci. U. S. A. 111, 9923–9928. doi:10.1073/pnas.1400592111

Rosenbrock, P., Martens, R., Buscot, F., Zadrazil, F., Munch, J.C., 1997. Enhancing the mineralization of [U-^{14}C]dibenzo-*p*-dioxin in three different soils by addition of organic substrate or inoculation with white-rot fungi. Appl. Microbiol. Biotechnol. 48, 665–670. doi:10.1007/s002530051113

Rouau, X., Foglietti, M.-J., 1985. Purification and partial characterisation of three endo-glucanases from *Dichomitus squalens*. Carbohydr. Res. 142, 299–314. doi:10.1016/0008-6215(85)85031-X

Rouau, X., Odier, E., 1986a. Production of extracellular enzyme by the white-rot fungus *Dichomitus squalens* in cellulose-containing liquid culture. Enzyme Microb. Technol. 8, 22–26. doi:10.1016/0141-0229(86)90005-0

Rouau, X., Odier, E., 1986b. Purification and properties of two enzymes from *Dichomitus squalens* which exhibit both cellobiohydrolase and xylanase activity. Carbohydr. Res. 145, 279–292. doi:10.1016/S0008-6215(00)90435-X

Ryvarden, L., 1991. Genera of polypores, nomenclature and taxonomy. Synopsis fungorum 5, 1–363.

Sato, S., Feltus, F.A., Iyer, P., Tien, M., 2009. The first genome-level transcriptome of the wood-degrading fungus *Phanerochaete chrysosporium* grown on red oak. Curr. Genet. 55, 273–286. doi:10.1007/s00294-009-0243-0

Sheldon, R.A., 2014. Green and sustainable manufacture of chemicals from biomass: state of the art. Green Chem. 16, 950–963. doi:10.1039/C3GC41935E

Sjöström, E., 1993. Wood chemistry: fundamentals and applications, Academic Press, San Diego, CA. 293 p.

Sturn, A., Quackenbush, J., Trajanoski, Z. 2002. Genesis: cluster analysis of microarray data. Bioinformatics. 18, 207–208. doi: 10.1093/bioinformatics/18.1.207

Šušla, M., Novotný, C., Erbanová, P., Svobodová, K., 2008. Implication of *Dichomitus squalens* manganese-dependent peroxidase in dye decolorization and cooperation of the enzyme with laccase. Folia Microbiol. 53, 479–485. doi:10.1007/s12223-008-0075-1

Šušla, M., Novotný, C., Svobodová, K., Susla, M., Novotný, C., Svobodová, K., 2007. The implication of *Dichomitus squalens* laccase isoenzymes in dye decolorization by immobilized fungal cultures. Bioresour. Technol. 98, 2109–2115. doi:10.1016/j.biortech.2006.08.007

Suzuki, H., MacDonald, J., Syed, K., Salamov, A., Hori, C., Aerts, A., Henrissat, B., Wiebenga, A., van Kuyk, P.A., Barry, K., Lindquist, E., LaButti, K., Lapidus, A., Lucas, S., Coutinho, P., Gong, Y., Samejima, M., Mahadevan, R., Abou-Zaid, M., de Vries, R.P., Igarashi, K., Yadav, J.S., Grigoriev, I.V., Master, E.R., 2012. Comparative genomics of the white-rot fungi, *Phanerochaete carnosa* and *P. chrysosporium*, to elucidate the genetic basis of the distinct wood types they colonize. BMC Genomics 13. doi:10.1186/1471-2164-13-444

Suzuki, H., Vuong, T. V, Gong, Y., Chan, K., Ho, C.-Y., Master, E.R., Kondo, A., 2014. Sequence diversity and gene expression analyses of expansin-related proteins in the white-rot basidiomycete, *Phanerochaete carnosa*. Fungal Genet. Biol. 72, 115–23. doi:10.1016/j.fgb.2014.05.008

Szabó, I.J., Johansson, G., Pettersson, G., 1996. Optimized cellulase production by *Phanerochaete chrysosporium*: control of catabolite repression by fed-batch cultivation. J. Biotechnol. 48, 221–230. doi:10.1016/0168-1656(96)01512-X

Tan, T.-C., Kracher, D., Gandini, R., Sygmund, C., Kittl, R., Haltrich, D., Hällberg, B.M., Ludwig, R., Divne, C., 2015. Structural basis for cellobiose dehydrogenase action during oxidative cellulose degradation. Nat. Commun. 6, 7542. doi:10.1038/ncomms8542

Temp, U., Eggert, C., 1999. Novel interaction between laccase and cellobiose dehydrogenase during pigment synthesis in the white rot fungus *Pycnoporus cinnabarinus*. Appl. Environ. Microbiol. 65, 389–395.

Toda, H., Nagahata, N., Amano, Y., Nozaki, K., Kanda, T., Okazaki, M., Shimosaka, M., 2008. Gene cloning of cellobiohydrolase II from the white rot fungus *Irpex lacteus* MC-

2 and its expression in *Pichia pastoris*. Biosci. Biotechnol. Biochem. 72, 3142–3147. doi:10.1271/bbb.80316

Tovar-Herrera, O.E., Batista-García, R.A., Sánchez-Carbente, M.D.R., Iracheta-Cárdenas, M.M., Arévalo-Niño, K., Folch-Mallol, J.L., 2015. A novel expansin protein from the white-rot fungus *Schizophyllum commune*. PLoS One 10, e0122296. doi:10.1371/journal.pone.0122296

Uzcategui, E., Ruiz, A., Montesino, R., Johansson, G., Pettersson, G., 1991. The 1,4-β-D-glucan cellobiohydrolases from *Phanerochaete chrysosporium*. I. A system of synergistically acting enzymes homologous to *Trichoderma reesei*. J. Biotechnol. 19, 271–285. doi:10.1016/0168-1656(91)90064-3

Vaaje-Kolstad, G., Westereng, B., Horn, S.J., Liu, Z., Zhai, H., Sørlie, M., Eijsink, V.G.H., 2010. An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides. Science. 330, 219–222. doi:10.1126/science.1192231

Vallim, M.A., Janse, B.J.H., Gaskell, J., Pizzirani-Kleiner, A.A., Cullen, D., 1998. *Phanerochaete chrysosporium* cellobiohydrolase and cellobiose dehydrogenase transcripts in wood. Appl. Environ. Microbiol. 64, 1924–1928.

van den Brink, J., de Vries, R.P., 2011. Fungal enzyme sets for plant polysaccharide degradation. Appl. Microbiol. Biotechnol. 91, 1477–1492. doi:10.1007/s00253-011-3473-2

Vanden Wymelenberg, A., Gaskell, J., Mozuch, M., BonDurant, S.S., Sabat, G., Ralph, J., Skyba, O., Mansfield, S.D., Blanchette, R.A., Grigoriev, I.V., Kersten, P.J., Cullen, D., 2011. Significant alteration of gene expression in wood decay fungi *Postia placenta* and *Phanerochaete chrysosporium* by plant species. Appl. Environ. Microbiol. 77, 4499–4507. doi:10.1128/AEM.00508-11

Vanden Wymelenberg, A., Gaskell, J., Mozuch, M., Kersten, P., Sabat, G., Martinez, D., Cullen, D., 2009. Transcriptome and secretome analyses of *Phanerochaete chrysosporium* reveal complex patterns of gene expression. Appl. Environ. Microbiol. 75, 4058–4068. doi:10.1128/AEM.00314-09

Vanden Wymelenberg, A., Gaskell, J., Mozuch, M., Sabat, G.G., Ralph, J., Skyba, O., Mansfield, S.D., Blanchette, R.A., Martinez, D., Grigoriev, I., Kersten, P.J., Cullen, D., 2010. Comparative transcriptome and secretome analysis of wood decay fungi *Postia placenta* and *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 76, 3599–3610. doi:10.1128/AEM.00058-10

Vanden Wymelenberg, A., Minges, P., Sabat, G., Martinez, D., Aerts, A., Salamov, A., Grigoriev, I., Shapiro, H., Putnam, N., Belinky, P., Dosoretz, C., Gaskell, J., Kersten, P., Cullen, D., 2006. Computational analysis of the *Phanerochaete chrysosporium* v2.0 genome database and mass spectrometry identification of peptides in ligninolytic cultures reveal complex mixtures of secreted proteins. Fungal Genet. Biol. 43, 343–356. doi:10.1016/j.fgb.2006.01.003

Vanden Wymelenberg, A., Sabat, G., Martinez, D., Rajangam, A.S., Teeri, T.T., Gaskell, J., Kersten, P.J., Cullen, D., 2005. The *Phanerochaete chrysosporium* secretome: database predictions and initial mass spectrometry peptide identifications in cellulose-grown medium. J. Biotechnol. 118, 17–34. doi:10.1016/j.jbiotec.2005.03.010

Wariishi, H., Valli, K., Gold, M.H., 1992. Manganese(II) oxidation by manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*. Kinetic mechanism and role of chelators. J. Biol. Chem. 267, 23688–23695.

Várnai, A., Huikko, L., Pere, J., Siika-aho, M., Viikari, L., 2011. Synergistic action of xylanase and mannanase improves the total hydrolysis of softwood. Bioresour. Technol. 102, 9096–9104. doi:10.1016/j.biortech.2011.06.059

Várnai, A., Siika-Aho, M., Viikari, L., 2013. Carbohydrate-binding modules (CBMs)

revisited: reduced amount of water counterbalances the need for CBMs. Biotechnol. Biofuels 6. doi:10.1186/1754-6834-6-30

Veneault-Fourrey, C., Commun, C., Kohler, A., Morin, E., Balestrini, R., Plett, J., Danchin, E., Coutinho, P., Wiebenga, A., de Vries, R.P., Henrissat, B., Martin, F., 2014. Genomic and transcriptomic analysis of *Laccaria bicolor* CAZome reveals insights into polysaccharides remodelling during symbiosis establishment. Fungal Genet. Biol. 72, 168–181. doi:10.1016/j.fgb.2014.08.007

Venugopal, P., Junninen, K., Linnakoski, R., Edman, M., Kouki, J., 2016. Climate and wood quality have decayer-specific effects on fungal wood decomposition. For. Ecol. Manage. 360, 341–351. doi:10.1016/j.foreco.2015.10.023

Westereng, B., Cannella, D., Wittrup Agger, J., Jørgensen, H., Larsen Andersen, M., Eijsink, V.G.H., Felby, C., 2015. Enzymatic cellulose oxidation is linked to lignin by long-range electron transfer. Sci. Rep. 5, 18561. doi:10.1038/srep18561

Westereng, B., Ishida, T., Vaaje-Kolstad, G., Wu, M., Eijsink, V.G.H., Igarashi, K., Samejima, M., Ståhlberg, J., Horn, S.J., Sandgren, M., 2011. The putative endoglucanase pcGH61D from *Phanerochaete chrysosporium* is a metal-dependent oxidative enzyme that cleaves cellulose. PLoS One 6. doi:10.1371/journal.pone.0027807

Whittaker, M.M., Kersten, P.J., Nakamura, N., Sanders-Loehr, J., Schweizer, E.S., Whittaker, J.W., 1996. Glyoxal oxidase from *Phanerochaete chrysosporium* is a new radical-copper oxidase. J. Biol. Chem. 271, 681–687. doi:10.1074/jbc.271.2.681

Vogel, C., Marcotte, E.M., 2012. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. Nat. Rev. Genet. 13, 227–232. doi:10.1038/nrg3185

Vogel, J., 2008. Unique aspects of the grass cell wall. Curr. Opin. Plant Biol. 11, 301–307. doi:10.1016/j.pbi.2008.03.002

von Ossowski, I., Ståhlberg, J., Koivula, A., Piens, K., Becker, D., Boer, H., Harle, R., Harris, M., Divne, C., Mahdi, S., Zhao, Y., Driguez, H., Claeyssens, M., Sinnott, M.L., Teeri, T.T., 2003. Engineering the exo-loop of *Trichoderma reesei* cellobiohydrolase, Cel7A. A comparison with *Phanerochaete chrysosporium* Cel7D. J. Mol. Biol. 333, 817–829. doi:10.1016/S0022-2836(03)00881-7

Vu, V.V., Beeson, W.T., Span, E.A., Farquhar, E.R., Marletta, M.A., 2014. A family of starch-active polysaccharide monooxygenases. Proc. Natl. Acad. Sci. 111, 13822–13827. doi:10.1073/pnas.1408090111

Wu, M., Beckham, G.T., Larsson, A.M., Ishida, T., Kim, S., Payne, C.M., Himmel, M.E., Crowley, M.F., Horn, S.J., Westereng, B., Igarashi, K., Samejima, M., Ståhlberg, J., Eijsink, V.G.H., Sandgren, M., 2013. Crystal structure and computational characterization of the lytic polysaccharide monooxygenase GH61D from the basidiomycota fungus *Phanerochaete chrysosporium*. J. Biol. Chem. 288, 12828–12839. doi:10.1074/jbc.M113.459396

Yoshida, M., Sato, K., Kaneko, S., Fukuda, K., 2009. Cloning and transcript analysis of multiple genes encoding the glycoside hydrolase family 6 enzyme from *Coprinopsis cinerea*. Biosci. Biotechnol. Biochem. 73, 67–73. doi:10.1271/bbb.80477

Zámocký, M., Hallberg, M., Ludwig, R., Divne, C., Haltrich, D., 2004. Ancestral gene fusion in cellobiose dehydrogenases reflects a specific evolution of GMC oxidoreductases in fungi. Gene 338, 1–14. doi:10.1016/j.gene.2004.04.025

Recent Publications in this Series

33/2015 Niina Idänheimo
The Role of Cysteine-rich Receptor-like Protein Kinases in ROS signaling in *Arabidopsis thaliana*
34/2015 Susanna Keriö
Terpene Analysis and Transcript Profiling of the Conifer Response to *Heterobasidion annosum s.l.* Infection and *Hylobius abietis* Feeding
35/2015 Ann-Katrin Llarena
Population Genetics and Molecular Epidemiology of *Campylobacter jejuni*
1/2016 Hanna Help-Rinta-Rahko
The Interaction Of Auxin and Cytokinin Signalling Regulates Primary Root Procambial Patterning, Xylem Cell Fate and Differentiation in *Arabidopsis thaliana*
2/2016 Abbot O. Oghenekaro
Molecular Analysis of the Interaction between White Rot Pathogen *(Rigidoporus microporus)* and Rubber Tree *(Hevea brasiliensis)*
3/2016 Stiina Rasimus-Sahari
Effects of Microbial Mitochondriotoxins from Food and Indoor Air on Mammalian Cells
4/2016 Hany S.M. EL Sayed Bashandy
Flavonoid Metabolomics in *Gerbera hybrida* and Elucidation of Complexity in the Flavonoid Biosynthetic Pathway
5/2016 Erja Koivunen
Home-Grown Grain Legumes in Poultry Diets
6/2016 Paul Mathijssen
Holocene Carbon Dynamics and Atmospheric Radiative Forcing of Different Types of Peatlands in Finland
7/2016 Seyed Abdollah Mousavi
Revised Taxonomy of the Family *Rhizobiaceae*, and Phylogeny of Mesorhizobia Nodulating *Glycyrrhiza spp*.
8/2016 Sedeer El-Showk
Auxin and Cytokinin Interactions Regulate Primary Vascular Patterning During Root Development in *Arabidopsis thaliana*
9/2016 Satu Olkkola
Antimicrobial Resistance and Its Mechanisms among *Campylobacter coli* and *Campylobacter upsaliensis* with a Special Focus on Streptomycin
10/2016 Windi Indra Muziasari
Impact of Fish Farming on Antibiotic Resistome and Mobile Elements in Baltic Sea Sediment
11/2016 Kari Kylä-Nikkilä
Genetic Engineering of Lactic Acid Bacteria to Produce Optically Pure Lactic Acid and to Develop a Novel Cell Immobilization Method Suitable for Industrial Fermentations
12/2016 Jane Etegeneng Besong epse Ndika
Molecular Insights into a Putative Potyvirus RNA Encapsidation Pathway and Potyvirus Particles as Enzyme Nano-Carriers
13/2016 Lijuan Yan
Bacterial Community Dynamics and Perennial Crop Growth in Motor Oil-Contaminated Soil in a Boreal Climate
14/2016 Pia Rasinkangas
New Insights into the Biogenesis of *Lactobacillus rhamnosus GG* Pili and the *in vivo* Effects of Pili

Helsinki 2016 ISSN 2342-5423 ISBN 978-951-51-2331-2