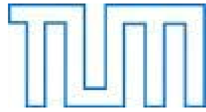

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Biochemical mechanisms of brown rot decay: A study on the mode of action of modified wood

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My brain surprises even me sometimes

- Professor Dumbledore (Harry Potter and the sorcerer's stone, J.K Rowling)

Summary

Non-durable wood in ground contact has traditionally been treated with toxic preservatives but these are now being questioned and to some extent restricted from use. In order to facilitate the sustainable use of wood in construction, other wood durability treatments must be developed and used. One of the promising alternatives to preservative treatment is wood modification. Modified wood is treated chemically and/or physically to enhance desired properties of the wood, among them durability. Understanding the mode of action against wood deteriorating organisms may facilitate efficient development of modified wood materials to increase their durability by allowing a more targeted approach. However, the exact mechanisms of how the wood modification techniques prevent biodeterioration have so far not been revealed.

Brown rot fungi cause the most destruction in wood construction in the Northern hemisphere, due to the preferential use of soft wood species in this region. Brown rot fungi degrade the wood polysaccharides, while modifying the lignin, by opening up the wood cell wall through induction of the formation of hydroxyl radicals inside the wood cell wall (chelator mediated Fenton (CMF) degradation) and subsequent enzymatic hydrolysis.

The aim of this thesis was to elucidate the mechanisms of decay resistance of acetylated, furfurylated, dimethylol dihydroxyethyleneurea (DMDHEU) treated and thermally modified wood to the brown rot fungus *Postia placenta*. The effects of acetylation, furfurylation, DMDHEU-treatment and thermal modification on fungal colonisation, moisture content, structural integrity and mass loss of the wood specimens and growth pattern of the fungi and its gene expression during exposure of materials treated in these ways to *P. placenta* were addressed. In acetylated wood, the ability of *P. placenta* to degrade acetyl was investigated. Furthermore, the ability of Fenton derived hydroxyl radicals and fungal cellulases to degrade polysaccharides in modified wood was studied. Finally, the results in this thesis as well as in the current literature were discussed based on the biochemical mechanisms of brown rot wood degradation in order to establish which mechanisms in the brown rot wood degradation process that possibly are inhibited by wood modification.

In the majority of the experiments, a decay test was run in which modified and untreated pine sapwood was exposed to the *P. placenta* for up to 300 days. Samples were harvested continuously for analysis of mass loss, moisture content, structural integrity, acetyl content and gene expression. Structural integrity was measured with the High Energy Multiple impact (HEMI) test, in which wood specimens are subjected to steel balls during shaking after which the resistance to impact milling (RIM) is calculated based on the sizes of the wood particles left. Acetyl content was analysed with gas chromatography and gene expression of selected genes involved in wood degradation was analysed with quantitative PCR (qPCR). The ability of hydroxyl radicals and fungal cellulases to degrade polysaccharides in modified wood was analysed in an *in vitro* test in which modified and untreated wood meal of pine sapwood was subjected to Fenton's reagent, fungal cellulases or both. Subsequently, the amount of reducing sugars released into the supernatant was measured spectrophotometrically using dinitrosalicylic acid (DNS).

Mass loss was lower than 3% on average and structural integrity maintained in acetylated and furfurylated wood treated to high levels and exposed to *P. placenta* for 300 days. Furthermore,

acetyl in acetylated wood was not degraded. In thermally modified wood, the rate of mass loss was very low compared to untreated wood up to 5% mass loss, after which the degradation rate increased considerably but was still lower than in untreated wood. Structural integrity in thermally modified wood was similar to that in untreated wood above 1% mass loss and when comparing samples with similar mass loss. Moisture content was lower in the modified wood materials both before and throughout the decay test. Mass loss of more than 3% were found in single samples with moisture content below 20%, which is a lower moisture content than what has previously been believed to allow degradation. Previous studies have not measured the above-mentioned parameters for time periods as long as in this work or with as high frequency as in this work, but results from single time-points are in agreement with previous studies of modified materials treated in the same way and to equal levels.

P. placenta was shown to colonise modified wood already during the first week of exposure and to up-regulate genes during the first two weeks of exposure. Colonisation and expression of genes in *P. placenta* growing on modified wood materials had previously only been reported for exposure times of two weeks and more, but gene expression up-regulation was observed in these studies. Comparing gene expression in thermally modified and untreated wood with the same mass loss showed that once degradation has started, the gene expression was similar in the two materials.

Fenton derived hydroxyl radicals and fungal cellulases were shown to degrade polysaccharides in acetylated wood meal *in vitro*. The efficacy was, however, lower than for untreated wood. The ability of hydroxyl radicals to degrade polysaccharides in modified wood has previously only been shown for DMDHEU-treated wood, but also in this material the efficacy was reduced. Previous studies show that fungal cellulases are able to degrade DMDHEU-treated and furfurylated wood. Unfortunately, the ability of hydroxyl radicals to degrade furfurylated wood could not be investigated in this work, since presumed degradation products of the poly(furfuryl alcohol) interfered with the method of analysis.

The research conducted for this thesis together with current research indicate that wood modification may interfere with a regulatory process in the formation or secretion of components necessary for wood degradation, with penetration and transportation of wood degrading agents through the wood cell wall or the sequestration and reduction of ferrous iron. A positive correlation between the reduction of equilibrium moisture content and decay resistance in modified wood materials has been frequently reported. A comprehensive review and evaluation of experimental data and established theories in the literature based on the biochemical mechanism of the brown rot wood degradation process has not been previously reported.

Zusammenfassung

Die herkömmliche Schutzbehandlung von nicht dauerhaftem Holz für den Einsatz im Erdkontakt wird aufgrund der giftigen, und teilweise bereits verbotenen Wirkstoffe zunehmend in Frage gestellt. Um eine nachhaltige Nutzung von Holz im wetterexponierten Anwendungen im Bauwesen zu ermöglichen, müssen alternative Schutzbehandlungen von Holz entwickelt und eingesetzt werden. Die Holzmodifizierung gilt als eine der vielversprechenden Alternativen zur klassischen Holzschutzbehandlung. Das Holz wird durch chemische und/oder physikalische Verfahren modifiziert, um gewünschte Eigenschaften, u.a. die Dauerhaftigkeit, zu verbessern. Das Verständnis der Wirkungsweise der Modifikationsverfahren gegen holzzerstörende Organismen ist für eine Weiterentwicklung und Optimierung der Modifikationstechnologien Voraussetzung. Allerdings wurden die genauen Mechanismen, wie die Holzmodifikationsverfahren einen biologischen Abbau verhindern, bis jetzt nicht ausreichend aufgezeigt.

Braunfäulepilze verursachen die größte Zerstörung an Holzkonstruktionen der nördlichen Hemisphäre aufgrund der dort vorzugsweisen Nutzung von Nadelholz. Braunfäulepilze bauen die Polysaccharide des Holzes ab, wohingegen Lignin nur modifiziert wird, indem sie die Zugänglichkeit der Zellwände über die Bildung von Hydroxylradikalen in der Zellwand einleiten *(Chelator-vermittelter Fenton (CMF) Abbau*), woran sich die enzymatische Hydrolyse anschliesst.

Ziel dieser Dissertation war es, die Mechanismen der verbesserten Widerstandsfähigkeit von acetyliertem, furfuryliertem, mit Dimethyloldihydroxyethylenurea (DMDHEU) behandeltem, sowie thermisch modifiziertem Holz gegen den Braunfäulepilz *Postia placenta* aufzuklären. Der Einfluss von Acetylierung, Furfurylierung und thermischer Modifizierung auf die Pilzbesiedlung, den Feuchtegehalt, die strukturelle Integrität und den Masseverlust der Holzproben sowie die Wachstumsmuster der Pilze und deren Genexpression beim Kontakt mit den modifizierten Hölzern wurde untersucht. Es wurde analysiert, inwieweit *P. placenta* fähig ist, in acetyliertem Holz das Acetyl abzubauen. Zudem wurde untersucht, ob durch Fenton-Reaktion erzeugte Hydroxylradikale und pilzliche Cellulasen in der Lage sind, Polysaccharide des modifizierten Holzes abzubauen. Abschließend wurden die experimentellen Ergebnisse dieser Arbeit mit den Erkenntnissen der aktuellen Literatur zu den biochemischen Mechanismen des Holzabbaus durch Braunfäule diskutiert, um Erklärungen für die möglichen Mechanismen für die Hemmung der Holzzerstörung an modifizierten Holz zu finden.

Bei den meisten Experimenten wurden Abbauversuche durchgeführt, bei denen modifiziertes und unbehandeltes Kiefernsplintholz dem Braunfäulepilz *P. placenta* für eine Dauer von 300 Tagen ausgesetzt wurde. Zur Analyse von Masseverlust, Feuchtegehalt, struktureller Integrität, Acetylgehalt und Genexpression wurden kontinuierlich Proben entnommen. Die strukturelle Integrität wurde anhand von High Energy Multiple Impact (HEMI) Tests bestimmt. Dazu wurden die Holzproben in einer Schüttelkammer durch die Einwirkung von Stahlkugeln zerkleinert und der Widerstand gegen die Schlagbeanspruchung (RIM) in Bezug auf die vorliegende Größe der Holzpartikel berechnet. Der Acetylgehalt wurde mittels Gaschromatographie bestimmt. Die Genexpression ausgewählter, am Holzabbau beteiligter Gene wurde anhand quantitativer PCR (qPCR) untersucht. In einem *in-vitro* Test wurde geprüft, inwieweit Hydroxylradikale und pilzliche Cellulase fähig sind, Polysaccharide in modifiziertem Holz abzubauen. Dazu wurde Mehl von modifizierten und unbehandelten Kiefernsplintholz einem Fenton-Reagens, pilzlicher Cellulase oder beidem ausgesetzt. Anschließend wurde die Menge an reduzierendem Zucker, der

in den Überstand freigesetzt wurde, mittels Dinitrosalicylic Säure (DNS) spektrophotometrisch bestimmt.

Der Masseverlust war durchschnittlich um 3% niedriger, und die strukturelle Integrität in stark modifiziertem acetylierten und furfurylierten Holz, das für eine Dauer von 300 Tagen *P. placenta* ausgesetzt war, wurde bewahrt. Außerdem konnte kein Abbau von Acetyl in acetyliertem Holz nachgewiesen werden. In thermisch modifizierten Holz war die Rate an Substanzabbau im Vergleich zu unbehandelten Holz bis zu 5% Masseverlust sehr gering, danach nahm die Abbaugeschwindigkeit beachtlich zu, war jedoch immer noch geringer als im unbehandelten Holz. Die strukturelle Integrität in thermisch modifizierten Holz war, bei vergleichbarem Masseverlust und ab einem Niveau von 1% Masseverlust ähnlich der von unbehandelten Holz. Der Feuchtegehalt in modifizierten Holz war sowohl vor als auch während des Abbauversuchs geringer. Ein Masseverlust von mehr als 3% wurde in einzelnen Proben mit einem Feuchtegehalt unter 20% gefunden, was unter dem Feuchteniveau liegt, der bisher für einen Abbau angenommenen wurde. In früheren Studien waren sowohl die Zeitdauer als auch die Frequenz der Messung niedriger, aber die Messungen für einzelne Zeitpunkte stimmen mit den Ergebnissen dieser Studien überein, wo auf gleiche Weise und in gleichem Grad modifiziertes Material untersucht wurde.

Eine Besiedlung des modifizierten Holzes *mit P. placenta* konnte bereits in der ersten Woche der Versuche nachgewiesen werden, einhergehend mit einer Hochregulierung der am CMF Abbau beteiligten Gene in den ersten zwei Wochen. Über Ansiedlung und Genexpression von auf modifizierten Holzmaterial wachsenden *P. placenta* wurde bislang nur für Befallszeiten von zwei oder mehr Wochen berichtet, jedoch war die Expression von CMF-bedingten Genen auch in diesen Studien hochreguliert. Ein Vergleich der Genexpression in thermisch modifizierten und unbehandelten Holz mit demselben Masseverlust hat gezeigt, dass die Genexpression in beiden Materialien ähnlich war, wenn der Abbau erst einmal begonnen hat.

Es wurde gezeigt, dass durch Fenton-Reaktion erzeugte Hydroxylradikale und Cellulasen Polysaccharide in acetylierten Holz *in-vitro* abbauen. Die Wirkung war jedoch geringer als in unbehandelten Holz. Die Fähigkeit, Polysaccharide durch Hydroxylradikale abzubauen, wurde bereits früher für DMDHEU-behandeltes Holz aufgezeigt, aber auch in diesem Material war die Wirkung reduziert. Frühere Studien zeigen, dass pilzliche Cellulasen mit DMDHEU behandeltes und furfuryliertes Holz abbauen. Jedoch konnte in dieser Arbeit die Fähigkeit der Hydroxylradikale, furfuryliertes Holz abzubauen nicht untersucht werden, da die mutmaßlichen Abbauprodukte des Poly(Furfurylalkohols) die Analysemethoden beeinträchtigten.

Die für diese Dissertation durchgeführten Untersuchungen, ergänzt durch aktuelle Forschungen weisen darauf hin, dass die Holzmodifizierung die Regulierungsprozesse bei der Bildung oder Sekretion von für den Holzabbau notwendigen Bestandteile, die Eindringung und den Transport von Abbausubstanzen durch die Holzzellwand, oder der Bindung und Reduzierung von Ferroeisen behindern können. Eine positive Korrelation zwischen der Reduzierung der Gleichgewichtsfeuchte und dem Abbauwiderstand in modifizierten Materialien wurde vielfach berichtet. Eine vergleichbar umfassende Auswertung und Analyse von Versuchsdaten sowie der etablierten Theorien aus der Literatur, basierend auf den biochemischen Mechanismen des Braunfäulebefalls, wurde bisher nicht vorgelegt.

Sammanfattning

Träs hållbarhet har traditionellt förbättrats med hjälp av giftiga impregneringsmedel men dessa ifrågasätts nu alltmer och vissa restriktioner har utfärdats. För att möjliggöra ett hållbart användande av trä i konstruktioner, måste andra träskyddsmedel användas istället. Ett av de mer lovande alternativen till impregnering är trämodifiering. Modifierat trä är behandlat kemiskt och/eller fysiskt för att förbättra önskade egenskaper, som t ex hållbarhet. För att effektivt och på ett mer riktat vis kunna förbättra hållbarheten på modifierade trämaterial är det viktigt att förstå de underliggande mekanismerna för materialens motståndskraft mot nedbrytning. Mekanismerna bakom modifierat träs motståndskraft mot svampangrepp har dock än så länge inte avslöjats.

Brunröte-svampar orsakar mest nedbrytning in träkonstruktioner på det norra halvklotet, på grund av den huvudsakliga användningen av barrved i den här regionen. Brunröte-svampar bryter ner träets polysackarider genom initiera bildandet av hydroxylradikaler inuti träcellväggen (chelator mediated Fenton (CMF) degradation) och på då vis att luckra upp träcellväggen och därefter hydrolysera dem enzymatiskt. Ligninet bryts inte ner under denna process men modifieras något.

Syftet med den här avhandlingen var att utröna mekanismerna bakom rötresistensen mot brunröte-svampen *Postia placenta* hos acetylerat, furfurylerat, dimethylol dihydroxyethyleneurea(DMHEU)-behandlat och termiskt modifierat trä. Effekten av acetylering, furfurylering, DMDHEU-behandling och termisk modifiering på svampens kolonisationsförmåga, fukthalt, strukturell integritet och massaförlust hos träprovet och svampens tillväxtmönster och dess genuttryck vid exponering av dessa material för *P. placenta* undersöktes. Dessutom undersöktes möjligheten för hydroxlradikaler (härstammande från Fenton-reaktionen) och svampars cellulaser att bryta ner polysackarider i modifierat trä. Slutligen diskuterades resultaten från den här avhandlingen och i den aktuella litteraturen utifrån de biokemiska mekanismerna bakom brunröte-svampens nedbrytningsprocess med syfte att fastslå vilka mekanismer i brunrötans nedbrytningsprocess som möjligen förhindras av trämodifiering.

I huvuddelen av experimenten utfördes ett rötförsök där modifierad och obehandlad splintved av furu utsattes för *P. placenta* i upp till 300 dagar. Proverna skördades kontinuerligt och analyserades för massaförlust, fukthalt, strukturell integritet, acetylinnehåll och genuttryck. Strukturell integritet mättes med High Energy Multiple Impact (HEMI)-testet vid vilket träprover skakas ihop med stålkulor och motstånd mot malning (resistance to impact milling (RIM)) räknas ut baserat på storleken av de kvarvarande träpartiklarna. Acetylinnehåll analyserades med gaskromatografi och genuttryck av utvalda gener analyserades med kvantitativ PCR (qPCR). Hydroxlradikalers och cellulasers förmåga att bryta ner polysackarider i modifierat träanalyserad i ett *in vitro*-försök där modifierat och obehandlat trämjöl av furu-splintved utsattes för Fentons reagens, cellulaser från svampar eller både och. Därefter kvantifierades mängden reducerande socker i supernatanten med dinitrosalicylsyra (DNS) i en spektrofotometer

Massaförlusten var mindre än 3% I medel och den strukturella integriteten oförändrad I acetylerat och furfurylerat trä med höga behandlingsnivåer vid exponering för *P. placenta* i 300 dagar. Acetyl i acetylerat trä bröts heller inte ner under detta försök. I termiskt modifierat trä var nedbrytningstakten låg jämfört med den i obehandlat trä upp till 5% massaförlust, varpå

nedbrytningstakten ökade väsentligt men den var fortfarande lägre än i obehandlat trä. Strukturell integritet i termiskt modifierat trä var likvärdig med den i obehandlat trä över 1% massaförlust vid jämförande av prover med likvärdig massaförlust. Fukthalten var lägre i modifierat trä både före och genom hela nedbrytningsförsöken. Massaförlust på mer än 3% uppmättes i enskilda prover som hade en fukthalt under 20%, vilket är en lägre fukthalt än vad som tidigare har trotts tillåta nedbrytning. I tidigare studier med modifierat trä av samma slag och samma behandlingsnivå har man inte mätt dessa parametrar under lika lång tid eller med så pass hög frekvens som i detta arbete.

P. placenta påvisades kunna kolonisera modifierat trä redan under första exponeringsveckan och upp-reglera gener involverade i CMF-nedbrytning under de två första exponeringsveckorna. *P. placentas* kolonisering och uttryck av gener i modifierat trä har tidigare bara studerats vid tidpunkter från två veckor och senare efter exponeringsstart, men även i dessa studier upp-reglerades gener involverade i CMF-nedbrytning jämfört med obehandlat trä. Jämförelse av genuttryck i termiskt modifierat och obehandlat trä med likvärdig massaförlust visade att när nedbrytningen har startar så är genuttryck liknande i dessa två material.

Hydroxylradikaler bildade genom Fenton-reaktionen och cellulaser från svamp påvisades kunna bryta ner polysackarider i acetylerat trä *in vitro*. Effektiviteten var däremot lägre än i obehandlat trä. Hydroxylradikalers förmåga att bryta ner polysackarider i modifierat trä har tidigare bara påvisats i DMDHEU-behandlat trä, men även här var effektiviteten lägre än i obehandlat trä. Tidigare studier visar att cellulaser från svamp kan bryta ner polysackarider i DMDHEU-behandlat och furfurylerat trä. Dessvärre kunde inte hydroxylradikalernas eller cellulasernas förmåga att bryta ner polysackarider i furfurylerat trä undersökas då förmodade nedbrytningsprodukter från poly(furfurylalkohol) störde analysmetoden.

Forskningen utförd för den här avhandlingen tillsammans med den aktuell alitteraturen indikerar att trämodifiering kan hindra i) en regulatorisk process i svampen som är involverad i bildandet eller utsöndrandet av kompontet nödvändiga för tränedbrytningen, ii) inträngning eller transport av tränedbrytande ämnen genom träcellväggen eller iii) bindandet och reducerandet av järn(III). En positiv korrelation mellan reducering av fukthalt vid jämvikt och rötresistens i modifierat trä har ofta rapporterats. En omfattande granskning och utvärdering av experimentella data och etablerade teorier från den aktuella litteraturen baserad på de biokemiska mekanismerna bakom brunrötans nedbrytningsprocess har inte tidigare rapporterats.

Papers on which this thesis is based

Paper I: Ringman R, Pilgård A, Brischke C, Windeisen L, Richter K (2017) Incipient brown rot decay in modified wood: patterns of mass loss, structural integrity, moisture and acetyl content in high resolution. International Wood Products Journal, 8(3): 172-182.

Paper II: Ringman R, Pilgård A, Richter K (2014) Effect of wood modification on gene expression during incipient *Postia placenta* decay. International Biodeterioration and Biodegradation, 86:86-91.

Paper III: Ringman R, Pilgård P, Kölle M, Brischke C, Richter K (2015) Effects of thermal modification on *Postia placenta* wood degradation dynamics: measurements of mass loss, structural integrity and gene expression. Wood Science and Technology, 50(2):385 - 397.

Paper IV: Ringman, R, Pilgård, A, Richter, K. (2015) In vitro oxidative and enzymatic degradation of modified wood. International Wood Products Journal, 6:36-39.

Paper V: Ringman R, Pilgård A, Brischke C, Richter K (2014) Mode of action of brown rot decay resistance in modified wood: a review. Holzforschung, 68:239–246.

Other publications

Peer-reviewed publications

Alfredsen, G., Ringman, R., Pilgård, A., Fossdal. C.G. (2015) New insight regarding mode of action of brown rot decay of modified wood based on DNA and gene expression studies: a review. International Wood Products Journal 6(1): 5–7.

Zelinka, S.L., Ringman, R., Pilgård, A., Engelund Thybring, E., Jakes, J.E., Richter, K. (2015) The role of diffusion in the decay resistance of modified wood. Accepted to a Special Issue of the International Wood Products Journals. Doi: 10.1080/20426445.2016.1161867

Conference proceedings

Zelinka, S.L., Ringman, R., Pilgård, A., Engelund Thybring, E., Jakes, J.E., Richter, K. (2015) The role of diffusion in the decay resistance of modified wood. European Conference on Wood Modification, 26-27 October, 2015, Helsinki, Finland. (Oral presentation by Rebecka Ringman)

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Alfredsen, G., Ringman, R., Pilgård, A., Fossdal , C. G. (2014) New insight regarding mode of action of brown rot decay of modified wood based on DNA and gene expression studies. European Conference on Wood Modification, 10-12 March, 2014, Lisbon, Portugal.

Rebecka, R., Pilgård, A., Richter, K. (2014) In vitro oxidative and enzymatic degradation of modified wood. European Conference on Wood Modification, 10-12 March, 2014, Lisbon, Portugal. (Oral presentation by Rebecka Ringman)

Ringman, R., Pilgård, A., Richter, K. (2013) Effects of wood modification on gene expression during incipient *Postia Placenta* decay. Proceedings of the 9th Meeting of the Nordic Baltic Network in Wood Material Science & Engineering (WSE). September 11-12, 2013, Hannover, Germany. (Oral presentation by Rebecka Ringman)

Ringman, R., Pilgård, A., Richter, K. (2013) The effect of wood modification and gene expression in incipient Postia placenta decay. Biodeterioration of Wood and Wood Products. 24-27 April, 2013, Tartu, Estonia. (Oral presentation by Rebecka Ringman)

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Abbreviations

CCA	Copper-chromium-arsenic
cDNA	Complementary DNA
CEN	European committee for standardisation
CMF degradation	Chelator mediated Fenton degradation
Cq	Quantification cycle
DDAC	Dicecyldimethylammoniumchloride
DMDHEU	Dimethylol dihydroxyethyleneurea
DNA	Deoxyribonucleic acid
DNS	Dinitrosalicylic acid
dNTP	Deoxynucleotide
DVS	Dynamic vapour sorption
EMC	Equilibrium moisture content
F	Fine fraction (HEMI)
FSP	Fibre saturation point
FT-IR	Fourier Transform Infrared Spectroscopy
GTP	Guanosine triphosphate
HEMI	High energy multiple impact
HPLC	High-performance liquid chromatography
IPCC	Intergovernmental Panel on Climate Change
MC	Moisture content
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
ML	Mass loss
n	number
NaAc	Sodium acetate
NMR	Nuclear magnetic resonance
OH-group	Hydroxyl group

P-layer	Primary cell wall layer
POD	Peroxidase
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
R^2	Coefficient of determination
RH	Relative humidity
RIM	Resistance to impact milling
RNA	Ribonucleic acid
S1-layer	First secondary cell wall layer
S2-layer	Second secondary cell wall layer
S3-layer	Third secondary cell wall layer
SYP	Southern yellow pine
TEM	Transmission electron microscopy
U	Enzyme unit (the amount of enzyme that catalyses the conversion of 1 micro mole of substrate per minute)
UMSP	Ultraviolet microspectrophotometry
UV	Ultra violet
WPG	Weight per cent gain

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1 Introduction

Forests and long-lived wood products play an important role as carbon sinks (Schlamadinger and Marland 1996). Use of wood in construction leads to lower CO_2 emissions than using aluminium, steel or concrete, which has been shown in several studies (Börjesson and Gustavsson 2000; Gustavsson et al. 2006; Gustavsson and Sathre 2006; Eriksson et al. 2007; Upton et al. 2008). Hence, growing wood and use it in construction would be beneficial for the climate.

Biodegradation, or the decomposition of wood by microbes, is a natural process that is essential for nutrient cycling in forest ecosystems (Eriksson et al. 1990). When decomposition occurs in wooden structures it can, however, lead to great economic losses. Schmidt (2006) estimated the costs of refurbishment to €3000 per square metre of living space. In the US, it has been estimated that every year as much as 10% of the harvested timber is used to replace decayed wood in service, resulting in extra costs of hundreds of millions of dollars (Zabel and Morell 1992).

In the future, the costs of replacing wood in construction may become even higher due to an increased deterioration rate caused by global warming. Emission of greenhouse gases from burning of fossil fuels is predicted to lead to a warmer climate than today (IPCC 2013). An increase in global temperature is further expected to enhance the biological deterioration and to shorten the service life of outdoor wood products in the parts of the world where the climate is expected to be both warmer and wetter (Brischke and Rapp 2010).

The majority of timber used in construction in the northern hemisphere is from coniferous species (Eaton and Hale 1993; Schwarze 2007). Brown rot basidiomycete fungi preferentially attack coniferous woods, including the two primary wood species in Sweden, *Picea abies* and *Pinus sylvestris* (Gilbertson and Ryvarden 1986). A large part of the biological deterioration of the timber in service is due to the action of brown rot fungi (Arantes and Goodell 2014). Brown rot attack causes faster loss of strength than white rot does, resulting in greater damage within a short period of time. In an evaluation of Norwegian buildings, Alfredsen et al. (2006) reported that 77% of the identified species of decay fungi were brown rot, 3% white rot, and 19% soft rot fungi.

Traditional wood preservatives contain metals and protect the wood from decay by killing the fungi (Eaton and Hale 1993; Rowell 2005). One of the most common preservatives is CCA, containing copper, arsenic and chromium (Rowell 2005). It has been shown that this preservative leaches out of the wood during service and affects the health of the wildlife, e.g. the renal function in mice (Hingston et al. 2000; Matos et al. 2010). Furthermore, health risks for humans have recently been demonstrated (Gress et al. 2014; Gress et al. 2015). Also newer preservatives, like the quaternary ammonium compound DDAC have been shown to be toxic to wildlife (Tatarazako et al. 2002). Restrictions and a growing environmental concern over toxic wood preservatives are the primary justifications for the development of new low environmental impact wood protection systems (KIFS 1998; Hingston et al. 2000; Ibach 2005; Townsend et al. 2005; Hill 2006).

One promising alternative to toxic preservatives is wood modification. For example, wood modified with acetic anhydride has been shown to be preferable to copper treated wood in a life cycle assessment analysis (van der Lugt and Vogtländer 2014). In contrast to traditional wood preservation, where the durability is achieved from the toxicity of the added chemicals (Eaton and Hale 1993; Rowell 2005), very little is known about the mode of action of the nontoxic wood modifications and even less about fungal response to modified wood. Understanding the decay resistance mechanism of modified wood will facilitate efficient development and improvement of modified wood materials. This, in turn, will lead to that modified wood systems might become an economically and functionally competitive alternative to preservative treated wood.

2 Wood

The following chapter is based primarily on text books. If nothing else is stated, the information was retrieved from (Eaton and Hale 1993; Rowell 2005; Schmidt 2006).

2.1 Wood microstructure and chemistry

Wood is a natural composite material with complex structures on several levels. The anatomical compositions differ between hardwoods and softwoods and to a lesser extent between species. Since the experiments in this thesis were all conducted on pine species (*Pinus* spp), the following details are referring to these species.

The innermost part of the tree trunk is called heartwood. In pine species, the heartwood is slightly darker than the surrounding sapwood. The heartwood differs from the sapwood in that it is denser, contains a higher amount of extractives and, in the living tree, lower amounts of moisture. Heartwood is formed as the sapwood expands and the innermost sapwood accumulates the heartwood specific chemicals, leaving the width of the sapwood band more or less uniform throughout the life of the tree. The width of the sapwood band is species specific; e.g. larch (*Larix occidentalis*) and sweet chestnut (*Castanea sativa*) have narrow sapwood bands while willow species (*Salix* spp) and pine species have wide sapwood bands. The sapwood is responsible for conducting the water (sap) from the roots to the needles. Outside the sapwood is the vascular cambrium, which produces both sapwood tissue and bark tissue. The inner bark (phloem), just outside the vascular cambrium, is the tissue through which sugars produced through photosynthesis are transported from the needles to the roots and growing parts of the tree. The outer bark is a physical barrier that protects the inner bark from damage.

The majority of cells in the sapwood are tracheids, which are very long cells arranged in the same direction as the tree trunk. Looking at a cross-section of a tree trunk, the tracheids look like the cross-section of a bundle of straws, only more rectangular. In the earlywood of tree rings, they are more thin-walled than in the latewood. The tracheids are connected through pits in the cell walls, resulting in that sap travelling through the tracheids has to zig-zag its way to the top of the tree. For the lateral transport of dissolved nutrients, there are ray parenchyma cells. The ray parenchyma cells are usually stacked on top of each other, forming a ray. Ray parenchyma cells are shorter and wider than the tracheids, but are also connected with pits. Special pits connect the ray parenchyma cells with the tracheids.

The tracheid cells in the sapwood (as in the heartwood and bark) are dead, which means they only consist of a cell wall, while the protoplast of the cells has disappeared. The empty void inside the cell is called the lumen, and it is thanks to that the cells are dead and empty that the sapwood is able to transport sap. The layer connecting two wood cells is called the compound middle lamella. Next to the middle lamella is the primary wall, followed by the secondary wall, which is divided into S1-S3. Each of the different parts is characterised by a different structure and chemical composition. For example, the S3-layer has the highest content of hemicelluloses, the S2-layer is very rich in cellulose and the S1-layer is the most lignin-rich layer of the secondary wall. The angle and arrangement of the cellulose microfibrils differ considerably between layers, contributing to the different characteristics of each layer.

Also the structure within each layer is important for understanding the characteristics of wood. Cellulose is a polymer of D-glucopyranose units linked by β-(1→4)-glucosidic bonds. However,

the building block in cellulose is actually cellobiose, since the repeating unit is this two-sugar unit. The length of a polymerised cellulose molecule is referred to as its degree of polymerisation. The cellulose chains are arranged in microfibrils in which hydrogen bonds give rise to crystallinity. Amorphous cellulose, hemicelluloses and lignin act as packaging around the microfibrils. As a metaphor, hemicelluloses and lignin may be compared to concrete, while cellulose microfibrils are the reinforcements, providing the tensile strength to the wood. Polysaccharides in wood may also be divided into accessible and non-accessible polysaccharides. Hemicelluloses, amorphous cellulose and the outward facing cellulose in the microfibrils are accessible to water as well as microorganisms and their degrading agents, while inward facing cellulose in the microfibrils is non-accessible.

2.1.1 Wood/water interactions and chemical transport in wood

Freshly cut wood (green wood) is saturated with water, which means that the wood cell walls are filled with hygroscopically bound water and some liquid water may also be found in the lumen (Engelund et al. 2012). As the wood dries, liquid water is removed from the larger voids and then the wood cell walls will become unsaturated with water. The transition between these two states is called the fibre saturation point (FSP), which in soft wood species is 30-40%, depending on the definition used (Engelund et al. 2012). At and below this point, in a wood sample in equilibrium, moisture is not found as capillary water in the lumen but only found inside the wood cell wall (Engelund et al. 2012). Water in the lumen is called free water, while water in the wood cell wall is called bound water, since it interacts with the wood polymers (Engelund et al. 2012). Previously, bound water was believed to exist in two states, freezing and non-freezing bound water, however, freezing bound water was recently found to be an artefact caused by the sample preparation (Zelinka et al. 2008). Bound water forms hydrogen bonds mainly with the hydroxyl groups of the wood constituents (Engelund et al. 2012). Most sorption sites are found in hemicelluloses followed by cellulose and lignin (Engelund et al. 2012). However, as mentioned above, hydroxyl groups in cellulose inside microfibrils are inaccessible to water and, therefore, not all of the hydroxyl groups will bind water even if the wood cell wall is saturated with water.

In oven dried wood, there are in general no voids or pores in the wood cell wall (Engelund et al. 2012). As moisture starts to accumulate in the wood cell wall, it therefore causes swelling of the material through occupying space between microfibrils and thereby forcing them apart (Engelund et al. 2012). The swelling of the wood cell walls is directly proportional to the macroscopic swelling of wood in the tangential direction (Engelund et al. 2012).

Transport of water in wood is mainly in the form of vapour diffusion, unless the wood is in contact with liquid water in which case capillary sorption occurs (Engelund et al. 2012). Water diffuses more than 10 times faster in the longitudinal direction than in the perpendicular direction in spruce and beech (Sonderegger et al. 2011). If no liquid water is present, water vapour diffuses through the lumen, is adsorbed on the wood cell wall, diffused as bound water through the wood cell wall and then desorbed on the other side into the adjacent lumen (Engelund et al. 2012). Models for the transport of water in wood have been developed (Eitelberger and Hofstetter 2011; Hozjan and Svensson 2011).

Chemical transport through the wood cell wall is essential for wood degradation (Koenigs 1974; Goodell et al. 1997). As mentioned above, when the wood is not in contact with liquid water or exposed to a relative humidity (RH) above 99.5%, there are insignificant amounts of liquid water

in the wood (Engelund et al. 2012). Since degradation occurs also in wood without contact with liquid water, transport of degradation agents in the wood cell wall is not dependent on a liquid aqueous medium (Jakes et al. 2013). Zelinka et al. (2008) proposed the percolation model, in which water at a certain RH is suggested to form a continuous network inside the wood cell wall through which chemical transport may occur. It was first proposed that this network consisted of freezing bound water, but when this was later proven to be an artefact, the plasticising hemicelluloses model was instead presented (Zelinka et al. 2008; Zelinka et al. 2012; Jakes et al. 2013). Hemicelluloses plasticise at room temperature and at 60% RH, which is a normal condition in the temperate zone (Cousins 1978; Kelley et al. 1987; Olsson and Salmén 2004). In this model, plasticised hemicelluloses are proposed to form a continuous gelatinous network, in which ions and degradation agents can be transported. The RH threshold for transport of ions through the wood cell wall was found to be 60-90% RH at 20°C depending on the ion, which is equivalent to 11-20% wood moisture content in the wood material (Zelinka et al. 2014).

2.2 Wood degradation and durability

All dead trees in nature will eventually be degraded biologically, just as all dead organic matter. This is an essential part of the circle of life, since it allows for recycling of nutrients. Wood is also degraded physically, mainly by weathering, i.e. the combined action of moisture, sunlight (especially UV-light), temperature, chemicals, and abrasion by windblown materials. Fire will of course also contribute to the degradation of wood. In forest ecosystems, turnover rates for complete degradation of wood may reach thousands of years, why the Basidiomycota play a crucial role in short-term conversion and degradation of wood lignocellulose (Lundell et al. 2014).

2.2.1 Biological wood degradation

For an infection to establish, survive and spread, the following criteria must be fulfilled: i) there must be a source of infection (spores or mycelium arriving from an established source), ii) there must be a suitable substrate (supplying nutrients and energy, such as wood), iii) environmental conditions must be suitable (temperature and moisture), iv) the aeration must be sufficient, v) there must be an interaction between microorganisms and vi) there must not be any poisonous substances, such as toxins from competing organisms or in the substrate (natural or added by man).

In nature, biological degradation of wood is carried out by several different kinds of organisms, working in concert. These usually appear in the wood in the following stages of successional invasion: i) bacteria, moulds and discolouring fungi in the surface regions, ii) staining fungi and soft rot fungi, penetrating into the wood through the ray parenchyma, iii) soft rot fungi penetrating the wood to greater depths, and iv) white and brown rot fungi causing extensive degradation throughout the wood specimen. The succession of fungi in wood in nature was investigated by Ottosson et al. (2014), who concluded that white rot fungi and fungi with high mycelia abundance were more often associated with succeeding species than brown rot fungi and fungi with low mycelial abundance.

2.2.1.2 Bacteria

Wood degrading bacteria are classified according to their way to attack wood: i) tunneling bacteria, ii) erosion bacteria, and iii) cavitation bacteria.

Tunneling bacteria

Tunneling bacteria cause a network of threadlike tunnels, originating from the margin of attack. The tunnels are mainly seen in the S2- and S3-layers of the cell walls and each is created by a single bacterium (Daniel et al. 2014). Attack is mainly seen in wood with high moisture content. Tunneling bacteria are able to degrade preservative treated wood, highly lignified wood, wood species with high extractive content and the high lignin content middle lamella regions (Daniel et al. 2014).

Erosion bacteria

Erosion bacteria degrade the wood cell wall from the S3-surface and eventually erode the wood cell wall to the middle lamella. This kind of degradation has been seen in untreated and preservative-treated hardwoods and softwoods in a wide range of environments (Eaton and Hale 1993). The ability of these bacteria to degrade highly lignified regions of the wood cell wall, suggest that they are ligninolytic. Their exact identity has not been resolved, but they are described as gram-negative and are taxonomically similar to tunnelling bacteria. Erosion bacteria may represent the only major decay in oxygen limiting conditions such as buried shipwrecks, terrestrial archaeological artefacts and building foundations (Daniel et al. 2014).

Cavitation bacteria

Cavitation bacteria form angular and often diamond shaped cavities, especially in the S2-layer in earlywood. This type of decay has been reported in in-ground CCA-treated pine posts in New Zealand and in softwood piling timber in the old city of Stockholm. The bacterial species causing this type of decay and the mechanisms are not fully understood.

2.2.1.2 Fungi

Discolouring fungi

Discolouring fungi include moulds, sapstain and bluestain fungi. These do not degrade the wood cell wall, but instead live off starch and sugars in the superficial parenchyma tissue. They do not affect the strength of the wood and their main effect is aesthetic. Some produce coloured spores and others release pigments that discolour the wood. Discolouring fungi are mainly members of the Ascomycota (Fig. 1) and Deuteromycota (an informal group of unrelated fungi with the common denominator that they so far have not been shown to use sexual reproduction).

Soft rot fungi

Soft rot fungi are just as the discolouring fungi members of the Ascomycota (Fig. 1) and Deuteromycota. Soft rot is mainly seen in wood where growth of the more competitive white and brown rot fungi is inhibited, such as wood with high moisture content or preservative treated wood. Soft rot, unlike white rot and brown rot, grows inside the wood cell wall. Characteristic for soft rot is rhombus-shaped cavities in the S2-layer, often lined up as pearls on a string. Soft rot degrade cellulose and hemicelluloses within close vicinity of the hyphae, while lignin is little affected. The intensive carbohydrate degradation leads to relatively large strength loss at low mass loss.

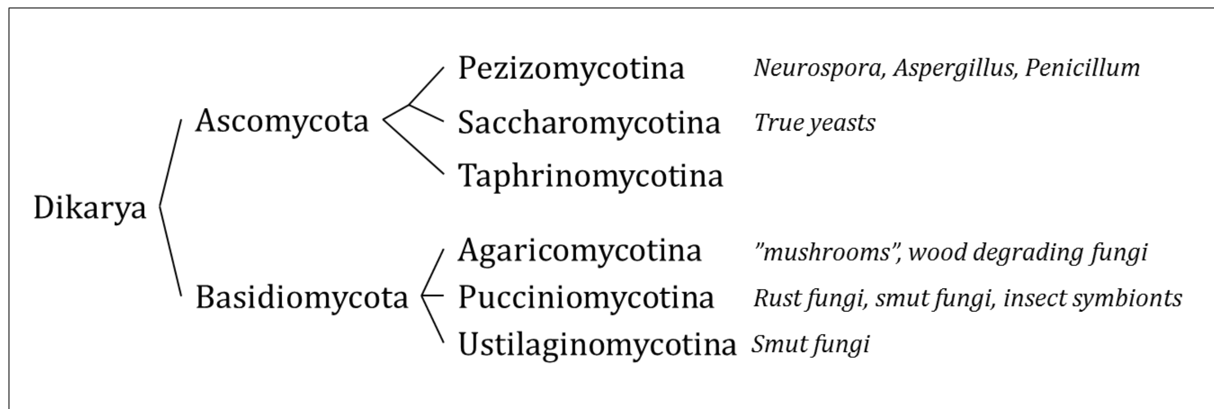

Figure 1. Tree of life of the higher fungi, based on Borkovich and Ebbole (Borkovich and Ebbole 2010).

The Basidiomycota

White and brown rot fungi belong mainly to the Basidiomycota (Fig. 1); however some of the higher ascomycetes have also been described as white rot fungi. As mentioned in chapter 1, brown rot fungi preferentially attack softwoods, while white rot fungi prefer hardwoods (Gilbertson and Ryvarden 1986). (Brown rot fungi are discussed further in chapter 3)

White rot fungi

Typically, white rot fungi belong to Agaricomycotina and are mainly polypores (Lundell et al. 2014). White rot is such named since the appearance of the wood becomes lighter, which is due to complete mineralisation of lignin (Daniel et al. 2014). White rot fungi, like all fungi, colonise wood via the ray canals that provide both rapid entrance and easily accessible nutrients (Daniel et al. 2014). The hyphae spread through pits or by the development of specialised bore hyphae that penetrate the wood cell wall (Daniel et al. 2014). White rot fungi degrades the wood cell wall close to the hyphae, using cellulases and a variety of oxidative enzymes, such as peroxidases and laccase (Daniel et al. 2014). Hemicelluloses, cellulose and lignin are degraded simultaneously in some species, while hemicelluloses and lignin degradation precedes cellulose degradation in others (sequential degradation) (Daniel et al. 2014). It has been reported that white rot fungi are able to degrade parts of the wood cell wall at a short distance (Daniel et al. 2014). Two extracellular systems have been proposed to mediate this decay, the manganese peroxidase system and the lignin peroxidase system who both are proposed to produce radicals (Daniel et al. 2014; Lundell et al. 2014). The first lignin peroxidases, and hence the ability to mineralise lignocellulose, evolved at the end of the Permo-Carboniferous era, possibly leading to the decrease in carbon deposit and thereby also a decrease in the formation of coal (Floudas 2012).

2.2.1.3 Wood inhabiting insects and marine borers

Boring beetles, marine borers and termites also degrade wood. They cause tunnels of significantly bigger size than bacteria and fungi and can degrade wood with lower moisture content than these species can. In beetles, it is mainly the larvae that degrade wood. Insects use their mouthparts to tear and chew the wood, which is ground even finer in the first part of the gut. In the second part of the gut, the carbohydrates, protein and lipids are degraded by the insects own or microbial enzymes.

2.2.2 Natural durability

The heartwood of some wood species are naturally more durable to biological degradation. These include e.g. *Quercus robur*, *Robinia pseudoacacia*, *Tectona grandis*, *Thuja plicata*. The heartwood is usually more durable than the sapwood, which is thought to depend mainly on the extractives of the heartwood. However, heartwood durability in e.g. redwood may differ between trees and there may also be parts of the heartwood within a tree that have lower durability (Davies et al. 2014).

Wood extractives that have been shown to have biocidal effect are e.g. terpenoids, tropolones, stilbenes and flavonoids. Mohareb et al. (2010) found that the decay resistance of *Cupressus litanica* is decreased by solvent extraction and that the isolated extractives inhibited *P. placenta* growth on agar plates. Chen et al. (2014) on the other hand found that aqueous crude extracts from *Acacia confuse* did not inhibit fungal growth on agar plates, but they did decrease decay resistance in wood blocks exposed to a brown rot fungi. In this case the extractives were found to possess ferrous ion chelating ability and contain 3,4-dihydroxybenzoic acid, which itself had excellent decay resistance ability when used to impregnate wood blocks. The authors suggested that 3,4-dihydroxybenzoic acid inhibits hydroxyl radical formation in the Fenton reaction by binding . However, treating non-durable wood with decay inhibiting extractives has not been thoroughly successful. Therefore, even though extractives are regarded to be significantly important for natural durability, other factors including density, nitrogen and starch content and lignin quantity and type may contribute.

3 Brown rot fungi

The contents of this chapter are based on the text books (Schmidt 2006; Borkovich and Ebbole 2010) if nothing else is stated.

3.1. Characteristics

In brown rot wood degradation, hemicelluloses and cellulose are degraded and metabolised whereas lignin is highly modified (Schwarze 2007; Yelle et al. 2011; Arantes et al. 2011). The efficient degradation of polysaccharides causes brittleness in the wood, cracking into cubes and finally crumbling into powder (Schwarze 2007). The dark colour of brown rotted wood, for which it is named, comes from the remaining lignin (Schwarze 2007). The definition of brown rot fungi is fungi that cause brown rot. This is a description based on how the wood is affected, and not on the fungal species causing it. This is equivalent to how deseases have been described by their symptoms rather than their cause, even though sometimes there are different causes for the same symptoms. In the same way, different brown rot fungi may use different means to achieve brown rot.

Brown rot fungi evolved from white rot fungi at at least four separate time points (Fig 2.) (Floudas 2012). This means that some brown rot fungi are more closely related to a white rot fungus than to another brown rot fungus. It also means that the way of causing brown rot may differ between different brown rot fungi. To transfer the description of the rot types to the rot fungi, white rot fungi have been described as fungi possessing multiple lignin-degrading peroxidases (PODs) and being able to degrade crystalline cellulose, while brown rot fungi lack these characteristics (Riley et al. 2014). However, it was recently shown that some rot fungi species lack PODs while being capable of both degrading crystalline cellulose and lignin and thereby causing white rot (Riley et al. 2014). Beside the difficulty to separate brown rot fungi from white rot fungi, there are also considerable differences between brown rot species. For example, brown rot fungi are often described as more or less aggressive or as high or low oxalic acid producers. Furthermore, the chelator mediated Fenton (CMF) degradation has so far only been described for brown rot fungi belonging to the Gloeophyllales, Polyporales and Boletales and whether brown rot fungi outside these orders use this mechanism to degrade wood is not known (Goodell et al. 1997; Shimokawa et al. 2004; Arantes and Milagres 2007; Arantes et al. 2011; Eastwood et al. 2011). From a wood perspective, using the terms white rot fungi and brown rot fungi are useful, but looking at the fungi one has to keep in mind that the white rot and brown rot fungi are both difficult to separate from each other and that each group is not a homogenous group of closely related species but rather several groups of species that may differ in some fundamental respects.

Brown rot fungi are basidiomycetes, which are filamentous fungi belonging to the Eufungi (higher fungi, see Fig. 1). In wood science they are referred to as rots in contrast to moulds that grow superficially on wood and do not degrade the wood cell wall. However, in mycology, brown rot fungi are considered moulds, which is another word for filamentous fungi and the contrary to yeasts which grow as single cells. Basidiomycetes belong to the subkingdom Dikarya, which have two nuclei in each cell in at least a part of their life cycle. The cells grow in a long chain that may split in two or more chains. Each cell is usually not completely divided from its neighbour, so that nutrients and water may be transported inside the hyphae from one end to the other. Growth occurs at the hyphal tip and this is also the part of the fungus that causes degradation of

wood. Brown rot fungi colonise wood via the ray parenchyma cells and subsequently spread in the longitudinal direction through pits (Daniel et al. 2014). The hyphae grow inside the lumens and close to the wood cell wall.

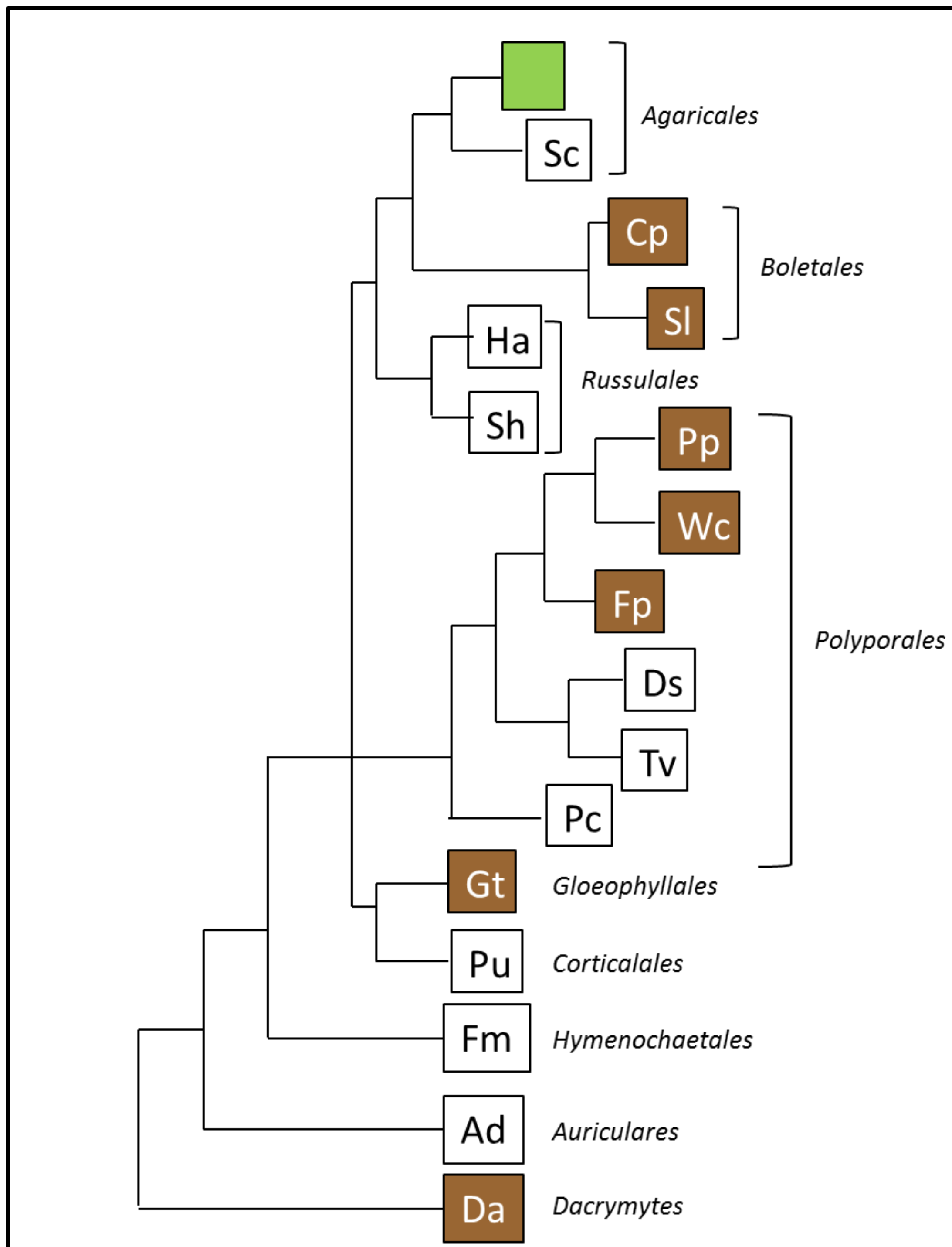

Figure 2. Tree of life of selected white and brown rot fungi, based on Floudas (Floudas 2012). Brown squares – brown rot fungi, white squares – white rot fungi, green square – mycorrhizal and soil saprotroph fungi. The main fungi described in this section are abbreviated as follows; Cp – *C. puteana*, Sl – *S. lacrymans*, Pp – *P. placenta*, Gt – *G. trabeum.*

Even though brown rot fungi are present all over the world, some species are more common in some parts than others. Furthermore, brown rot fungi are the most spread in the temperate

zone of the Northern hemisphere due to their preference for softwood species (Schwarze 2007). The dominating use of softwoods in construction in this part of the world is probably also the reason why brown rot is the most frequent type of fungal wood degradation in these countries (Eaton and Hale 1993; Schwarze 2007). In the Southern hemisphere, brown rot fungi are common also in hardwoods (Schwarze 2007).

Below a closer description of four brown rot fungi is given. These cause high amounts of damage to wood in construction and therefore have been the most studied with respect to degradation mechanisms and the most used in durability standards (AWPA E10 1991; EN 113 1996). Their phylogenetic interrelationships can be seen in Fig. 2.

3.1.1 *Postia placenta*

P. placenta is known under many different names, including *Poria placenta*, *Oligoporus placenta* and *Rhodonia placenta*. Schmidt (Schmidt 2006) claims that the most legitimate name of the species *Postia placenta* is *Oligoporus placenta*, while Niemelä et al. (2005) concludes that *Rhodonia placenta* is the correct name. *Rhodonia placenta* is also the current name according to Index Fungorum (http://www.speciesfungorum.org). However, *Postia placenta* is frequently used in biochemical and molecular contexts, why this is the name chosen to be used in this thesis.

P. placenta is a polypore fungus that can be found in the coniferous zone all around the world. It preferentially degrades softwoods and is one of the most common indoor decay fungi in many parts of Europe. The mycelium spreads over the substrate and the species is mainly found in damp buildings. The optimum moisture content for *P. placenta* is around 45%, but it may survive in dryness. It can resist high temperatures and several strains have been shown to be copper resistant.

The genome of *P. placenta* has been sequenced and this species has therefore been used in previous molecular investigations on e.g. brown rot gene expression in modified wood (Martinez et al. 2009; Alfredsen and Fossdal 2010; Schmöllerl et al. 2011; Vanden Wymelenberg et al. 2011; Pilgård et al. 2012).

3.1.2 *Coniophora puteana*

C. puteana, a polypore, is called the Cellar fungi due to its preference to humid constructs (Lundell et al. 2014). It mainly attacks softwoods but may also be seen on hardwoods, including durable species like oak (Eaton and Hale 1993). Its optimum moisture content is 50-60% and it tolerates cold (Eaton and Hale 1993; Lundell et al. 2014).

3.1.3 *Serpula lacrymans*

The bolete *S. lacrymans* is commonly known as Dry rot and is the most common cause of brown rot in construction in Europe. It mainly occurs in old or water damaged buildings and usually in the cellar or the ground floor (Lundell et al. 2014). *S. lacrymans* prefers softwoods but it can also be seen on hardwoods, especially light coloured ones. It is known for its ability to colonise and degrade wood with relatively low moisture content (21%), but the optimum is 30-80% (Eaton and Hale 1993). *S. lacrymans* can withstand alkaline environments and is therefore able to grow through plaster and cement (Eaton and Hale 1993). Its hyphae are temperature resistant in dry wood and the fungus can resist dryness if the moisture is slowly removed.

3.1.4 *Gloeophyllum trabeum*

G. trabeum has a broad moisture optimum of 30-50% and that also tolerates drying (Eaton and Hale 1993). It is a strong destroyer of coniferous structural timbers, stumps, stored and finished timber and window construction. It may be found in houses that have suffered moisture damage or that have an incorrect structure of the roofing and in building elements such as the façade, outside doors and balconies.

3.2. Brown rot wood degradation

3.2.1 Adaptation to the substrate and growth phases of microorganisms

When a microorganism is inoculated in a new substrate or experience a change in other environmental factors, it will have to adapt to the new situation (Prescott et al. 1999). A change in pH, heat, moisture etc. will lead to an immediate change in gene expression, inactivation and activation of enzymes, transporters etc. A change in nutrient source will cause the equivalent adaptations once the change has been noticed by the microorganism (Prescott et al. 1999; Aro et al. 2005). For example, presence of glucose represses the expression of genes involved in any other carbon metabolism, while the lack of glucose de-represses the expression of these genes and certain metabolites may induce the expression of genes involved in a related carbon metabolism pathway (Prescott et al. 1999; Aro et al. 2005).

The following description of the growth phases of microorganisms in liquid culture is based on (Prescott et al. 1999; Baranyi and Roberts 2000; Madigan et al. 2000; Rolfe et al. 2012). Depending on the severity of the change in environmental factors etc., the time to adapt to the new conditions will vary. During this time, growth is detained and therefore this phase is called the lag phase (Fig. 3). Once the microorganisms have adapted to the new conditions, exponential growth will occur; this is called the logarithmic phase. When nutrients are depleted or e.g. the pH has become inadequate due to carbon metabolism, the growth declines and the microorganisms enter the stationary phase.

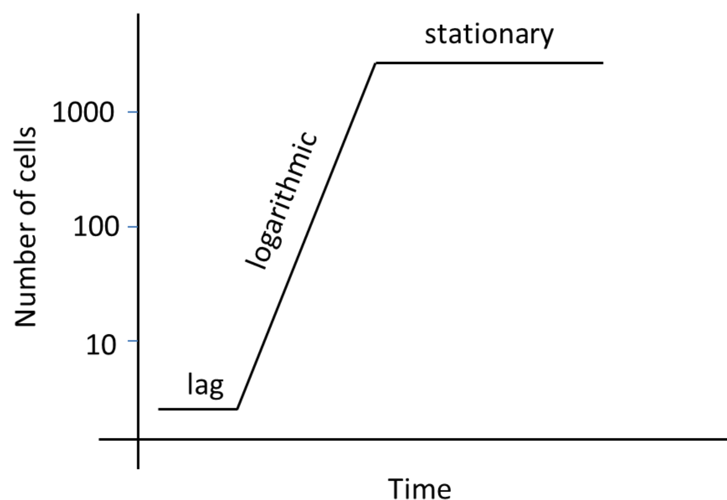

Figure 3. Schematic figure of the growth phases of microorganisms in liquid culture. Numbers are arbitrary. (Based on Prescott et al. 1999)

Whether filamentous fungi undergo these growth phases also in solid substrates such as wood has not been thoroughly investigated. However, *Penicillum chrysosporum* was shown to go through lag, logarithmic and stationary phase like phases when grown on a solid food substrate, while *Physisporinus vitreus* has been modelled to go through similar phases when growing in wood (Fuhr et al. 2011; Arquiza and Hunter 2014). If the growth phases of microorganisms in liquid culture could be applied on wood degrading fungi, the lag phase would be the amount of time it takes for the fungi to adapt to the wood material. Depending on the type of the wood material, the length of the lag phase may differ. Since nutrient metabolism is needed for exponential growth to occur, the logarithmic phase would be characterised by mass loss and

expression of genes involved in wood degradation. When the wood is depleted of nutrients, the growth rate of the fungi will decrease and this may be equivalent to the stationary phase.

3.2.2 Chelator mediated Fenton (CMF) degradation

3.2.2.1 Characteristics of brown rot decay

As described in section 3.1, brown rot fungi lack PODs and are unable to degrade lignin. However they efficiently degrade the polysaccharides in wood and are able to modify the lignin (Eaton and Hale 1993; Yelle et al. 2008; Yelle et al. 2011; Arantes et al. 2011; Arantes et al. 2012). Unlike most other types of wood degrading microorganisms, brown rot fungi degrade the interior of the wood cell wall at a distance, starting with the middle lamella, P and S1-layers even though the hyphae is in the lumen (Irbe et al. 2006; Fackler et al. 2010). The S3 layer remains intact throughout the early stages of decay in some decay types, which has been proposed to lead to a limited permeability of enzymes into the S2 layer (Highley et al. 1985; Eriksson et al. 1990; Kleman-Leyer et al. 1992). The formation of hydroxyl radicals only inside the wood cell wall and the delayed degradation of the S3 layer may protect the hyphae from oxidative injuries. Hemicelluloses are probably degraded before cellulose because of their higher accessibility, which is due to that they surround the cellulose fibrils and are amorphous compared to the mainly crystalline cellulose (Zabel and Morell 1992; Curling et al. 2002a; Irbe et al. 2006; Fackler et al. 2010).

3.2.2.2 The biochemical process of the chelator mediated Fenton degradation

To be able to degrade the wood cell wall at a distance from the hyphae, brown rot fungi must possess a degradation mechanism that consists of molecules that are small enough to penetrate the intact wood cell wall, i.e. significantly smaller than enzymes (Cowling 1975; Reese 1977; Murmanis et al. 1987; Srebotnik et al. 1988; Daniel et al. 1989; Flournoy et al. 1991; Jellison et al. 1991; Schwarze and Spycher 2005). The prevailing model is based on the Fenton reaction and was proposed in 1974, while addition of chelators to the model was done in 1997 (Koenigs 1974; Goodell et al. 1997).

The majority of the work on the degradation mechanisms in brown rot fungi has been performed in *G. trabeum*, although some mechanisms have also been confirmed in *P. placenta*, *S. lacrymans, Wolfiporia cocos* and other species (Goodell et al. 1997; Shimokawa et al. 2004; Martinez et al. 2009; Arantes et al. 2011). The following is a general description of brown rot CMF degradation, mainly based on *G. trabeum*.

Brown rot fungi induce the production of hydroxyl radicals within the polymer matrix by the secretion of phenolate and hydroquinone iron reductants, hydrogen peroxide and oxalic acid (Goodell et al. 1997). Oxalic acid is thought to sequester oxidised forms of Fe^{3+} from the environments after which the complex diffuses into the wood cell walls. The hydrogen peroxide and iron reductants secreted by the fungus have also been demonstrated to be transported through the wood cell walls (Goodell et al. 1997; Arantes et al. 2012). As described in section 2.2, the water in wood is bound at the EMC where decay may occur. The chemical transport through the wood cell walls is therefore not dependent on a liquid, aqueous media but may instead be conducted in a continuous network consisting of e.g. bound water or plasticised hemicelluloses (Engelund et al. 2012; Zelinka et al. 2012; Jakes et al. 2013). Inside the higher pH wood cell wall, the fungal iron reductants sequester Fe^{3+} from the iron-oxalate complexes and reduce it to Fe^{2+} at a point in the wood cell wall at which the concentration of oxalic acid and the pH are adequate

for this reaction (Goodell et al. 1997; Arantes et al. 2009b; Arantes et al. 2012). After the iron is reduced in the high pH and low oxalate concentration environment of the wood cell wall, it reacts with H_2O_2 in the Fenton reaction to form the hydroxyl radical (Fenton 1894; Goodell et al. 1997). The hydroxyl radical is the most powerful oxidant in living systems and has a half-life of 10^{-9}s, both of which are reasons for why the reaction needs to take place inside the wood cell wall rather than close to the hyphae (Arantes et al. 2012). Other reactive oxygen species such as FeIV = O may also form, as was recently reported (Enami et al. 2014). The hydroxyl radicals cause a one-electron transfer from the cellulose, leading to cleavage of the polymer chain (Kirk et al. 1991; Hammel et al. 2002; Arantes et al. 2012). Oxidative modification of lignin can result in several alterations to the lignin moieties, including demethylation, hydroxylation and side chain oxidation (Kirk and Adler 1970; Yelle et al. 2011; Arantes et al. 2012).

3.2.2.3 Oxalic acid

Oxalic acid has been shown to be formed by *P. placenta* growing on cellulose, while levels on glucose medium were low (Ritschkoff et al. 1995). The activity of enzymes in the glyoxalate cycle was found to correspond to the biosynthesis of oxalic acid and, additionally, isocitrate lyase and malate dehydrogenase were shown to have an important role in the formation of oxalic acid from oxaloacetate (Munir et al. 2001a; Munir et al. 2001b). In most fungi, the glyoxalate cycle is involved in gluconeogenesis (glucose synthesis) in glucose-deficient media (Munir et al. 2001b). However, in wood degrading fungi these enzymes may be expressed constitutively, as shown in the brown rot fungus *Fomotopsis palustri* (Munir et al. 2001b). It was proposed by the authors that this fungus uses the glyoxalate cycle to produce oxalic acid, possibly to use in wood degradation.

The levels of oxalic acid are down-regulated through degradation of oxalic acid by oxalate dehydrogenase to formic acid and, subsequently, degradation of formic acid by formate dehydrogenase to carbon dioxide and water (Hastrup et al. 2012). *G. trabeum*, which accumulates lower amounts of oxalic acid than *P. placenta*, *C. puteana* and *S. lacrymans*, was shown to have a higher activity of oxalic acid degrading enzymes (Hastrup et al. 2012). Furthermore, oxalic acid was more rapidly mineralised by *G. trabeum* than by other brown rot species (Espejo and Agosin 1991). Hastrup et al. (2012) concludes that the fact that *G. trabeum* caused similar amounts of decay with significantly lower amounts of oxalic acid, supports the theory that decay mechanisms may deviate between brown rot species, especially since *G. trabeum* is phylogenetically distant from *C. puteana* and *S. lacrymans* (Fig. 2).

3.2.2.4 Iron reductants

Although various mechanisms have been proposed to explain the reduction of Fe^{3+} to Fe^{2+} during brown rot wood degradation, the most accepted mechanism today is by means of fungal low molecular weight aromatic compounds (Arantes et al. 2012). These compounds are commonly of phenolate and hydroquinone origin and several of them have been shown to be produced by brown rot fungi; for example, the iron reductant 2,5-dimethoxyhydroquinone (2,5-DMHQ) that has been found in cultures of *P. placenta*, *S. lacrymans* and various *Gloeophyllum* species (Goodell et al. 1997; Paszcynski et al. 1999; Shimokawa et al. 2004; Suzuki et al. 2006; Arantes et al. 2011). Fungal iron reductants have been localised to the S2-layer during *G. trabeum* decay, thus showing they are sufficiently small to be able to penetrate the wood cell wall (Jellison et al. 1991).

2,5-dimethoxyhydroquinone has been shown to reduce Fe^{3+} to Fe^{2+} while simultaneously generating a semiquinone radical. The semiquinone radical may subsequently reduce O_2 to ·OOH, which in turn can yield hydrogen peroxide or reduce additional Fe^{3+} to Fe^{2+}, in which case a quinone is also formed (Kerem et al. 1999; Paszczynski et al. 1999; Newcombe et al. 2002). As an alternative hypothesis, it has been proposed that lignin degradation products may be able to reduce Fe^{3+} to Fe^{2+} (Filley et al. 2002; Goodell et al. 2006). This is supported by the fact that demethylation of lignin is proportional to holocellulose loss during brown rot wood degradation (Filley et al. 2002).

Just as oxalic acid, iron reductants are formed as bi-products in the fungus' metabolism, but exactly which enzymes and biosynthesis pathways are responsible for the increased formation and/or secretion of these molecules during wood degradation are not known (Martinez et al. 2009; Vanden Wymelenberg et al. 2010). However, the *G. trabeum* quinone reductase (QRD) has been shown to be able to regenerate hydroquinone descending iron reductants (Paszcynski et al. 1999; Jensen et al. 2002; Qi and Jellison 2004). Since the *G. trabeum* QRD is intracellular, the oxidised iron reductant would have to be transported back to the hypha for the reduction. Alternatively, the iron reductants might continuously reduce Fe^{3+} until they are mineralised (Goodell et al. 1997; Arantes and Milagres 2006). A putative homologue to the *G. trabeum* QRD was identified in *P. placenta* (Martinez et al. 2009).

3.2.2.5 Hydrogen peroxide

Extracellular hydrogen peroxide may be produced by alcohol oxidase since it has a preference for methanol, a product that is potentially available from the demethylation of lignin during degradation (Martinez et al. 2009). The *P. placenta* alcohol oxidase has been shown to be up-regulated in the presence of cellulose and wood compared to glucose (Martinez et al. 2009; Vanden Wymelenberg et al. 2010).

However, the involvement of alcohol oxidase in wood degradation has been questioned. Even though *G. trabeum* alcohol oxidase has been localised to the secondary cell wall layer of wood fibres in liquid culture, it is doubtful that alcohol oxidase can penetrate the intact wood cell wall (Daniel et al. 2007).

3.2.2.6 Laccase

Postia placenta produce higher amounts of oxalic acid than *G. trabeum*, which may result in hydroquinones being unable to sequester and reduce Fe^{3+} in iron-oxalate complexes (Wei et al. 2010). Mixing hydroquinones, chelated ferric iron and laccase led to the formation of Fenton's reagent and subsequently hydroxyl radicals (Guillen et al. 2000). Laccases were originally thought to be present only in white rot, but it was shown that they exist also in brown rot fungi (Lee et al. 2004). For example, *P. placenta* has been described to harbour two to four putative laccase genes in its genome (Martinez et al. 2009; Vanden Wymelenberg et al. 2010; Riley et al. 2014). Laccase activity was found in aspen wood exposed to *P. placenta* and the *P. placenta* laccase was able to oxidise hydroquinones (Wei et al. 2010). It was estimated that the amount of radicals produced by laccase *in vivo* presumably would have an impact on the decay activity (Wei et al. 2010).

The mechanism used in the methoxyhydroquinone/laccase system is still unclear. Methoxyhydroquinones may be oxidized by laccase into semiquinones, which are expected to better reductants of ferrous iron than methoxyhydroquinones (Wardman 1989). However, it has

also been suggested that the main role of the *P. placenta* methoxyhydroquinone/laccase system is to produce hydrogen peroxide (Wei et al. 2010).

3.2.2.7 Lignin modification

Brown rot fungi circumvent lignin mineralization and degrade the polysaccharides in a straightforward manner (Koenig et al. 2010; Martinez et al. 2011). Brown rotted lignin is polymeric (Kirk 1975; Niemenmaa et al. 2008; Yelle et al. 2008), but its structure is modified substantially by the action of the hydroxyl radicals (Yelle et al. 2008; Arantes et al. 2009a; Martinez et al. 2011; Yelle et al. 2011; Arantes et al. 2011). Lignin modification can be detected in the outer parts of the wood cell wall as increases in the carbonyl group content and demethylation (Eriksson et al. 1990; Filley et al. 2002; Fackler et al. 2010). Lignin modification may contribute to the formation of microcapillary pathways that allow enzymes to penetrate the wood cell wall (Arantes et al. 2012). Yelle et al. (2008) showed that the lignin side chains were completely removed during *G. trabeum and* proposed that lignin is completely degraded during degradation followed by repolymerisation. As mentioned in section 3.2.2.4, it has been suggested that fragments of oxidatively remodelled lignin in brown-rotted wood may contribute to the formation of hydroxyl radicals as an integral part of the redox processes (Xu and Goodell 2001; Filley et al. 2002; Goodell et al. 2006). The strong relationship between lignin demethylation and polysaccharide degradation suggests that they are mechanistically interlinked (Filley et al. 2002). However, no water-soluble lignin products with Fe^{3+}-reducing activities have been detected in brown-rotted wood (Aguiar et al. 2013).

3.2.2.8 Gene regulation in CMF degradation

The regulation of genes involved in the CMF degradation is complicated and remains poorly investigated. Most of the molecules involved in the CMF degradation have been suggested to be secondary metabolites; therefore, the enzymes that produce them may also be expressed when the fungi is not degrading wood (Goodell et al. 1997; Munir et al. 2001b).

In *G. trabeum* there are two *qrd* genes. QRD1 has been proposed to be mainly involved in detoxifying quinones during wood decay and perhaps also to play a role in the quinone redox cycle, while QRD2 was proposed to play an important role when the mycelium is stressed (Cohen et al. 2004). This is based on quantitative PCR (qPCR) studies in which *G. trabeum* was subjected to heat and chemical stress and growth media with and without wood. Examination of the *qrd* promoter sequences showed that *qrd2* contains likely antioxidant, xenobiotic, and heat shock elements while these are absent in *qrd1*. In the *P. placenta* genome only one *qrd* has been identified (Martinez et al. 2009). The regulation of this gene is currently unknown.

A review of gene regulation in filamentous fungi showed that the regulation of laccases is complex and diverse (Janusz et al. 2013). Laccase promoter regions were shown to contain heat shock, antioxidant, and metal responsive as well as stress response elements. The particular regulation of laccases in brown rot fungi has not been described.

3.2.3 Enzymatic wood degradation

3.2.3.1 Cellulose degrading enzymes in brown rot fungi

In contrast to CMF degradation, enzymatic degradation causes compositional changes in the wood cell wall (Fackler et al. 2010). In white rot fungi, the cellulose degradation generally works as follows: i) the endoglucanases cut the cellulose chains internally by hydrolysis of the glycosidic bond, providing ends for ii) the exoglucanases, which cleave off cellobiose units from

the ends of the polysaccharide chains, and finally iii) intracellular, membrane bound and extracellular β-glucosidases hydrolyse the cellobiose to glucose (Aro et al. 2005; Baldrian and Valášková 2008). However, in brown rot fungi, exoglucanases are absent in most species (Baldrian and Valášková 2008). For example, when sequencing the *P. placenta* genome, no genes encoding for exoglucanases were found (Martinez et al. 2009). Processive endoglucanases have been suggested to be able to fill the function of exoglucanases in brown rot fungi, by both cleaving cellulose internally and releasing oligosaccharides before detaching from the polysaccharide (Baldrian and Valášková 2008). However, the *P. placenta* endoglucanases lack cellulose binding domains (Vanden Wymelenberg et al. 2010), a structural feature that confers processivity on some endoglucanses (Zhou et al. 2004). Furthermore, the putative *P. placenta* glycosyl hydrolases found by Martinez et al. (2009) belong to glycosyl hydrolase families which are generally non-processive (Martinez et al. 2009). Ryu et al. (2011) have shown that there are no highly processive cellulases in *P. placenta* or at least none that is similar to known cellulases. However, the *P. placenta* endoglucanases produce higher levels of soluble cellobiose compared to most endoglucanases (Ryu et al. 2011). Since brown rot fungi produce a lot of reactive oxygen species, which modifies the sugar units of cellulose, it is possible that having processive cellulases may not be an advantage for them as processive cellulases do not hydrolyze these modified sugars very well (Ryu et al. 2011). Sequencing the genome of *P. placenta* showed that this fungus possesses only two potential endoglucanases (Martinez et al. 2009). Furthermore, only four of the *P. placenta* glycosyl hydrolase genes with potential cellulase activity were substantially upregulated on lignocellulose medium compared to glucose medium (Vanden Wymelenberg et al. 2010)

β-glucosidases in brown rot fungi are relatively nonspecific and cleave xylose, mannose and galactose units in addition to cellobiose (Baldrian and Valášková 2008). There are several β-glucosidases in *P. placenta* (Martinez et al. 2009).

Another class of cellulose degrading enzymes consists of the lytic polysaccharide monooxygenases (LPMOs). One group of LPMOs, auxiliary activities 9 (AA9, formerly classified as glycosyl hydrolase family 61), have been shown to promote lignocellulose degradation; however, the exact mechanism is unclear (Harris et al. 2010). Busk and Lange (Busk and Lange 2015) mainly found AA9 encoding genes in plant cell wall degrading asco- and basidiomycetes, with up to 40 AA9 encoding genes in the ascomycete *Chaetomium globosum.* In the brown rot fungi studied, however, only a few AA9 encoding proteins were found. Vanden Wymelenberg et al. (2010) found two glycosyl hydrolase family 61 encoding genes in the genome of *P. placenta*. It has been shown that LPMO's can use lignin as an electron donor and, even though this may be more important in white rot fungi than in brown rot fungi, it provides another link between the degradation of polysaccharides with degradation and/or modification of lignin (Westereng et al. 2015).

3.2.3.2 Gene regulation in enzymatic wood degradation

The regulation of cellulases and hemicellulases has primarily been studied in the ascomycetes (to which soft rot and some of the white rot fungi belong, see Fig. 1) (Ilmén et al. 1997; Aro et al. 2001; Aro et al. 2003; Aro et al. 2005; Amore et al. 2013). The carbon metabolism in many microorganisms is regulated by carbon catabolite repression, through which genes involved in degradation of less favourable carbon sources are repressed by the presence of more favourable ones, such as glucose (Borkovich and Ebbole 2010). Carbon catabolite repression has been found also in wood-degrading ascomycetes (Ilmén 1997; Aro et al. 2005). In the absence of a

more favourable carbon source, cellulases in ascomycetes are de-repressed (Ilmén 1997; Aro et al. 2005; Amore et al. 2013). In the absence of glucose, a low, consecutive expression of cellulases was seen in *Trichoderma reesei* (Ilmén 1997). The authors concluded that the expression was not starvation induced since it was not triggered by lack of carbon or nitrogen. Genes encoding proteins involved in degradation of less favourable carbon sources are commonly induced by a metabolite from this carbon source (Borkovich and Ebbole 2010). Filamentous fungi grown on wood and cellulose have been shown to induce their expression of cellulase encoding genes; however, the inducing metabolite seems to differ between species (Ilmén et al. 1997; Ilmén 1997; Aro et al. 2005; Znameroski et al. 2012; Amore et al. 2013).

Protein regulation may also occur at post-transcriptional and post-translational levels (Borkovich and Ebbole 2010). These include capping and splicing of the mRNA and reversible and irreversible modifications of the protein. For extracellular proteins, such as cellulases, regulation of the secretory pathway may also contribute.

4. Modified wood

4.1 Wood modification and properties of modified wood

As described in the chapter 1, toxic preservatives have traditionally been used to enhance durability in wood. Due to their general toxicity, alternatives have been developed (Hingston et al. 2000; Matos et al. 2010). Wood modification is by definition non-toxic during production, service and at end-of-service disposal (Hill 2006). It involves a chemical or physical treatment of the wood, leading to enhanced properties such as improved durability (Hill 2006). In this thesis the following wood modifications were investigated: thermal modification, acetylation, and furfurylation and dimethylol dihydroxyethyleneurea (DMDHEU) treatment. These materials were chosen for their commercial availability; however, DMDHEU-treated wood has recently been withdrawn from the market.

Correlations between the equilibrium moisture content (EMC), treatment level and decay resistance were found for thermally modified, acetylated and furfurylated wood (Tjeerdsma et al. 1998; Ibach and Rowell 2000; Epmeier et al. 2004; Rowell et al. 2007; Lande et al. 2008; Rapp et al. 2008). A reduction of EMC with 40% was shown to correspond to the level of treatment that significantly improved decay resistance for all of the herein studied modified wood materials (Thybring 2013).

It has been reported that moisture content below 23-25% will protect wood from decay (Cardias Williams and Hale 2003; Hill and Ormondroyd 2004; Thybring 2013). This correlates with the WPG level seen to inhibit decay in modified wood for several different kinds of wood modifications (Thybring 2013). However, in a recent paper by Meyer et al. (2015), *G. trabeum* was shown to degrade *P. sylvestris* sapwood at 15% moisture content, which corresponds to the threshold for ion movement in wood proposed by Zelinka et al. (2008; 2014) (see section 2.1.1).

4.1.1 Thermal modification

Thermal modification is perhaps the oldest way to modify wood to improve durability. Thermal modification involves the wood being subjected to a mild pyrolysis at approximately 160-240°C for several hours (Seborg et al. 1953; Stamm 1956; Kollmann and Fengel 1965; Militz 2002). In most studies, a temperature of 200°C or more was needed to obtain maximum durability (Hakkou et al. 2006; Rowell et al. 2009; Calonego et al. 2010). Free hydroxyl-groups on the wood polymers decrease during heat treatment and this leads to a decrease in hygroscopicity (Kollmann and Fengel 1965; Burmester 1975; Phuong et al. 2007; Almeida et al. 2009). However, neither decrease in hygroscopicity nor in free hydroxyl-groups correlates with increased resistance against decay by white rot fungi (Hakkou et al. 2006; Paul et al. 2006). Increased decay resistance instead correlates with mass loss due to heat treatment, which occurs at temperatures above 200°C and is mainly due to degradation of hemicelluloses into carbon dioxide (Hakkou et al. 2006; Paul et al. 2006). At these temperatures, modifications of the wood polymers occur, among which auto-condensation of lignin and lignin cross-linking with polysaccharides are leading to dimensional stability (Tjeerdsma et al. 1998; Mouras et al. 2002; Hakkou et al. 2006; González-Peña et al. 2009; Šušteršic et al. 2010; Mohareb et al. 2011). Modifications of the wood polymers also lead to a decrease in EMC, and the reversion of these modifications by KOH reverses both EMC and decay resistance to the same level as for untreated wood (Rapp et al. 2008).

During heat treatment, extractives are formed in the wood. These were proposed to be toxic to fungi, but this was later refuted (Kamdem et al. 2000; Kamdem et al. 2002; Mazela et al. 2003; Hakkou et al. 2006; Peters et al. 2009; Mohareb et al. 2011).

4.1.2 Acetylation

Acetylation of wood was studied already over 70 years ago (Tarkow 1945). During acetylation of wood, wood is reacted with acetic anhydride, which results in that acetyl groups replace hydroxyl groups on the wood constituents (Rowell 2005). Acetylated wood has an improved decay resistance and lower EMC and FSP than untreated wood has (Rowell et al. 1994; Larsson Brelid et al. 2000; Hill et al. 2005; Hill 2006). However, it is not clear which property is the most important for decay resistance. Rowell et al. (2009) proposed that decay resistance may be achieved in acetylated wood by a lack of water molecules at the glycosidic bonds which was suggested to inhibit enzymatic hydrolysis of the polysaccharides. Papadopoulos and Hill (Papadopoulos and Hill 2002), on the other hand, have shown that for *Pinus nigra*, acetyl bulking of the wood cell wall is more important for moisture exclusion than blocking of hydroxyl groups (Hill 2009). However, for a number of other wood species the results are less clear and in some cases even show the opposite (Hill et al. 2009). However, studies of *Pinus nigra* has shown that this wood specie allows for the acetyl to be more evenly distributed than in *P. sylvestris*, a characteristic known to be important for the effect of acetyl on decay resistance (Hill and Ormondroyd 2004; Habu et al. 2006).

4.1.3 Furfurylation

Furfurylation of wood involves impregnation of the wood with furfuryl alcohol and subsequent curing during which polymerised furfuryl alcohol (poly(furfuryl alcohol)) is formed (Goldstein 1960). The mode of action of furfurylated wood has not been extensively studied. It has, however, been shown that decay resistance increases with increasing weight per cent gain (WPG) as well as with increasing EMC (Lande et al. 2004; Esteves et al. 2010). From the chemical characteristics of furfural alcohol and furfurylated wood, bulking is a possible explanation for the increased decay resistance. According to Nordstierna et al. (2008), furfuryl alcohol binds a model lignin compound which suggests that furfuryl alcohol may also bind to lignin. It has not been shown whether the number of accessible hydroxyl groups is affected in furfurylated wood as they are in acetylated and thermally modified wood.

4.1.4 Treatment with dimethylol dihydroxyethyleneurea

Treatment with DMDHEU involves immersion in a DMDHEU solution and subsequent curing, during which cross-links between the wood constituents may be formed (Yasuda et al. 1994; Krause et al. 2003). Indications of cross-linking within the wood cell walls after DMDHEU treatment have been found, but the mechanisms for the reactions are unknown (Yasuda et al. 1994). DMDHEU treatment improves decay resistance and decreases EMC. It is clear that the change in free hydroxyl groups does not contribute to decay resistance in DMDHEU-treated wood, since hydroxyl groups are actually added through the modification (Dieste et al. 2009b). So far, none of the conferred characteristics of DMDHEU treated wood has been correlated to decay resistance.

4.2 Theories of mode of action of modified wood

The mode of action of modified wood has long been discussed and a number of theories have been presented. The major established theories on the mode of action of modified wood are compiled in table 1, paper IV, and discussed below.

The first theories on the mode of action of modified wood mainly concerned the enzymatic degradation process. For example, it was proposed that the reason why fungi are not able to degrade modified wood is because their enzymes no longer can recognise their substrates, the polysaccharides (Rowell 2005). Cellulases and hemicellulases have been shown to be less efficient in thermally modified, DMDHEU-treated and furfurylated wood (Lekounougou et al. 2008; Venås 2008; Verma and Mai 2010). In furfurylated wood, however, the furfuryl alcohol does not bind to polysaccharides and the reduced enzyme efficacy seen in this material must therefore be due to another mechanism. An alternative theory to the decreased enzyme efficacy that has been put forward is that the enzymatic hydrolysis requires a water molecule while the moisture content is lowered in modified wood (Rowell 2005). Especially in acetylated and thermally modified wood, there are fewer water binding sites on the polysaccharides and therefore water may not be immediately available for the enzymes at the glycosidic bonds. Another explanation for the recorded decrease in enzyme efficacy in acetylated and DMDHEU-treated wood might be the reduction in pore number and size, which has been proposed to lead to inaccessibility of the cellulases to the wood polymers (Hill et al. 2005; Dieste et al. 2009a). But since the discovery of the importance of CMF degradation of cellulose it has instead been suggested that micropore blocking might reduce the rate at which fungal reductants needed for oxidative degradation penetrate the wood cell wall (Goodell et al. 1997; Hill et al. 2005; Arantes et al. 2012).

Another theory that has been proposed is that the extensive loss of hemicelluloses seen in thermally modified wood as well as the heavy modification of the hemicelluloses in acetylated wood, in itself leads to decay resistance (Irbe et al. 2006; Rowell et al. 2009). Brown rot fungi degrade hemicelluloses before cellulose and some researchers consider that this order of degradation is essential and that fungi will not degrade cellulose if there are no hemicelluloses to degrade first (Curling et al. 2002a; Weiland and Guyonnet 2003; Boonstra et al. 2007; Rowell et al. 2009). Rowell et al. (2009) even proposed that arabinose, which is the only L-pentose sugar found in wood, is a key compound that triggers the chain reactions of degradation, and that its absence would prevent the initiation of degradation.

In recent years, the attention has instead been turned to the moisture exclusion of the modified wood materials and what effects this might have on the fungal decay processes (Zelinka et al.; Papadopoulos and Hill 2002; Dieste et al. 2009b; Thybring 2013). This may also be a consequence of the general acceptance of the brown rot degradation process and the importance of the CMF degradation (Goodell et al. 1997; Arantes et al. 2012). The dominating theory states that the moisture content of modified wood is reduced to such an extent that there are no longer sufficient amounts of water in the wood cell wall to allow chemical transport (discussed in section 2.1.1) (Zelinka et al.). Inhibition of transport of CMF metabolites through the wood cell wall would lead to that no Fenton reaction could occur inside the wood cell wall (Goodell et al. 1997; Arantes et al. 2012). Without the oxidative loosening of the wood cell wall, the fungal cellulases will not be able to penetrate it (Cowling and Brown 1969; Koenigs 1974; Schmidt et al. 1981; Flournoy et al. 1991; Arantes et al. 2012).

4.3 Biochemical mechanisms of brown rot wood degradation and its inhibition in modified wood

The brown rot degradation of modified wood and its inhibition may be discussed from the point of view of the biochemical mechanisms of brown rot wood degradation, in which case the

following steps should be considered: colonisation, transcription of necessary genes, translation of these genes into proteins, secretion of necessary enzymes and metabolites, their penetration of and diffusion through the wood cell wall, formation of hydroxyl radicals inside the wood cell wall and depolymerisation and hydrolysis of the polysaccharides (Goodell et al. 1997; Aro et al. 2001; Baldrian and Valášková 2008). The current knowledge on how wood modification affects the different steps in the brown rot wood degradation process is presented below.

4.3.1 Colonisation

As described in section 4.1, wood modification reduces the EMC and may alter the pH of wood (Yasuda et al. 1994; Larsson-Brelid 1998; Lande et al. 2008; Rapp et al. 2008). Nevertheless, brown rot fungi colonise acetylated, furfurylated, DMDHEU-treated and thermally modified wood (Verma et al. 2008; Alfredsen and Fossdal 2010; Schmöllerl et al. 2011; Pilgård et al. 2012). Verma et al. (2008) used calorimetry to show that *C. puteana* colonised DMDHEU-treated wood. Schmöllerl et al. (2011) measured *P. placenta* DNA levels in acetylated, furfurylated and thermally modified wood after 2, 14 and 26 weeks of incubation. Fungal DNA could be detected in the modified wood materials already after two weeks, but the levels were lower than in the control throughout the experiment. These results were supported by the findings of Pilgård et al. (2012), who examined Southern Yellow Pine (SYP: *Pinus elliotti*, *Pinus palustri*, *Pinus echinata*, *Pinus taeda*, *Pinus rigida* and *Pinus serotine*) with three different levels of acetylation. Thus, brown rot fungi are not killed by wood modification and even capable of colonisation, despite changes in the pH and moisture content.

4.3.2 Transcription

The genes involved in wood degradation are up-regulated in cellulose and wood-containing media compared to glucose-containing medium and are also expressed in modified wood (Martinez et al. 2009; Alfredsen and Fossdal 2010; Vanden Wymelenberg et al. 2010; Schmöllerl et al. 2011; Pilgård et al. 2012). As described in section 3.2.1, enzymes involved in the metabolism of a carbon source other than glucose, are repressed in the presence of glucose and induced in the presence of metabolites derived from the degradation of that carbon source (Aro et al. 2005). The higher expression of cellulases in wood compared to glucose is therefore probably due to the absence of free glucose in wood and/or induction by cellulose degradation metabolites. In a similar manner, genes involved in CMF degradation may be expressed at a higher level in wood than in glucose due to the fungus experiencing a nutritional stress. Up-regulation due to nutritional stress could also explain the higher expression of CMF related genes in modified wood compared to untreated wood, since nutrients in modified wood are more difficult to acquire. In this context it should be stressed that up-regulation of genes involved in degradation of wood caused by exposure of the fungi to untreated or modified wood is probably not due to that the fungi recognises the wood as a nutrient source but should rather be described as a scouting response.

Alfredsen and Fossdal (2010) studied the gene expression of *P. placenta* at 2, 4 and 8 weeks of colonisation in furfurylated *P. sylvestris* sapwood, and the primary finding was that the genes related to CMF degradation (e.g., alcohol oxidase, QRD and a laccase-like gene) generally were expressed at higher levels in furfurylated wood than in untreated pine. However, the genes studied that are involved in the enzymatic degradation, including one endoglucanase and one β-glucosidase, displayed different expression patterns depending on the gene and no conclusion of the effect of wood modification on the expression of these genes could be made. These results were supported by the findings of Schmöllerl et al. (2011), who found that alcohol oxidase, a

laccase-like gene and a peroxidase had higher levels of expression in acetylated, furfurylated and thermally modified *P. sylvestris* sapwood specimens than in untreated pine at 2 and 14 weeks of incubation with *P. placenta*. Even after 36 weeks of exposure of acetylated SYP specimens to *P. placenta*, the expression of alcohol oxidase, QRD and the laccase-like gene was higher than in untreated SYP after 4 weeks (Pilgård et al. 2012). Together these results indicate that *P. placenta* expresses the genes needed for wood degradation also in modified wood and that the response of *P. placenta* when encountering modified wood might be to up-regulate the expression of the CMF degradation machinery.

4.3.3 Translation and secretion

The enzymes involved in the degradation of wood, such as the cellulases and xylanases, are translated into proteins and are also secreted into the lumen in thermally modified wood (Elisashvili et al. 2012; Irbe et al. 2014). In a study by Elisashvili et al. (2012), the enzyme activities of *C. puteana* cellulase and xylanase in thermally modified *P. sylvestris* were equivalent to that in untreated pine. However, because the mass loss was considerably lower in the thermally modified wood, the amount of fungi was probably also lower. Since enzyme activity was measured per specimen and not per fungal mass, this result indicated that the amount of enzyme produced by the fungus was higher in the thermally modified wood than in the untreated pine. Irbe et al. (2014), on the contrary, found lower activity of the *C. puteana* cellulase and xylanase in thermally modified hardwood (*Betula* species, *Populus tremula* and *Alnus incana*) than in untreated wood of the same species. However, since the mass loss was much lower in the thermally modified wood than in the untreated wood also in this experiment, the enzyme activity per fungal mass is unclear. In both studies it is interesting to see that in some cases considerable enzyme activity could be seen in thermally modified wood even at very low mass loss levels (0.1-1%). Neither studies on the translation or secretion of brown rot cellulase in other types of modified wood materials nor studies that showed whether the enzymes or metabolites involved in CMF degradation were formed in the modified wood materials were found in the literature; similarly, no studies were found on the secretion of CMF metabolites in modified wood. If these steps are inhibited by wood modification, it is possible that the inhibition is conducted through a regulatory mechanism in the fungus.

4.3.4 Penetration of and diffusion through the wood cell wall

As described in section 4.2, the pores in the wood cell walls are reduced in number and in some cases in size by wood modification (Hill et al. 2005; Dieste et al. 2009a; Zauer et al. 2013; Kekkonen et al. 2014). However, no experiment has demonstrated whether the size of the pores in modified wood is large enough for the CMF metabolites to pass through. Furthermore, although it has been suggested that moisture reduction lead to the inhibition of diffusion of the CMF metabolites through the wood cell walls, there is no experimental evidence to support this conclusion (Papadopoulos and Hill 2002; Boonstra and Tjeerdsma 2006).

The ability of cellulases and other related enzymes to penetrate the wood cell walls in modified wood and to be transported through the cell wall remains to be investigated; however, this ability may be of less importance because CMF degradation creates pathways for the cellulases to pass through (Cowling and Brown 1969; Koenigs 1974; Schmidt et al. 1981; Flournoy et al. 1991; Arantes et al. 2012). On the other hand, a longer and/or more severe CMF attack may be required in modified wood materials than in untreated wood to achieve sufficiently large pores for the cellulases to pass through.

4.3.5 Formation of radicals and depolymerisation and hydrolysis of polysaccharides

The pH is important for the reaction in which insoluble Fe^{3+}, located in the lumen or bound to the wood components, is sequestered by oxalic acid, transported into the wood cell wall and then reduced to Fe^{2+} by fungal iron reductants (Arantes et al. 2009b). It has been proposed that the pH is affected in acetylated wood and therefore hydroxyl radical formation may be inhibited (Larsson-Brelid 1998). In *in vitro* experiments, Fenton derived radicals formed in the supernatant depolymerised DMDHEU-treated wood and caused mass loss, however, to a much lower extent than in untreated specimens (Verma and Mai 2010; Xie et al. 2014). Therefore, it can be concluded that if radicals are produced inside the wood cell wall *in vivo*, the polysaccharides in the modified wood would be depolymerised. No *in vivo* study to elucidate whether the formation of hydroxyl radicals is induced by brown rot CMF metabolites in modified wood was found in the literature.

The fungal cellulases hydrolyse polysaccharides in DMDHEU-treated and furfurylated wood (Venås 2008; Verma and Mai 2010), even though they were less efficient in the modified wood materials than in untreated wood. The efficacy of the cellulases was restored in DMDHEU-treated wood when beforehand pre-treated with Fenton's reagent, simulating the CMF degradation that precedes the enzymatic degradation in vivo (Verma and Mai 2010). In conclusion, the enzymatic degradation was not inhibited by wood modification, only less efficient.

5 Knowledge gap

One important aspect to keep in mind when evaluating reported findings in any area is that one can only see what the available and applied methods allow us to see and therefore, in a sense, what researchers are looking for. There is always a risk that what is now considered a fact might be disputed in the future.

With that said, the wood structure is well studied by now and both structure and composition is well known (Eaton and Hale 1993; Rowell 2005). However, wood is an organic material and therefore there inevitably will be differences not only between species but also between individuals and even within individuals. When it comes to wood-water interactions, researchers deviate on the definition of FSP, the kinetics of sorption in wood, etc. (Engelund et al. 2012). Chemical transport in wood has not been thoroughly studied as yet. A couple of models have been proposed (see section 2.1.1) but they have neither been verified nor refuted (Zelinka et al. 2008; Jakes et al. 2013). So far, mainly ion transport has been studied, and even though conclusions of transport of bigger molecules have been drawn from this, the transport of degradation agents has not been studied (Jakes et al. 2013).

As mentioned in section 3.1, the definition of brown rot fungi is not clear-cut (Riley et al. 2014). That means, using a certain species of brown rot fungi as a model organism for all brown rot fungi is not feasible. To be able to draw conclusions for all brown rot fungi, several species with different characteristics need to be examined.

The model of the brown rot degradation, in which CMF degradation works in concert with enzymatic degradation, has been supported by numerous studies, but has not been proven in sufficient detail (Goodell et al. 1997; Kerem et al. 1999; Jensen Jr. et al. 2001; Cohen et al. 2002; Hammel et al. 2002; Jensen et al. 2002; Arantes and Milagres 2006; Suzuki et al. 2006; Arantes and Milagres 2007; Martinez et al. 2009; Arantes et al. 2009b). Furthermore, most of the work has been done on *G. trabeum* and even though some of the results have been confirmed also for other fungal species, it is unknown to what extent the wood degradation mechanism may differ between different species (Goodell et al. 1997; Shimokawa et al. 2004; Martinez et al. 2009; Arantes et al. 2011).

However, the major question mark dealt with in this thesis is of course the mode of action of modified wood. As described in chapter 4, this has been extensively studied but the exact mechanism of the decay resistance in modified wood has not been revealed as yet (Tjeerdsma et al. 1998; Hakkou et al. 2006; Hill 2006; Rowell et al. 2007; Rapp et al. 2008; Rowell et al. 2009; Verma et al. 2009). Most decay tests performed on modified wood have been carried out according to conventional durability standards and the results show whether the modified wood material has an increased durability compared to untreated wood or not (Larsson Brelid et al. 2000; Papadopoulos and Hill 2002; Lande et al. 2004; Verma et al. 2009). However, it does not reveal how that durability is achieved. Studies of durability of modified wood generally look at mass loss at one time point why no previous studies have monitored the dynamics of the colonisation and eventual decay in modified wood. Furthermore, the wood characteristics measured after decay are mainly mass loss and, to some degree, structural integrity (Larsson Brelid et al. 2000; Papadopoulos and Hill 2002; Lande et al. 2004; Brischke et al. 2008). Characteristics like moisture content and composition as well as a more detailed study of structural integrity may provide information on how the brown rot fungi and the modified wood

affect one another. The dynamics of the brown rot colonisation and decay may show to what extent the wood is affected before substantial mass loss can be detected and help in service life predictions since the rate of decay is crucial for such estimations. Furthermore, it has not been investigated to what extent the fungi are able to degrade the modification chemicals and, if they do degrade the modification chemicals, in what stage of the colonisation/degradation process that occurs.

More than that, it is not known which part(s) of the brown rot degradation process is inhibited by wood modification. Even though gene expression studies that have been performed indicate that while genes involved in CMF degradation are generally up-regulated in modified wood, only a few genes have been studied so far (Alfredsen and Fossdal 2010; Schmöllerl et al. 2011; Pilgård et al. 2012). Previously, gene expression during the first two weeks of exposure has not been analysed. Furthermore, in previous studies (Alfredsen and Fossdal 2010; Schmöllerl et al. 2011; Pilgård et al. 2012) gene expression in modified wood materials is generally compared to the gene expression in untreated wood after the same time of exposure. At, for example, eight weeks of exposure of miniblock samples to *P. placenta*, there might be no or very little degradation in the modified wood material while untreated wood typically has lost 20-50% of its mass. That means the two materials are in very different stage of degradation and therefore would be expected to have differing gene expression. Comparing gene expression (as well as other characteristics) at the same stage of decay might be more informative when studying the mode of action in modified wood. However, an obstacle in the research of the brown rot decay resistance of modified wood is the difficulty to measure the amount of CMF degradation.

Even though it has been shown that Fenton derived hydroxyl radicals are able to degrade polysaccharides in DMDHEU-treated wood, it is not known whether they are able to cleave polysaccharides in other kinds of modified wood (Verma and Mai 2010; Xie et al. 2014). Furthermore, it is not known whether fungal cellulase efficacy can be restored in these wood materials as it is in DMDHEU treated wood.

In addition, it is not known whether wood modification inhibits translation of CMF related proteins, secretion of CMF molecules, penetration or diffusion of CMF molecules through the wood cell wall or the Fenton reaction or any of the reactions leading up to it inside the wood cell wall. Neither have there been any previous general comparative reviews of the modes of action of different wood modification methods.

There are some theories of how some of the mechanisms in the brown rot degradation process may be inhibited by wood modification, but no conclusive experimental evidence has so far been provided that explains the mode of action (Papadopoulos and Hill 2002; Rowell 2005; Hill et al. 2005; Hill 2006; Boonstra et al. 2007; Rapp et al. 2008; Rowell et al. 2009). Neither have the established theories been evaluated on the basis of how the brown rot degradation process works.

6 Objectives

The aim of this thesis was to elucidate the mechanisms behind the decay resistance to the brown rot fungus *Postia placenta* in acetylated, furfurylated, DMDHEU-treated and thermally modified wood. In particular the following questions were addressed:

1. Does *P. placenta* colonise acetylated, furfurylated, DMDHEU-treated and thermally modified wood during the first two weeks of exposure?
2. How do acetylation, furfurylation, DMDHEU-treatment and thermal modification affect the kinetics of wood mass loss and the fungal growth pattern during *P. placenta* wood degradation?
3. What are the moisture conditions in acetylated, furfurylated, DMDHEU-treated and thermally modified wood prior to and during degradation?
4. Are hydroxyl radicals and fungal cellulases able to respectively depolymerise and hydrolyse acetylated and furfurylated modified wood?
5. How do wood acetylation, DMDHEU-treatment and thermal modification affect gene expression in *P. placenta*?
6. Is *P. placenta* able to degrade acetyl in acetylated wood?
7. Is *P. placenta* able to decrease structural integrity in acetylated, furfurylated, DMDHEU-treated and thermally modified wood?
8. Based on results in this thesis as well as the current literature, what *P. placenta* degradation mechanism is inhibited by wood acetylation, furfurylation, DMDHEU-treatment and thermal modification and which of the established theories on the effects of wood modification is the most plausible?

7 Overview of subprojects

7.1 Paper I

Paper I: Ringman R, Pilgård A, Brischke C, Windeisen L, Richter K (2017) Incipient brown rot decay in modified wood: patterns of mass loss, structural integrity, moisture and acetyl content in high resolution. International Wood Products Journal, 8(3): 172-182.

7.1.1 Summary

Fungi growing in liquid culture undergoes three separate phases in which they i) adapt to the new environment, ii) grow unrestrictedly and exponentially, and iii) are inhibited to increase in number/mass due to lack of nutrients etc. Filamentous fungi have been shown to exhibit similar growth phases in a solid food substrate and have been modelled to grow in this way also in solid wood.

Wood acetylation and furfurylation are promising decay protection treatments and possible substitutes to preservative treatments. In modified wood with high treatment levels, fungi cause no or little mass loss but the reason for this has not been fully explained. To be able to efficiently develop and improve these materials as well as predict the service-life of modified wood, understanding their mode of action is important. One aspect of this is the effect of acetylation and furfurylation on the growth pattern of wood degrading fungi.

The aim of this study was to find out whether brown rot fungi undergoes the same growth phases in solid wood as in liquid culture and study the effect of acetylation and furfurylation on the growth pattern. This was done through high-frequent monitoring of mass loss over 300 days of exposure of acetylated and furfurylated wood to the brown-rot fungi *Postia placenta*. To complement the mass loss results, which mainly reflect the enzymatic degradation, structural integrity and acetyl content (in the acetylated wood) was measured in an attempt to detect signs of the chelator mediated Fenton degradation.

Mass loss results of the untreated wood indicated that the fungi in this material go through phases similar to the phases seen in liquid cultures. In the modified wood materials did little mass loss, a degradation rate 100 times lower than in the untreated wood during exponential growth and no or little loss of structural integrity suggest that the fungi in the modified wood samples were still adapting to the new environment. On the other hand, the fact that mass was lost at all suggests that degradation did occur and that the fungi were growing exponentially but at a very slow rate. Maintained levels of acetyl in the acetylated wood throughout the prolonged decay test shows that the treatment level was not affected by exposure to *P. placenta*.

7.1.2 Contribution of the authors

Rebecka Ringman, together with Annica Pilgård, developed the research idea, designed the experiment, prepared and started the decay test and supervised harvest, preparation of samples and the analyses thereof. Rebecka Ringman also compiled the results and wrote the paper.

Christian Brischke managed the structural integrity tests and Elisabeth Windeisen managed the acetyl content analysis. Annica Pilgård and Klaus Richter supervised the research and all co-authors collaborated in scientific discussions and critical reading of the manuscript.

7.2 Paper II:

Paper II: Ringman R, Pilgård A, Richter K (2014) Effect of wood modification on gene expression during incipient Postia placenta decay. International Biodeterioration and Biodegradation, 86:86-91.

7.2.1 Summary

P. placenta has been shown to express genes involved in CMF degradation at high levels in modified wood materials between 2 and 36 weeks (Alfredsen and Fossdal 2010; Schmöllerl et al. 2011; Pilgård et al. 2012). The aim of this study was to investigate the reaction of *P. placenta* in terms of gene expression of selected genes upon the encounter of acetylated, DMDHEU-treated and thermally modified wood during the first two weeks of exposure by means of qPCR.

The investigated *P. placenta* genes presumed to be involved in oxidative degradation (QRD Ppl124517 and alcohol oxidase Ppl118723) were expressed at higher levels in acetylated, DMDHEU-treated and thermally modified wood than in untreated wood. For the investigated genes involved in enzymatic degradation (endoglucanase Ppl103675 and β-glucosidase Ppl112501), the levels of expression in modified woods were equal to or lower than those in untreated wood.

The results indicate that the response of *P. placenta* upon the encounter of modified wood is to up-regulate the expression of the CMF degradation machinery.

7.2.2 Contribution of the authors

Rebecka Ringman prepared and executed the decay test, prepared the samples for analysis, coordinated the analyses, compiled the results and wrote the paper. Annica Pilgård developed the research idea, designed the experiment, evaluated qPCR data and, together with Klaus Richter, supervised the research, participated in scientific discussions and critically read the manuscript.

7.3 Paper III:

Ringman R, Pilgård P, Kölle M, Brischke C, Richter K (2015) Effects of thermal modification on Postia placenta wood degradation dynamics: measurements of mass loss, structural integrity and gene expression. Wood Science and Technology, 50(2):385-397.

7.3.1 Summary

Previous studies showing that *P. placenta* expression of genes involved in CMF degradation is up-regulated in modified compared to untreated wood, analysed samples at the same time points but with different amounts of mass loss and hence in different stages of decay (Alfredsen and Fossdal 2010; Schmöllerl et al. 2011; Pilgård et al. 2012)(Paper II). The aim of this study was to compare gene expression, moisture content and structural integrity in thermally modified wood with untreated wood in the same stages of decay. Through this we hoped to answer the following questions: i) Before mass loss can be detected, is the wood affected by CMF degradation?, ii) once active decay has started, are there any differences in the degradation patterns between the two materials?

High-frequency monitoring of mass loss over 120 days in thermally modified wood exposed to *P. placenta* showed a delay in the onset of degradation compared to untreated wood and once the degradation had started the rate was lower. Thermally modified wood did not inhibit expression of wood degradation related genes before mass loss was detected and was similar to that in untreated wood once mass loss could be detected. Comparing gene expression as well as strength loss at the same stage of decay rather than at the same time after exposure, showed smaller differences in decay patterns between thermally modified and untreated wood than previous results indicate. The results suggest that, once degradation has started, the fungi degrade thermally modified wood similarly to untreated wood.

7.3.2 Contribution of the authors

Rebecka Ringman, together with Annica Pilgård, developed the research idea, designed the experiment, prepared and started the decay test and supervised harvest, preparation of samples and the analyses thereof. Rebecka Ringman compiled and evaluated the results and wrote the paper. Martina Kölle harvested samples, prepared them for qPCR, ran qPCR and Christian Brischke managed the structural integrity tests. Annica Pilgård assessed the reliability of the qPCR-results and, together with Klaus Richter, supervised the research and participated in scientific discussions. All co-authors contributed with critical reading of the manuscript.

7.4 Paper IV:

Paper IV: Ringman, R, Pilgård, A, Richter, K. (2015) In vitro oxidative and enzymatic degradation of modified wood. International Wood Products Journal, 6: 36-39.

7.4.1 Summary

Fungal cellulases have been shown to be less efficient in modified wood than in untreated wood (Lekounougou et al. 2008; Venås 2008). However, Verma and Mai (Verma and Mai 2010) showed that cellulase efficacy is partly restored in 1,3-dimethylol-4,5-dihydroxyethyleneurea (DMDHEU) treated wood by pre-treatment with Fenton’s reagent, simulating the oxidative degradation phase in initial brown rot decay.

In this study, we examined whether Fenton derived hydroxyl radicals and cellulases are able to cleave polysaccharides in furfurylated and acetylated wood and to what extent enzyme efficacy is increased by oxidative pre-treatment of these materials. Treated and untreated wood meal was incubated with Fenton’s reagent, fungal cellulases or both and the supernatant was analysed for reducing sugars released from the wood meal using dinitrosalicylic acid (DNS).

Fungal cellulases were able to degrade polysaccharides in acetylated wood. Cellulase efficacy was increased after treatment with Fenton’s reagent, but not to the same extent as in untreated wood. This indicates that Fenton derived hydroxyl radicals facilitates cellulase degradation also in acetylated wood, possibly by depolymerisation of polysaccharides and/or removal of acetyl groups from wood polymers.

Reacting furfurylated wood meal with Fenton’s reagent turned the supernatant dark brown and the results from the DNS measurements indicated extremely high levels of reducing sugars. However, DNS does not only detect reducing sugars but also other aldehydes. We hypothesised that Fenton derived hydroxyl radicals depolymerised the polymerised furfuryl alcohol. Additionally, the results showed that fungal cellulases were able to degrade cellulose in furfurylated wood.

These results show that if brown rot fungi are able to form hydroxyl radicals inside the wood cell wall in acetylated wood, they would be able to degrade it. The inhibition must therefore be earlier in the degradation process.

7.4.1 Contribution of the authors

Rebecka Ringman developed the research idea, designed the experiment, performed the experimental work, evaluated and compiled the results and wrote the paper. Annica Pilgård and Klaus Richter supervised the research, participated in scientific discussions and critically read the manuscript.

7.5 Paper V:

Ringman R, Pilgård A, Brischke C, Richter K (2014) Mode of action of brown rot decay resistance in modified wood: a review. Holzforschung, 68:239–246.

7.5.1 Summary

Several theories of how wood modification inhibits decay has been put forward, among which the following are the most discussed: i) fungal enzyme inefficacy due to non-recognition (Rowell 2005), ii) fungal enzyme inefficacy due to lack of water at glycosidic bonds (Rowell 2005; Rowell et al. 2009), iii) reduced flow of fungal molecules into the wood cell wall due to micropore blocking (Hill et al. 2005), and iv) inhibition of diffusion of fungal molecules due to insufficient amounts of moisture in the wood cell wall (Papadopoulos and Hill 2002; Boonstra and Tjeerdsma 2006).

This review provided a comparative evaluation of established theories related to the mode of action of brown rot fungi resistance in modified woods. The evaluation focused on the biochemical mechanisms of brown rot wood degradation and was limited to acetylated, furfurylated, DMDHEU-treated and thermally modified wood.

The main conclusion was that only one theory provides a consistent explanation for the initial inhibition of brown rot degradation in modified wood, i.e., moisture exclusion via the reduction of cell wall voids. Other proposed mechanisms – such as enzyme non-recognition, micropore blocking, and reducing the number of free hydroxyl groups – may reduce the degradation rate when cell wall water uptake is no longer impeded.

7.5.2 Contribution of the authors

Rebecka Ringman performed the literature review, evaluated and compiled the results and wrote the paper. Annica Pilgård, Christian Brischke and Klaus Richter supervised the research, contributed in scientific discussions and critically read the manuscript.

8 Methods

All methods are thoroughly described in the attached papers. This chapter is a joint general description and discussion of the materials and methods used and applied in the experimental research of this thesis.

8.1 Wood material, wood treatments and fungal strain

In the experiments described in this thesis, sapwood from either *P. sylvestris* or SYP (*Pinus elliotti*, *Pinus palustri*, *Pinus echinata*, *Pinus taeda*, *Pinus rigida* and *Pinus serotine*) was used. Due to its anatomy, pine sapwood is very permeable and possible to treat with the wood modifications used in this work. Pine wood is also one of the most common wood materials used in building construction in the Northern hemisphere and, being a softwood specie, a preferred substrate of brown rot fungi (Eaton and Hale 1993; Schwarze 2007).

Modification treatments were carried out as described in papers I, II, III and IV by Christian Brischke, Pia Larsson-Brelid and Andreas Treu. In the experiment in paper II, the wood was treated as boards and subsequently specimens of 10 x 5 x 30 mm^3 (the long side in the fibre direction and the tree rings at 45° in the cross-section) were cut out of the boards. In paper I the specimens (10 x 5 x 30 mm^3) were instead cut out of untreated boards and treated as individual specimens. In paper III rods of approx. 10 x 5 x 300 mm^3 were cut out of untreated boards, heat-treated and then cut into 10 x 5 x 30 mm^3 specimens. In unpublished results shown in Fig. 4, from a decay test with specimens cut out of acetylated boards, the variation in mass loss between specimens is large. Using individually treated specimens, we were able to reduce the variation (Paper I: Fig. 2A; Paper III: Fig. 1). Furthermore, we knew the WPG of each individual specimen, which was useful in the interpretation of the results. The variation seen in Fig. 4 is probably due to uneven treatment of the acetylated board.

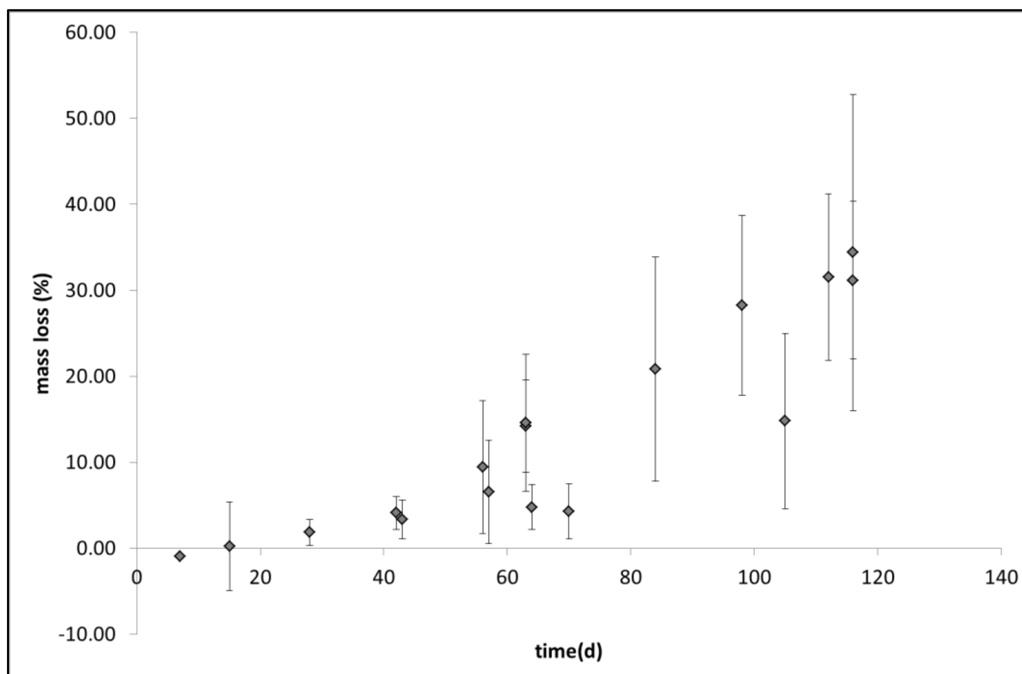

Figure 4. Mass loss of miniblock specimens cut out of acetylated boards.

P. placenta (strain FPRL 280) was used in all decay tests described in this thesis. *P. placenta* was chosen as model fungus since its genome is sequenced (Martinez et al. 2009), however the strain sequenced was MAD-698-R. The FPRL 280 strain is originally European, while the MAD-698-R strain was isolated in the US. It has been shown that these strains differ somewhat in e.g. aggression (Thaler et al. 2012). In this study we chose to use the FPRL 280 strain since it is more relevant in Europe and it had previously been used in studies that we wanted to be able to compare our results with (Alfredsen and Fossdal 2010; Schmöllerl et al. 2011; Pilgård et al. 2012). Primer sequences were based on the MAD-698-R strain. Even though it is unclear to what extent the sequences deviate between the strains, the primers will match FPRL 280 transcripts isolated from both treated and untreated wood with equal effectivity and the results can thus be compared.

8.2 Decay tests and subsequent mechanical, biochemical and molecular analyses

8.2.1 Decay test

The decay tests were carried out as miniblock tests, described by Bravery (Bravery 1979), with minor alterations. Schematic flowcharts of agar and soil decay tests are shown in Fig. 5. Annica Pilgård assisted in supervising and Anja Viehler, Martina Kölle, Petra Arnold, Angelica Full and Monica Rinas assisted in the execution of the decay tests.

The specimens were leached according to EN 84 (1996) and acclimatised in 20°C and 65% RH for two weeks. The EMC after acclimatisation was recorded for each specimen in paper III. The specimens were sterilised with gamma radiation and placed two by two in Petri dishes either containing inoculated agar (paper II) or sterile soil (autoclaved twice at 120°C for 20 min) in which case the specimens were subsequently inoculated with 1 ml *P. placenta* liquid culture (papers I and III). The liquid culture was prepared by inoculating malt medium with plugs from a malt agar plate covered with *P. placenta* mycelium and incubating it in 22°C and 75% RH without stirring. Prior to inoculation of the wood specimens in the soil plates, the liquid culture was homogenised with an electric stir. Inoculated plates were incubated in 22°C and 75% RH for up to 300 days and harvested at regular intervals. At harvest, the external mycelia was gently removed and discarded while the specimens were either dried (103°C, 18h) or shock-frozen in liquid nitrogen and stored in -80°C, depending on the purpose of the specimen.

Soil was used in decay tests planned to run longer than 8 weeks. Both substrates will start to dry after this time point, but soil is possible to re-wet with sterile water. Addition of sterile water was performed every four weeks in the experiments in papers I and III. The amount of water added equalled the weight loss of the petri dish.

The miniblock test used in these experiments (Bravery 1979) was originally developed for durability tests of preservative treated wood materials. Due to the crucial difference in mode of action between preservative treated wood and modified wood, it has been suggested that there is a need for a different standard method to test durability in modified wood (Junga and Militz 2005; Militz and Lande 2009). Unfortunately, no such standard has as yet been developed. However, in this work, the miniblock test was not used as a durability test but only as a way to cause rot in the wood specimens in a controlled fashion. For example, the limit of 3% mass loss used in the European standard EN113 as an indication of durability was not considered in these experiments.

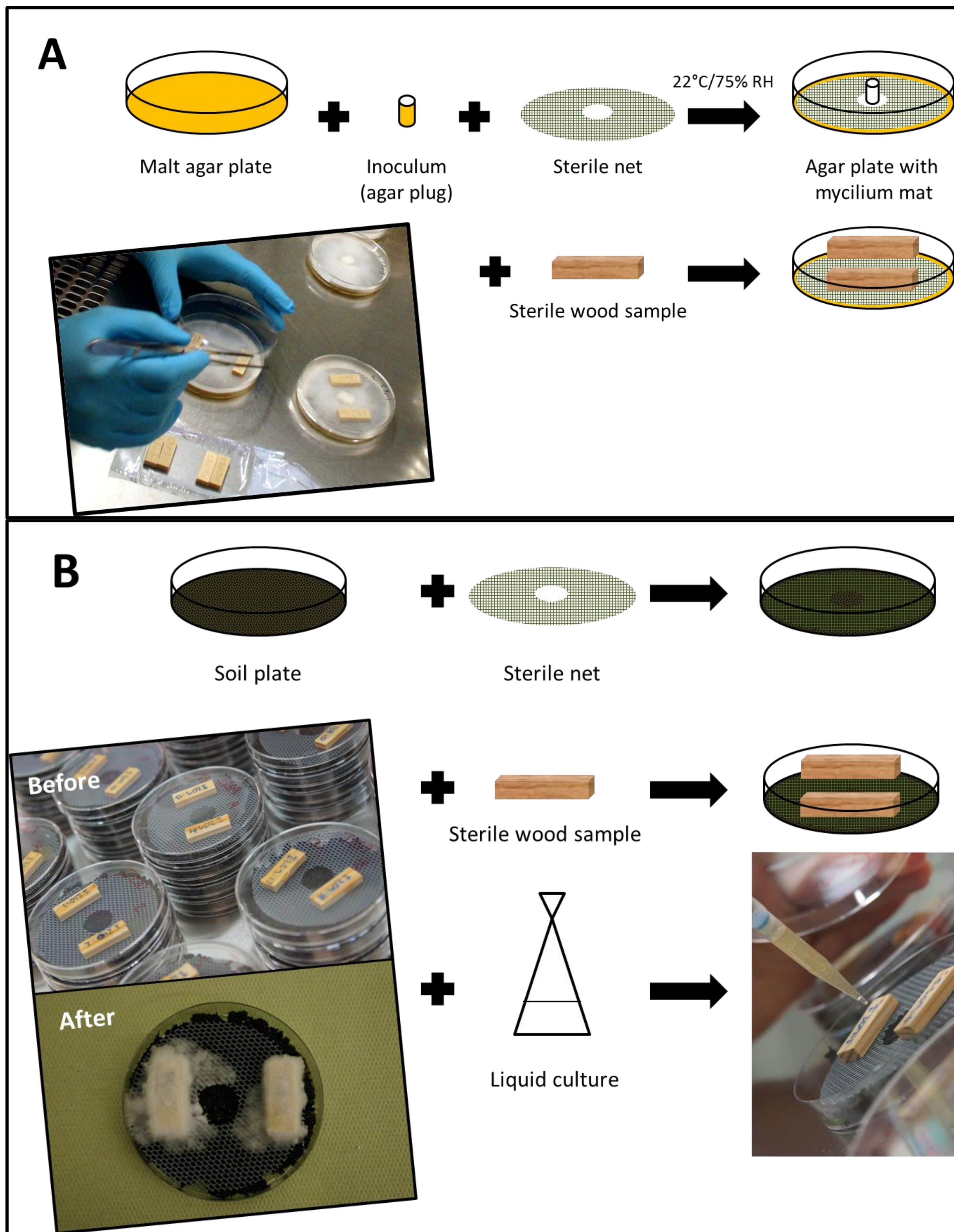

Figure 5. Schematic illustration of the assemblage of A) an agar plate decay test and B) a soil plate decay test

8.2.2 Mass loss and moisture content

Before drying in 103°C for 18h and weighing for mass loss, specimens were weighed for moisture content. In the first decay tests run for this thesis (paper II), four replicates were used for both mass loss/moisture content and biochemical/molecular analyses. Annica Pilgård assisted in supervision and Anja Viehler, Martina Kölle and Petra Arnold in execution of these analyses.

Due to high variation in mass loss seen in a pre-test (Fig. 4, see section 8.1), eight replicates were used for mass loss in the subsequent decay test (papers I and III). Weighing was done manually on a scale with three decimals. Specimens were kept dry in a dessicator after drying. The variation seen in mass loss in the pre-test (Fig. 4) were first suspected to be due to differences in fungal load, but the variation in untreated wood was always lower than in treated wood in all the decay tests in this thesis (Paper I: Fig. 1A; Paper III: Fig. 1). This might be due to that the untreated specimens were not exposed to fungi as long as the treated specimens (since variation increases with time) but could also indicate that the main cause of the variation is rather different levels of treatment in the different specimens than difference in fungal load. Treatment level is affected by anatomical properties such as annual ring width and latewood content, tree attributes and growth conditions (Zimmer et al. 2014). In these experiments, the structural differences were not large enough to cause major variation in mass loss in the untreated wood, but may have been large enough to cause uneven treatment. A solution to this problem might therefore be to choose the material to be used carefully according to the criteria described by Zimmer et al. (2014).

8.2.3 Structural integrity (HEMI)

Structural integrity was determined by Christian Brischke, Andreas Tenz and Paul-Simon Schroll with the High energy multiple impact (HEMI) test in papers I and III (Brischke et al. 2006; Rapp et al. 2006). The development and optimisation of the HEMI test have been described by Rapp et al. (2006). For the untreated, thermally modified and acetylated specimens, the structural integrity specimens were selected by random out of the eight replicates harvested at the same time, but for the furfurylated specimens, four specimens with relatively similar mass loss were selected. Selecting according to mass loss gives less variation in mass loss and therefore makes it easier to interpret the results. The four specimens from each harvest and treatment were cut in three and the now 12 smaller specimens were shaken together in a heavy-impact ball mill with seven steel balls of various but specified sizes. The fragments were fractionated and the following values were calculated: i) the degree of integrity (I), which is the ratio of the mass of the 12 biggest fragments to the mass of all fragments after crushing, ii) the fine fraction (F), which is the ratio of the mass of fragments under 1 mm (mesh size) to the mass of all fragments, multiplied by 100, and iii) the resistance to impact milling (RIM), which is calculated from I and F as follows:

$$RIM = (I - 3 \cdot F + 300)/4 \ \ [\%]$$

The threefold weighing of the fine fraction was according to earlier studies (Rapp et al. 2006) and can finely distinguish between different intensities of fungal decay. To ensure that RIM varies between 0 and 100 %, the constant, 300, was added.

This method addresses the structural integrity of the wood and therefore gives an indication of the depolymerisation of the cellulose. The advantages of HEMI are small variances, high

reproducibility of results, short time for specimen preparation, and a small number of specimens needed (Rapp et al. 2006). Furthermore, for the experiments performed for this thesis, the possibility to measure change in structural integrity in miniblock specimens was important. However, the method does not distinguish between different types of strength, such as bending strength, tensile strength and compression strength.

8.2.4 Acetyl content

Acetyl content analysis was performed by Elizabeth Windeisen and Andreas Tenz. Prior to chemical analyses, the specimens were cut into small pieces and then ground with a ball mill (MM 400 Retsch GmbH, Haan, Germany) under cooling with liquid nitrogen and finally air-dried overnight. The moisture content of the air-dried wood specimens was determined separately by drying at 105 °C. The determination of acetyl groups was carried out according to Månsson and Samuelsson (Månsson and Samuelsson 1981) by means of an aminolysis with pyrrolidine and subsequent gas chromatography analysis on a GC 2010 (Shimadzu) equipped with a BP5 (SGE) or a HP-5 (Agilent-Technologies) capillary column.

8.2.5 Gene expression analyses

The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were developed and published to encourage better experimental practice and allowing more reliable and unequivocal interpretation of qPCR results (Bustin et al. 2009). The MIQE guidelines were followed in this thesis.

Preparation of samples and execution of qPCR analyses were assisted by Annica Pilgård, Anja Viehler and Martina Kölle. Wood powder from frozen samples was produced using a ball mill (MM 400 Retsch GmbH, Haan, Germany). Total RNA was isolated from the wood samples and DNA was removed using MasterPure™ RNA Purification Kit (Epicenter), according to the manufacturer's instructions. The RNA was not checked for *P. sylvestris* contamination, since the samples were prepared for qPCR with *P. placenta* specific primers. RNA concentration from equivalent samples had been measured with a Nanodrop in a method development pre-study and concluded to be so low that the highest volume prescribed for cDNA synthesis was needed. Random RNA samples were analysed with gel electrophoresis. The ribosomal bands were intact which indicated that the rest of the RNA also had good integrity.

RNA from each sample was converted into cDNA using TaqMan Reverse Transcription Reagents (using Oligo d (T)16) (Applied Biosystems) with a Rotor-gene (Qiagen) according to the manufacturer's protocol but with 10 times the standard dNTP concentration. The increase in dNTP concentration was due to the low concentration of RNA in the samples and increased the possibility of dNTP binding to RNA.

Quantitative real-time PCR (Rotor-gene, Qiagen) was performed on cDNA samples using Rotor-gene SYBRGreen PCR kit (Qiagen) according to the manufacturer's protocol. From each cDNA sample, originating from one wood sample, three technical replicates were run. Primer sequences for target genes and endogenous controls are listed in table 1 in paper II and table 1 in paper III. The amplicon length was approx. 100bp for all genes. The thermocycle programme was optimised for each set of primers. The annealing temperature was adjusted to fit to the melting temperature of the primers and the time was changed as needed to improve the outcome. Elongation temperature was set according to the manufacturer's protocol and the time was adjusted for low template concentration.

Rotor-gene Q series softwear (Qiagen) was used to evaluate the samples. Runs were evaluated from their reaction efficiency and R^2-value. Runs with reaction efficiency and R^2-values strikingly deviating from 1 were not used. Runs were also evaluated from the Cq-value of the no template control samples. Only samples with 1000 times higher concentration than the no template control samples were analysed. In controls using *P. sylvestris* primers, no product was formed.

Standard curves were produced from cDNA using the primers of the gene in question. The concentration of the amplified target genes were measured with SYBRGreenI in a fluorometer. The original concentration of each mRNA transcript was calculated in each technical replicate. Mean concentration of the target gene was calculated for each wood sample, and was normalised through division by the corresponding amount of a reference gene (see below). Significance ($p < 0.05$) was calculated using independent two-sample t-tests assuming unequal variances.

To be able to compare expression levels between different replicates, time-points and materials, the expression of the genes of interest were related to the expression of a reference gene. Normalising to a reference gene controls for variation in extraction yield, reverse-transcription yield and efficiency of amplification (Bustin et al. 2009). Furthermore, it compensates for the differences in fungal mass in the different wood specimens, i.e. the final value is the expression per fungal mass and not per specimen. In this work, β-tubulin was used as a reference gene, which, along with α-tubulin and actin, is commonly used as a reference gene in a variety of species (Wan et al. 2011; Zhuang et al. 2015; Dankai et al. 2015). Even though the MIQE guidelines (2009) recommend the use of three reference genes, it was decided to use only one in this work. This decision was based on the results of a pre-study showing the same expression pattern for two α-tubulins as for β-tubulin but a stronger signal was found for the latter and hence it is more reliable when it comes to controlling and monitoring variations. However, no biological system or process can be expected to be absolutely stable and therefore some variation in expression of the reference gene may occur. Careful validation the reference gene(s) used is therefore crucial for minimising inaccuracy due to normalisation.

All preparation steps, such as RNA extraction and cDNA synthesis but also handling and storage, may contribute to some degradation of the RNA. The amount RNA detected in the qPCR analyses compared to the original amount of RNA is called the recovery rate. Pilgård et al. (2010) showed that furfurylation and acetylation affected the recovery rate of DNA extracted from wood samples. The recovery rate in furfurylated wood was 60% and in acetylated wood 55% of that from untreated wood. The recovery rate was measured by adding a known amount of foreign DNA to the milled wood before extraction was begun. Measuring the foreign DNA after extraction showed how much DNA had been lost during the process. Presumably, the chemicals added to the modified wood materials were released during the extraction and interfered with the extraction chemicals or reacted with the DNA. In the work of this thesis, recovery rate was not addressed *per se*, since the reference gene is degraded to the same extent as the gene of interest.

qPCR shows the level of gene expression of the genes for which corresponding primers are used. The disadvantage of this method is that the genes to be analysed must be selected before the analysis. Next-generation sequencing (high-throughput sequencing) instead gives the levels of

expression of all genes expressed in a specimen. However, this is a more expensive method that also requires much more data analysis. qPCR is therefore a both cheaper and faster tool as long as the genes of interest can be easily chosen with confidence.

As mentioned in section 8.1, *P. placenta* was chosen as model fungus in this work since its genome is sequenced (Martinez et al. 2009); however, the strain sequenced was MAD-698-R. In this work, the MAD-698-R DNA sequence was used to design the primers for the qPCR-experiments, which means they may not have been perfect matches. Sequencing the genome of FPRL 280 would be beneficial in future studies, especially if high-throughput sequencing is to be used.

8.3 *In vitro* degradation of wood powder

The degradation tests were performed according to Verma and Mai (Verma and Mai 2010) with minor changes. For *in vitro* simulation of CMF degradation followed by enzymatic degradation, wood powder produced using a ball mill (MM 400 Retsch GmbH, Haan, Germany) was incubated in 100 mM sodium acetate (NaAc) buffer (pH 5.0) with Fenton's reagent (0.5 mM $(NH_4)Fe(SO_4)^2$ and 1% H_2O_2). The tubes were centrifuged and the supernatant was removed. The wood powder was washed with sterile deionised water and Kieralon antiperox (BASF, Limburgerhof, Germany) was used to neutralise any remaining oxides. The wood powder was again washed with sterile deionised water. NaAc buffer and 28 U of Celluclast 1.5L (Sigma-Aldrich Sweden, Stockholm, Sweden) were added and the tubes were incubated on a shaker.

In order to ensure the same experimental conditions, also samples only treated with either Fenton's reagent or fungal cellulases were washed according to the routine above. Treatment with Kieralon antiperox was however shown not to affect cellulase activity (data not shown) and therefore this step was not performed for samples treated with only Fenton's reagent or only cellulases. For *in vitro* simulation of CMF degradation, washed wood powder was incubated in 12.5 ml NaAc buffer with Fenton's reagent (0.5 mM $(NH_4)Fe(SO_4)^2$ and 1% H_2O_2). For *in vitro* simulation of enzymatic degradation, washed wood powder was incubated in 12.5 ml NaAc buffer with 28 U of Celluclast 1.5L.

To ensure that the degradation seen was due to hydroxyl radicals and not hydrogen peroxide, $(NH_4)Fe(SO_4)^2$ was added before hydrogen peroxide. If hydrogen peroxide is added first, the hydrogen peroxide itself may cause oxidative damage on the wood particles.

The pH of NaAc buffer inoculated with wood meal was 5.05 ±0.05 for all wood treatments as well as for untreated wood. Hence, possible pH alterations in the modified wood materials did not affect the efficacy of the Fenton and/or cellulase reactions.

Reducing sugar content was assayed according to Miller (Miller 1959) and Verma and Mai (Verma and Mai 2010). The tubes were centrifuged and the supernatant was transferred to a new tube. When necessary, the samples were diluted in NaAc buffer. DNS solution (0.63% 3,5-dinitrosalicylic acid, 0.57% phenol, 0.5% $NaHSO_3$, and 2.14% NaOH) was added and the tubes were incubated at 100°C for 10 min. The tubes were subsequently cooled down to room temperature and the absorbance was measured with a spectrophotometer at 540 nm. A standard curve was prepared from a glucose solution.

The method was chosen in order to facilitate comparison of our results with the results of Verma and Mai (Verma and Mai 2010). The major disadvantage with the method is the lack of

possibility to distinguish between the aldehydes on the reducing sugar and other aldehydes, since the DNS reacts with all aldehydes. This turned out to be a problem in our study, since poly(furfuryl alcohol) probably was degraded into furfurals by the Fenton reaction, causing extremely high levels of aldehydes compared to untreated wood. The dark brown colour of the Fenton treated furfurylated wood can be seen in Paper IV: Fig. 2. Unpublished results on thermally modified wood showed that a similar problem may arise also when analysing this material. Furfurals are formed during the heating process and are probably released upon treatment with Fenton's reagent (Hill 2006).

9 Results and discussion

9.1 Objective 1 - Ability of brown rot fungi to colonise modified wood

The aim of this objective was to investigate whether *P. placenta* was able to colonise acetylated, DMDHEU-treated and thermally modified wood during the first two weeks of exposure. To fulfil this aim, fungal presence in the wood material was detected by qPCR-analysis of fungal mRNA.

In paper II, *P. placenta* was shown to be present in acetylated, DMDHEU-treated and thermally modified wood after 2 days while at 6 days, the levels of gene expression were significantly different from zero (Paper II: Fig. 2). In paper III, gene expression was first measured at 7 days and the results indicate fungal presence in thermally modified wood at this time point (Paper III: Fig. 4).

Modified wood is by definition non-toxic and should hence not kill fungi (Hill 2006). It is, however, theoretically possible that the change in moisture content and pH seen in modified wood could inhibit colonisation (Schmidt 2006). Presence of fungi in modified wood beyond two weeks of exposure was previously shown through DNA-measurements, calorimetry and ergosterol/chitin analyses, proving that modified wood does not kill fungi and fulfils the requirements for colonisation (Verma et al. 2008; Alfredsen and Fossdal 2010; Pilgård et al. 2010; Schmöllerl et al. 2011; Pilgård et al. 2012).

RNA measurements do not show how much fungi there is in a sample. To investigate the amount of fungus, DNA measurements would be an option. Using biochemical methods instead, measuring chitin and ergosterol, will quantify the amount of fungal biomass in the sample. Chitin reflects the amount of total fungal biomass in the sample, while ergosterol is believed to show the living fungal biomass (Braid and Line 1981; Pasanen et al. 1999). Eikenes et al. (2005) concluded that qPCR of DNA, being the most sensitive quantitative detection method for microbes, is suitable during initial and early decay, after which the DNA-based estimates were saturated in comparison to estimates using chitin and ergosterol assays. The authors suggest that this is due to that in advanced stages of decay parts of the mycelium undergo autolysis during which DNA may be degraded at a higher rate than ergosterol and chitin. If the purpose of the measurements is to estimate the amount of degradation in a sample in the later stages of decay, measuring ergosterol and chitin might be beneficial since these correlate well with mass loss. However, measuring DNA content would still show the amount of living and, presumably, actively degrading fungi.

The findings in this work support previous studies by showing that *P. placenta* was able to colonise the modified wood materials. Furthermore, the findings in this thesis show that *P. placenta* was able to colonise the modified wood materials within the first week of exposure. Together with previous results, the results in this work show that wood modification does not kill the fungi or inhibit it from colonising wood, but instead inhibits fungal degradation through some other mechanism (Verma et al. 2008; Alfredsen and Fossdal 2010; Schmöllerl et al. 2011; Pilgård et al. 2012). Hence, neither is the wood modification itself toxic to the fungi nor is the change in moisture content and pH sufficient to inhibit colonisation.

In conclusion, qPCR analysis of *P. placenta* mRNA in acetylated, DMDHEU-treated and thermally modified wood exposed to *P. placenta* showed that *P. placenta* was able to colonise these materials already during the first week of exposure, which had not been previously shown.

9.2 Objective 2 - Effect of acetylation, furfurylation and thermal modification on the kinetics of wood mass loss and the fungal growth pattern

The aim of this objective was to investigate the effect of acetylation, furfurylation and thermal modification on the growth pattern of *P. placenta*. The interpretation of the results were based on the assumption that fungi in solid substrates go through the same growth phases as fungi in liquid culture and this assumption was also experimentally tested. Growth was measured indirectly by mass loss measurements.

The mass loss data of paper I was plotted logarithmically (Paper I: Fig. 2). For untreated wood, it was possible to detect three different stages in the mass loss curve similar to the phases seen in liquid fungal cultures; the lag, logarithmic and stationary phase. Unfortunately, many of the samples in the lag phase had negative mass loss and did therefore not show in the logarithmic graph. For acetylated and furfurylated wood, there seems to be an increase in degradation rate up to approximately 150 days, however the rate is 100 times lower than in the logarithmic phase of fungi in the untreated wood. The logarithmic mass loss curve based on the mass loss data of the thermally modified wood specimens in paper III (unpublished, Fig. 6) resembled that of untreated wood, although thermally modified wood had a longer lag phase and a smaller slope in the logarithmic phase.

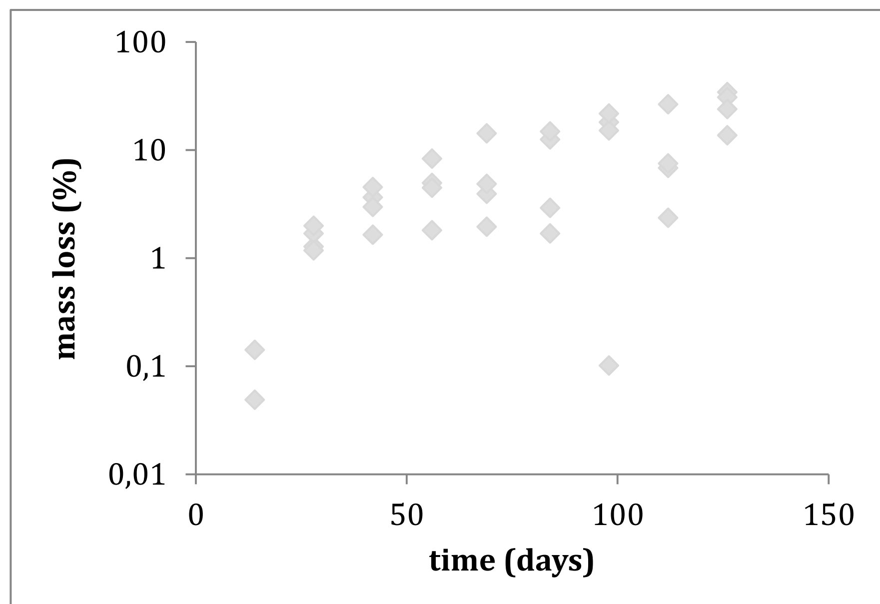

Figure 6. Logarithmic representation of the mass loss data for thermally modified wood in Paper III

The mass loss data for the acetylated and furfurylated wood in paper I, shows that at the treatment levels used in this experiment, less than 3% mass loss (on average) was lost during the prolonged exposure to *P. placenta* of 300 days. Single species laboratory decay tests with this time span and the harvesting frequency used in this work has not been previously reported for modified wood.

As described in section 3.2.1, microorganisms in a liquid culture go through three distinct growth phases; i) the lag phase where they adapt to the new environment, ii) the logarithmic phase where they grow exponentially and iii) the stationary phase where nutrient depletion and other factors inhibit exponential growth (Prescott et al. 1999; Baranyi and Roberts 2000; Madigan et al. 2000; Rolfe et al. 2012). *Penicillum chrysosporum* was shown to go through lag, logarithmic and stationary phase like phases when grown on a solid food substrate, while *Physisporinus vitreus* has been modelled to go through similar phases when growing in wood (Fuhr et al. 2011; Arquiza and Hunter 2014). No experimental studies on growth phases of wood degrading fungi growing in solid untreated or modified wood were found in the literature.

If the growth phases of microorganisms in liquid culture are applicable on filamentous fungi growing in wood, the lag phase might be equivalent to the time it takes for the fungi to adapt to the environment provided by the wood material. For example, the absence of glucose will lead to an up-regulation of genes involved in wood degradation, as described in section 3.2.1 (Aro et al. 2005; Martinez et al. 2009). At the end of the lag phase, the fungi might start the chelator mediated Fenton (CMF) degradation. No change in composition will be noticed, but there will be a change in structure (Fackler et al. 2010). Using CMF degradation, the fungi will depolymerise cellulose chains and hemicelluloses and modify lignin through induction of the Fenton reaction in which hydroxyl radicals are formed (Fenton 1894; Goodell et al. 1997; Arantes et al. 2012). The depolymerisation of the wood cell wall polysaccharides will lead to a loss in strength, which is noticeable before mass loss can be detected (Wilcox 1978; Winandy and Morrell 1993; Curling et al. 2002b; Fackler et al. 2010; Maeda et al. 2014). Once the CMF degradation has opened up the wood structure sufficiently, the enzymatic degradation machinery will start degrading the wood constituents and thus the fungi are now in the logarithmic phase. Enzymatic degradation will further reduce the strength of the wood while also causing mass loss (Curling et al. 2002b; Brischke et al. 2008; Fackler et al. 2010). When the fungi have degraded the wood material to such an extent that all available nutrients are depleted, the fungi has reached the stationary phase in which the degradation rate will flatten out.

In a liquid culture, fungal growth is measured as the change in fungal biomass. In wood, where the mycelium cannot be extracted, fungal biomass is determined by assays of cell constituents such as ergosterol, total extractable liquid phosphates, nucleic acids and chitin as well as indicators of biological activity such as guanosine triphosphate (GTP), enzyme and respiratory activities (Lena et al. 1994). To be reliable, an indicator of fungal biomass must correlate to mycelium increase and be independent of growth conditions. In a solid substrate, measuring the loss of mass of the substrate is an indirect measurement of the growth of the fungi (Mohebby et al. 2003; Verma et al. 2008).

The results obtained from the untreated wood supports the proposed model that depicts that filamentous fungi growing in wood go through a lag, logarithmic and a stationary phase (Paper I: Fig. 2) (Fuhr et al. 2011). It is, however, important to note that all measurements were done on the substrate and not on the fungi and therefore it is possible that the increase in fungal biomass does not show the same pattern as the decrease in the substrate mass.

Since the fungi seem to undergo the lag, logarithmic and stationary phases in untreated wood, it was assumed that they undergo these phases also in modified wood. The question was in which growth phase the fungi were in these experiments. The results based on the mass loss data for thermally modified wood in paper III, indicate that decay is initially inhibited by thermal modification, leading to a prolonged lag phase. Similarly, the degradation rate in the acetylated and furfurylated wood specimens were 100 times lower during the time period when mass loss increased than during logarithmic phase in untreated wood, which may suggest that the fungi in these specimens were in the lag phase (Paper I: Fig. 2). Based on current knowledge of the brown rot degradation process and chemical transport in wood cell walls (discussed in sections 2.1.1 and 3.2), it may be hypothesised that during the lag phase the fungi are either i) spreading into the wood specimen and finally reaching enough areas with sufficient moisture levels for degradation to become exponential, or ii) reducing the modification level using low molecular weight compounds and thus increasing the moisture level, presumably starting in areas with treatment levels below the proposed decay threshold (Thybring 2013). The question remains

why the increase in mass loss eventually flattens out and, of course, what kind of mass is lost. The samples were leached before inoculation and therefore there should be only small amounts of nutrients and non-polymerised modification chemicals in the lumen. The untreated as well as acetylated and furfurylated non-inoculated control specimens had a mass loss of 0.5-1% up to 23 weeks of incubation (Paper I: Fig. 1A). It is possible that this is at least partly due to evaporation of volatile substances. The mass loss seen in the modified specimens is however 2-3 times higher than in the non-inoculated controls.

Since the mass loss during this time frame slowly increased up to 5% in the thermally modified specimens and up to approximately 2% in the acetylated and furfurylated wood specimens (Fig. 6; Paper I: Fig. 2), it is also possible that the fungi in these materials were already in the logarithmic phase. If the fungi in the acetylated and furfurylated specimens are in the logarithmic phase during the time frame where the degradation rate is very low, the flattening out of the mass loss curve in Paper I: Fig. 2 may be due to that the fungi are beginning to die, due to e.g. starvation. Furthermore, if the fungi are in the logarithmic phase, the mass loss seen is probably wood cell wall hemicelluloses and celluloses degraded by CMF and enzymatic degradation. If the degradation takes place mainly in areas where the modification level is locally very low, the fungi may go into starvation when the low treatment areas have been mainly degraded.

The degradation rate in the thermally modified specimens in paper III increased after 20 days of exposure to *P. placenta* and appears to be exponential for the remainder of the experiment. The rate was, however, lower than for untreated wood in the logarithmic phase. A reduced degradation rate in the logarithmic phase may be due to a decrease in diffusion rate caused by lower moisture content and smaller pores and perhaps decreased enzyme efficacy caused by lack of hydrogen at the glycosidic bonds and impaired recognition of the wood polymers (Hill et al. 2005; Rowell et al. 2009; Zelinka et al. 2014). Furthermore, in chemically modified wood, a reduced degradation rate may be due to that the hydroxyl radicals also react with the modification chemicals.

In a complex solid material, such as wood, it is not unlikely that the fungi alternate between restricted and unrestricted (i.e. exponential) growth. This may be due to stepwise invasion in the longitudinal direction (Fuhr et al. 2011). In modified wood, it may also be due to that the fungi will degrade areas with locally lower treatment levels faster than ones with higher treatment levels. Maybe, *P. placenta* in the acetylated and furfurylated samples in this experiment degraded the low treatment areas exponentially during the first part of the decay experiment, but then went into a new lag phase when these areas were more or less degraded. In that case it would be possible that the fungi would have started exponential degradation of areas with higher treatment levels once it had overcome the inhibition by the modification and if the test had been run longer. This may be the case in the experiment with thermally modified wood in paper III, only here the transitions between the first logarithmic, the second lag and the second logarithmic phase were indistinctive.

In summary, *P. placenta* growing in untreated wood was shown to go through similar growth phases as fungi in liquid culture. A growth pattern divided into these growth phases has been modelled for a white rot fungus (Fuhr et al. 2011), but previous experimental evidence for growth phases in any wood degrading fungi could not be found in the literature. The growth phases of the fungi growing in the acetylated, furfurylated and thermally modified wood were

more difficult to interpret. They may suggest that wood modification causes a prolonged lag phase, the length of which may depend on the treatment level. It is also possible that the fungi oscillate between the lag and logarithmic phases, thus depleting the most easily accessible nutrients first and then adapting to be able to degrade nutrients less accessible. Furthermore, the results show that at the treatment levels used in paper I, acetylated and furfurylated wood lost less than 3% mass during the 300 day long exposure to *P. placenta*. Mass loss measurements over such long time periods and/or with such high frequency as in this work has not been previously reported for modified wood.

9.3 Objective 3 - Moisture conditions in modified wood prior to and during brown rot degradation

The aim of this objective was partly to confirm that EMC and moisture content after harvest in the specimens used were equivalent to specimens in comparable studies and hence that the decay tests were reliable, and partly to investigate the effect of acetylation, furfurylation, DMDHEU-treatment and thermal modification on these parameters. Equilibrium moisture content after acclimatisation in 20°C and 65% RH for three weeks was measured in all samples used in paper I and the moisture content at harvest was recorded during all decay tests.

For the specimens in paper I, EMC (at 65% RH, 20°C) in untreated wood was on average 11%, while in acetylated and furfurylated on average 3% and 5% respectively. The correlation between mass loss and EMC in acetylated wood was quite high (R^2=0.64). However, in furfurylated wood there was only a weak correlation between mass loss and EMC (R^2=0.31 (Fig. 7)).

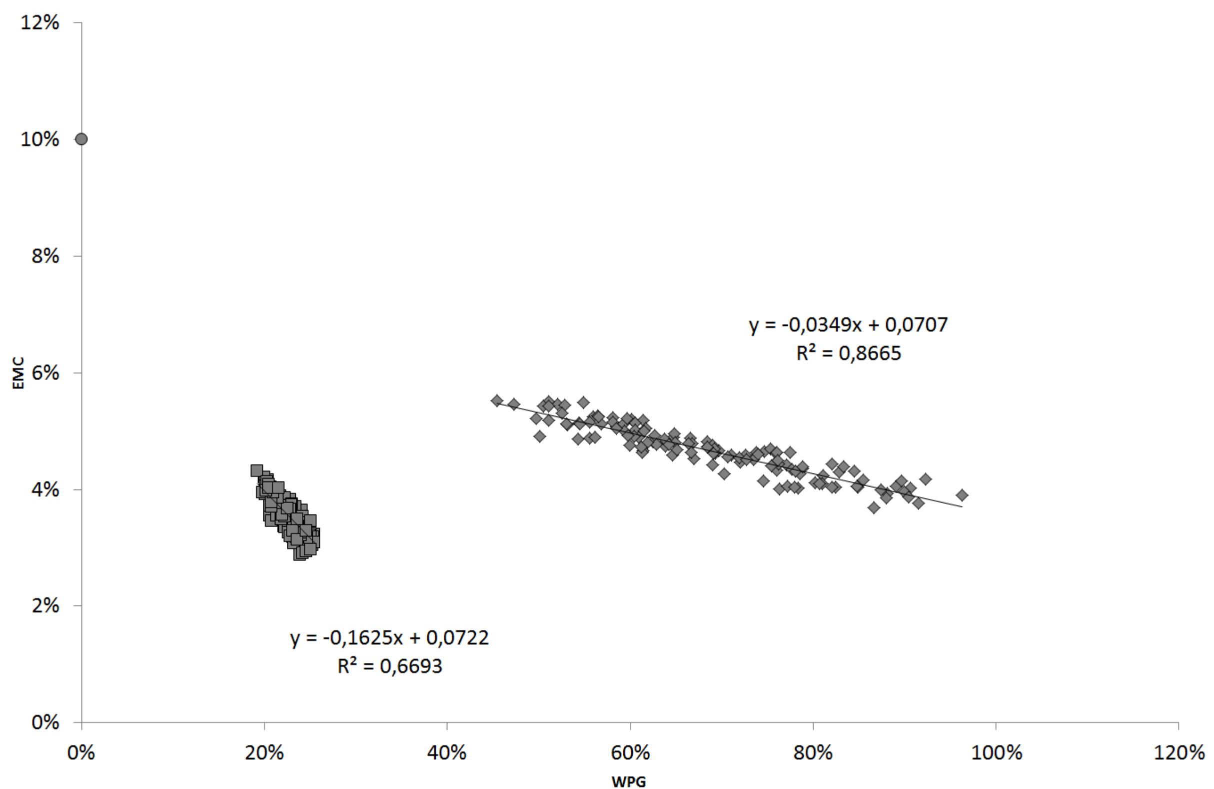

Figure 7. EMC in per cent of dry weight of acetylated (squares, left), furfurylated (diamonds, right) and untreated (circles) samples against weight per cent gain (WPG).

The moisture contents of the specimens in papers II was not published and are therefore shown in Fig. 8. Moisture content in the untreated specimens in papers I and III had an average moisture content of 49% over the whole decay test, ranging from 22% to 91%. The average mositure content one week after exposure was 40%. In paper II, the average moisture content over the whole decay test was 49%, ranging from 24% to 127%. In all three papers, moisture content in untreated wood increased with both mass loss and time.

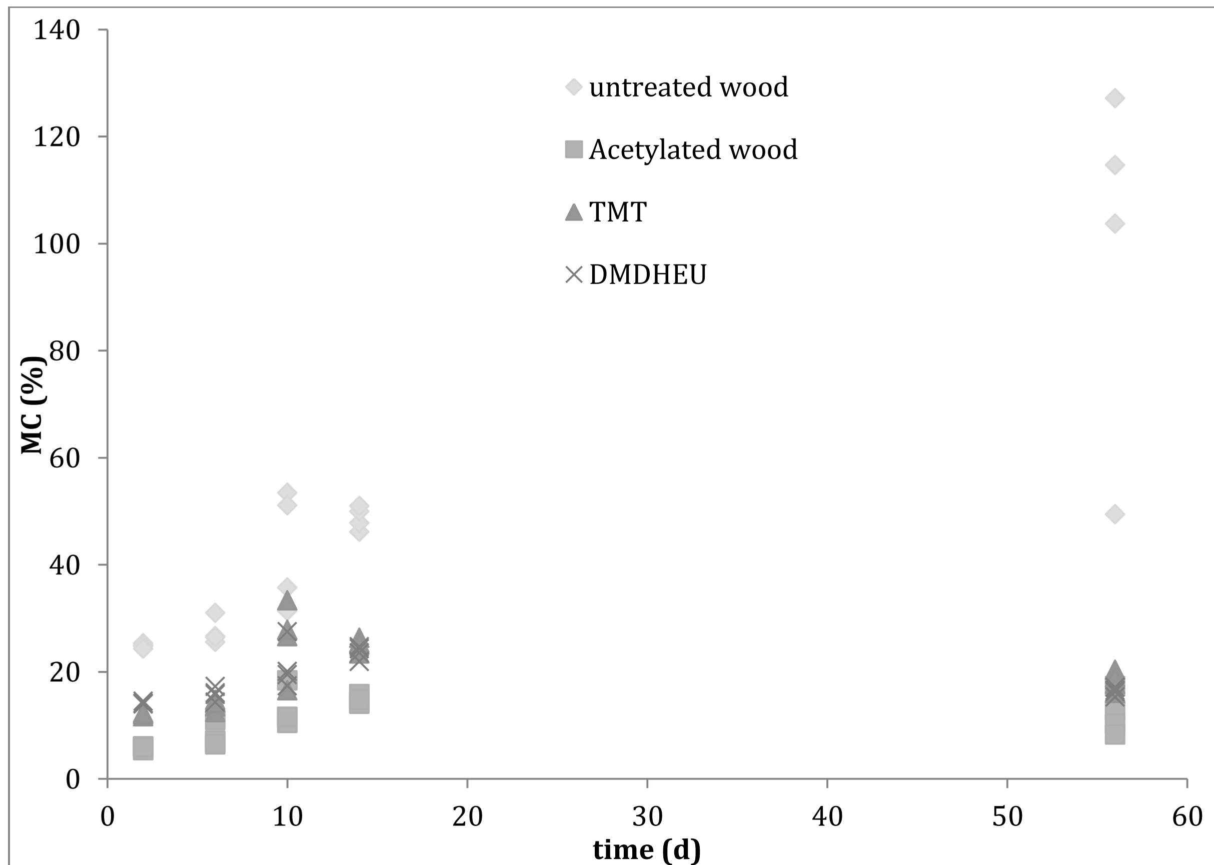

Figure 8. Moisture content (%) of untreated, acetylated, thermally modified and DMDHEU-treated wood exposed to *P. placenta* in an agar decay test (Paper II)

The moisture content in the modified wood specimens was lower than the untreated wood throughout all of the decay tests (Paper I: Fig. 1B; Paper III: Fig 2; Fig. 8). In paper I, acetylated and furfurylated wood had a constant moisture content throughout the decay test (300 days) with averages of approximately 25% over the period of the whole decay test, while in paper II the moisture content increased over time in acetylated, thermally modified and DMDHEU-treated wood. In both experiments, mass loss did in general not exceed 2%. Moisture content stayed lower in the thermally modified wood samples than in untreated wood up to 35% mass loss in the decay test in paper III (Paper III: Fig. 2). There seemed to be a lag in the moisture content increase in thermally modified wood up to 10% mass loss compared to the increase in moisture content in untreated wood at the same mass loss.

The above presented results on EMC in untreated and furfurylated wood are in accordance with results by Esteves et al. (2010), who found EMC at 65% RH in untreated wood to be 12.9% and in furfurylated wood (39.8% WPG) 7.3%. The correlation between WPG and EMC in modified wood materials has been extensively reported (Yasuda et al. 1994; Tjeerdsma et al. 1998; Rowell

et al. 2007; Lande et al. 2008; Thybring 2013). In the present study, the correlation between mass loss and EMC in acetylated wood was quite high, R^2=0.64, but in furfurylated wood the correlation was weak, R^2=0.31 (Fig. 7). At the WPG used in paper I, the wood cell wall was already saturated with poly(furfuryl alcohol) and the added furfuryl alcohol was accumulated in the lumen, which does not influence moisture content at 65% RH (Thygesen et al. 2010). Previous studies showed that at approximately 30% WPG, additional furfuryl alcohol will localise to the lumen due to that the wood cell wall is saturated with furfuryl alcohol (Thybring 2013).

In a previous study, moisture content in acetylated wood with approximately 20% WPG was 10-50% after four weeks and 5-70% after 28 weeks (Alfredsen and Pilgård 2014). Although the variation was higher, these results are in general in accordance with the results reported in paper I. Schmöllerl et al. (Schmöllerl et al. 2011) reported the moisture content in acetylated wood (23% WPG) to be approximately 45% after 2 weeks, 20% after 14 weeks and 15% after 26 weeks. During the same time period the moisture content in furfurylated wood (37% WPG) varied between 25-35%. However, in Schmöllerl et al. (2011) no water was added to the soil which may have caused the samples to dry out during the test.

Moisture content after decay was calculated from the wet and dry weight after harvest. This measurement quantifies the total amount of water inside the specimen, i.e. both in the wood cell wall and in the lumen. At the RH used in the decay tests in this thesis (70% +/- 5%), all of the moisture should theoretically be in the wood cell wall (Thygesen et al. 2010). However, during decay the moisture content may be locally very high due to water generation in the degradation process. The exact location of the water in the specimens is therefore unclear.

The moisture content in the wood samples was lower in the experiment using agar in paper II (Fig. 6), than in the experiments using soil (Paper I: Fig. 1B; Paper III: Fig. 2). This is probably mainly due to that in the soil decay tests, the fungi were added as liquid culture, while in the agar decay tests, the fungi were already growing on the agar and no liquid was added (see section 8.2.1). However, the moisture content of the non-inoculated control samples in paper I had higher moisture content than the untreated samples in figure 5 after 2 days of exposure, even though no liquid was added to the controls, which suggests that there might have been more reasons for that the specimens in the soil decay tests were wetter than the specimens in the agar decay test than just the inoculation method. In future tests, dry inoculation methods would be preferable.

The reason for the maintained moisture content in the modified wood specimens in paper I and the increasing moisture content in the modified specimens in paper II may again be due to the mode of inoculation. In the soil tests, the modified wood samples may already have been saturated with water through addition of liquid culture, while in the agar decay test the modified wood samples absorbed moisture from the surrounding air and/or water was transported into the samples by the fungi. This is supported by the fact that the moisture content of acetylated wood in paper II is 5% at 2 days of exposure, while moisture content of acetylated wood in paper I is 21-57% after 6 days of exposure.

It is possible that the samples had dried somewhat before harvest, why moisture levels may have been higher when degradation actually occurred. However, this should not have affected the results during the first two weeks in the decay test in paper II, and in the decay tests in

papers I and III, the soil plates were watered regularly to minimize drying of the samples. The minor increase in moisture content between 14 and 56 days in untreated wood in the paper II experiment and the fact that all modified wood samples had a moisture content below 20% (Fig. 6), indicate that the agar plates were starting to dry out even though the test was only run for eight weeks. The suitability of agar plates also in short tests could therefore be questioned. A substrate possible to re-wet, such a soil, or perhaps containers with less moisture leakage may solve the problem.

The large span in EMC in acetylated and furfurylated specimens highlights the difference in treatment levels in the modified specimens and the magnitude of its effect. A more homogenous set of specimens, based on anatomical features, and treatment levels would probably have decreased the variation in EMC and would probably also have reduced the variation in mass loss (Fig 7; Paper I: Fig. 1A; Paper III: Fig. 1). The poor correlation between EMC and WPG in the furfurylated specimens may be due to that at the WPGs used in this study, the wood cell wall was already nearly saturated with poly(furfuryl alcohol).

In all experiments, the standard deviation of the moisture content was quite large. As an example, moisture content in acetylated samples harvested after 6 days of exposure to *P. placenta* ranged between 21% and 57% (Paper I: Fig. 1B). The high variation between the replicates makes the mean value less representative of the true mean. However, the number of observation points was large and a clear trend for each treatment is detectable. The observed variation in untreated wood was likely mainly affected by differences in mass loss between replicates, while in modified wood samples (that had lost no or little mass) the variation was probably mostly representing variable treatment results. In the thermally modified wood samples in paper III, the variation was probably a combination of both.

The lag in moisture content increase in the thermally modified wood in paper III may be due to the cross-links between lignin and polysaccharides formed during thermal modification that reduces the swelling capacity of the wood (Weiland and Guyonnet 2003; Hakkou et al. 2005; Boonstra and Tjeerdsma 2006). Perhaps there are still enough cross-links in thermally modified wood up to 10% mass loss to inhibit swelling. However, water is also produced as a bi-product in the degradation, which means that if the net moisture content of thermally modified wood stays constant during degradation up to 10% mass loss, water should in theory be forced out of the sample. But again, these are probably quite small amounts.

In Fig. 9, the moisture content values from paper I and III are plotted against mass loss. 15% (red) and 20% (green) moisture content are marked with horizontal lines and 1% (red) and 3% (green) mass loss are marked with vertical lines. In untreated wood, no samples with moisture content below 20% had a mass loss over 1%. However, for furfurylated, acetylated and thermally modified wood there are specimens with moisture content below 20% that have a mass loss of more than 1%. Even if the limit for decay considered in EN 113 of 3% mass loss is applied here, there are samples with moisture content below 20% that according to the standard are being degraded (EN 113 1996). However, very few samples show mass losses of more than 1% at a moisture content below 15%. Comparison with untreated wood at this low moisture content could unfortunately not been made since no untreated samples had a moisture content below 15%. The results in Fig. 9 indicate that *P. placenta* degradation can occur at a lower moisture content than previously believed (Cardias Williams and Hale 2003; Hill and Ormondroyd 2004; Hill 2009; Thybring 2013). They also imply that the proposed diffusion

threshold of approximately 15% may be limiting in degradation of modified wood while other parameters are more important in untreated wood.

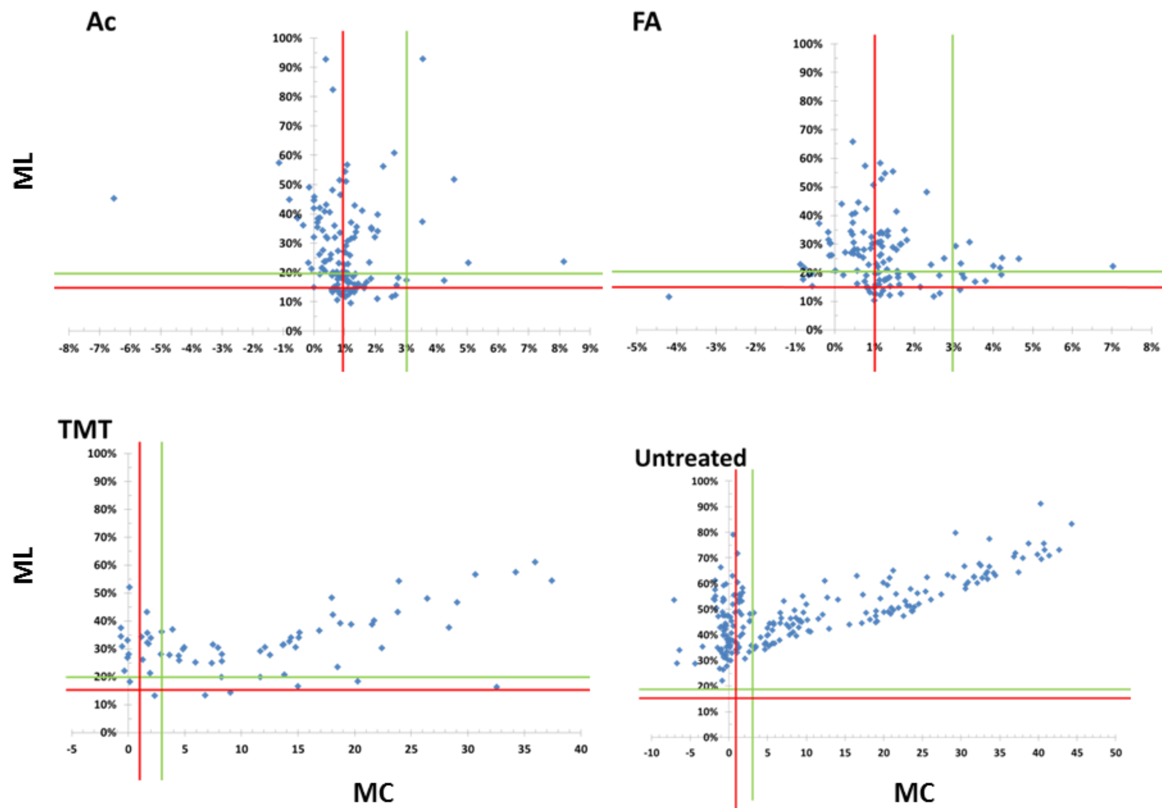

Figure 9. Moisture content (MC) in acetylated (Ac), furfurylated (FA, thermally modified (TMT) and untreated wood plotted against mass loss (ML). Vertical lines mark 1% (red) and 3% (green) mass loss and horizontal lines mark 15% (red) and 20% (green) moisture content. (Data from papers I and III)

In summary, these results show that the EMC/WPG ratio of the modified wood materials in paper I and the moisture content after decay of both untreated and modified wood specimens in all decay tests were in agreement with previous results and hence the decay tests may be considered reliable. Furthermore, the results indicate that degradation may occur in modified wood at lower moisture contents than previously believed.

9.4 Objective 4 - Ability of hydroxyl radicals and cellulases to degrade polysaccharides in modified wood

The aim of this objective was to investigate whether hydroxyl radicals and fungal cellulases are able to depolymerise and hydrolyse acetylated and furfurylated wood meal. This experiment was an *in vitro* study mimicking i) CMF degradation, in which polysaccharides were depolymerised using Fenton's reagent (hydrogen peroxide and an Fe^{2+} salt), ii) enzymatic degradation, in which polysaccharides were degraded using fungal cellulases and iii) the whole brown rot degradation process by first treating the wood meal used with Fenton's reagent and subsequently with fungal cellulases. To analyse the amount of damage caused by the different treatments, the amount of reducing sugars released to the supernatant was measured using DNS (see section 8.3).

For all wood treatments as well as untreated wood, treatment with only Fenton's reagent gave the lowest amount of reducing sugars released to the supernatant, treatment with only cellulases a medium amount and the combined treatments the highest amount (Paper IV: Fig. 1). The amount of reducing sugars released from the acetylated wood was lower than from untreated wood for all treatments (Paper IV: Fig. 1).

When treating furfurylated wood with Fenton's reagent, the supernatant turned brown after treatment with DNS and showed extremely high values in the spectrophotometer (Paper IV: Fig. 2). However, fungal cellulases were shown to be able to degrade furfurylated wood.

The same experiment has been performed by Verma and Mai (Verma and Mai 2010) on DMDHEU-treated wood meal. In their study, fungal cellulases were shown to be able to degrade DMDHEU-treated wood meal without pre-treatment, but since cellulase efficacy was increased by treatment with Fenton's reagent, the authors concluded that Fenton derived hydroxyl radicals were able to depolymerise polysaccharides in DMDHEU-treated wood. The ability of fungal cellulases to degrade furfurylated wood has previously been demonstrated by Venås (Venås 2008). The ability of fungal cellulases to degrade polysaccharides in acetylated wood as well as the ability of hydroxyl radicals to depolymerise polysaccharides in acetylated and furfurylated wood has not previously been reported.

The components needed for the Fenton reaction to occur, were all in the supernatant in the experiment in paper IV. Due to the reactivity of these compounds, the Fenton reaction presumably occurred in the supernatant outside the wood particles. This leads to that the hydroxyl radicals probably mainly reacted with wood constituents on the surface of the wood particles. Furthermore, this experiment does not say anything about the ability of the reagents to diffuse through the wood cell wall or to react inside the wood cell wall. The only conclusion that can be drawn is that if hydroxyl radicals would be formed inside the wood cell wall of modified wood *in vivo*, they would be able to degrade the polysaccharides. The same can be assumed for the cellulases, since they are too big to be able to penetrate the intact wood cell wall (Cowling 1975; Murmanis et al. 1987; Flournoy et al. 1991). In the combined treatment they may have been able to diffuse a little further into the wood particles than in the treatment with only cellulases, since the Fenton reaction may have been able to open up the wood cell wall to some extent.

The DNS analysis was designed to measure reducing sugars (Miller 1959), i.e. the end sugar of a polysaccharide. This measurement will show the number of polysaccharides in the sample and give an estimation of the number of cleavages caused by the fungi. However, the reducing sugar is an aldehyde and DNS can react with other aldehydes as well. Treatment of furfurylated wood with Fenton's reagent apparently caused release of other aldehydes. To circumvent the problem with other types of aldehydes released, another analysis method needs to be used. FT-IR was tried as an alternative method on the remaining wood meal (measuring the glycosidic bonds and hemicelluloses in relation to lignin according to Fackler et al. (2010)), but did not have sufficient resolution for this experiment.

In the experiment reported in this thesis as well as in Verma and Mai (Verma and Mai 2010), treating the wood meal with only with Fenton's reagent did not cause much degradation, which is in accordance with the theory that the hydroxyl radicals merely depolymerise the polysaccharide chains. The reason for that reducing sugars were released at all is probably due

to the fact that the concentration of hydroxyl radicals may have been higher in this experiment than it is in vivo.

Treating acetylated wood meal with fungal cellulases showed that these are able to hydrolyse polysaccharides in acetylated wood (Paper IV: Fig. 1). Treating with Fenton's reagent followed by fungal cellulases, which increased the cellulase efficacy compared to treatment with only cellulases, indicated that the polysaccharides in modified wood can be depolymerised by hydroxyl radicals. However, the levels of reducing sugars released into the supernatant were still lower than in untreated wood after both treatments. The same was shown for DMDHEU-treated wood (Verma and Mai 2010). It seems possible that lower levels of released reducing sugars with Fenton's reagent are due to the hydroxyl radicals reacting also with the modification chemical(s). When treating modified wood meal with cellulases, lower levels of released reducing sugars may be due to the acetyl groups being physically hindering the access of the cellulases to the polysaccharides. It could thus be that i) the acetyl groups are only blocking a part of the hydrolysis sites while the accessible parts of the polymers may still be hydrolysed or ii) the acetyl groups does not inhibit hydrolysis completely but only makes it less efficient and thus slower. The increased efficacy of the cellulases seen after pre-treatment with Fenton's reagent may then (partly) be due to de-acetylation of the polysaccharides by action of the hydroxyl radicals. This aspect may be investigated by measuring release of acetyl into the supernatant with e.g. gas chromatography.

As described in section 8.3, we concluded that the reason for the high levels of aldehydes in the supernatant after treatment of furfurylated wood with Fenton's reagent was probably that poly(furfuryl alcohol) had been degraded by the hydroxyl radicals into furfurals. However, we were able to show that furfurylated wood could be hydrolysed by fungal cellulases, which supports previous studies by Venås (Venås 2008).

In summary, the experiment in paper IV showed that fungal cellulases were able to degrade acetylated wood, as had previously been shown only for DMDHEU-treated and furfurylated wood (Venås 2008; Verma and Mai 2010). Furthermore, the results indicate that Fenton derived hydroxyl radicals were able to depolymerise polysaccharides also in acetylated wood, as previously shown for DMDHEU-treated wood (Verma and Mai 2010). Unfortunately, the same could not be shown for furfurylated wood due to a presumed release of furfurals into the supernatant. However, these results instead suggest that the poly(furfuryl alcohol) was itself degraded by the hydroxyl radicals, an aspect insufficiently investigated in the literature.

9.5 Objective 5 – Effect of wood modification on *Postia placenta* gene expression

The aim of this sub-objective was to study *P. placenta* expression of genes involved in wood degradation when growing on modified wood materials in order to investigate i) how early after inoculation expression of these genes could be detected and ii) whether the differences previously seen were due to samples having been in different stages of decay. To this end, *P. placenta* gene expression was studied in acetylated, DMDHEU-treated and thermally modified wood with emphasis on the first two weeks of exposure, in paper II. In paper III, gene expression was studied in thermally modified wood and the results were compared to untreated wood with approximately the same level of mass loss.

Gene expression was analysed using qPCR of selected genes, as described in section 8.2.5. The genes were selected based on which genes had been studied in previous research, to facilitate

comparison (Alfredsen and Fossdal 2010; Schmöllerl et al. 2011; Pilgård et al. 2012). In these studies, the genes had been selected based on the knowledge of what genes are needed or thought to be needed during CMF and enzymatic degradation and the primers were designed using the sequenced genome available at http://www.jgi.doe.gov/Postia (Martinez et al. 2009; Alfredsen and Fossdal 2010; Pilgård et al. 2012).

In paper II, expression of genes presumably involved in CMF degradation (alcohol oxidase Ppl118723 and QRD Ppl124517) and enzymatic degradation (β-glucosidase Ppl 112501 and endoglucanase Ppl103675) was analysed in acetylated, DMDHEU-treated, thermally modified and untreated SYP sapwood at 2, 6, 10, 14 and 56 days of exposure to *P. placenta*. The results showed that genes involved in CMF degradation (especially alcohol oxidase) were expressed at higher levels in the modified wood materials than in untreated wood already during the first two weeks of exposure, but most significantly at 14 days. The genes involved in enzymatic degradation on the other hand, were expressed at similar levels as in the untreated wood, except for at 56 days where the expression of these genes was up-regulated in the untreated wood. However, it should be noted that the samples were compared after the same time of exposure, which means that after 14 days the modified and untreated wood samples were in completely different stages of decay.

In the experiment in paper III, no significant differences could be detected in alcohol oxidase (Ppl118723) and laccase (Ppl111314) gene expression between thermally modified wood and untreated (Paper III: Fig. 4). The median value of expression of QRD (Ppl124517) was 10-100 times higher in thermally modified wood samples than in untreated samples with negative mass loss, but there was no significant difference due to large variation of the measured data. At 1.5% mass loss expression of QRD was only 3 times as high in the thermally modified wood samples as in the untreated, but this difference was statistically significant. β-glucosidase (Ppl112501) and endoglucanase (Ppl103675) had similar gene expression levels all through the experiment. There were neither significant differences between time points or treatments nor signs of trends.

In previous studies of fungal gene expression in modified wood, gene expression was analysed at time points from 14 days on and compared with the expression in untreated wood after the same time of fungal exposure (Alfredsen and Fossdal 2010; Schmöllerl et al. 2011; Pilgård et al. 2012). The results indicated that genes involved in CMF degradation were up-regulated in the modified wood materials while genes involved in enzymatic degradation was not consistently affected by the wood modification. However, since the modified wood materials in these studies had no or little mass loss even after exposure for up to 36 weeks, while the untreated wood had lost 50% mass after only approximately 8 weeks, the compared samples were in different stages of decay.

In the gene expression studies in this thesis as well as in previous studies, the expression of only a limited number of genes has been analysed. To be able to draw solid conclusions on the expression of genes involved in CMF end enzymatic degradation, more genes need to be analysed. To help choosing more genes, a transcriptome sequencing using high-throughput sequencing could be of value. This method identifies and quantifies all expressed mRNAs at a given time-point. Drawbacks are that next-generation sequencing is a much more costly and time-consuming method. High-throughput sequencing may therefore rather be used to screen

for interesting genes that subsequently can be analysed in more detail with qPCR (e.g. doing a time series).

The results from papers II and III show that at least some genes presumed to be involved in CMF degradation are expressed in modified wood during the first two weeks of exposure; detection of expression of some of these genes could be made already after two days. High expression of genes involved in CMF degradation in spite of no or little mass loss indicates that modified wood inhibits a mechanism in brown rot wood degradation that is downstream of gene expression. However, in theory, CMF degradation may still occur even without detectable mass loss. This would nevertheless affect other properties of the wood, such as strength. Brown rot gene expression during the first two weeks of growing on modified wood has not previously been reported.

The irregular expression pattern of QRD both along time and across treatments led us to question the role of this protein in *P. placenta*. *G. trabeum* has two QRDs, of which one has been suggested to be involved in quinone reduction during wood decay while the other may be involved in stress defence (see section 3.2.2.4) (Cohen et al. 2004). We therefore suggested in paper II that the induction of QRD seen in the modified wood materials could be the result of some kind of stress caused by the wood modification. In paper III, QRD was the only one of the CMF related genes studied that was expressed at considerably higher levels in thermally modified wood than in untreated wood, which supports the theory of a different role for this protein. Wood modification affects moisture content, nutrient availability and perhaps also other parameters important for the well-being of the fungi and may therefore induce a stress response (Militz 1993; Rowell et al. 1994; Militz 2002; Hakkou et al. 2006; Schmidt 2006). Whether this is true also for the *P. placenta* QRD could be investigated by subjecting *P. placenta* to the same kinds of stress conditions used by Cohen et al. (2004) and measuring its gene expression profile.

In conclusion, the expression of genes involved in wood degradation was seen already during the first week of exposure in acetylated, DMDHEU-treated and thermally modified wood, which has not been previously shown. The previously reported induction of CMF related genes could not be seen for laccase and alcohol oxidase in thermally modified wood when compared to untreated wood in the same stage of decay (paper III) while genes involved in the enzymatic degradation were expressed at similar levels in both modified and untreated wood, just as previously reported (Alfredsen and Fossdal 2010; Schmöllerl et al. 2011; Pilgård et al. 2012).

9.6 Objective 6 - Ability of brown rot fungi to degrade acetyl in acetylated wood

The aim of this sub-objective was to investigate the ability of *P. placenta* to degrade acetyl in acetylated wood. The amount of acetyl groups was determined in wood meal from acetylated wood samples that had been exposed to *P. placenta* for different amounts of time (by means of an aminolysis with pyrrolidine and subsequent GC analysis, as described in section 8.2.4 and paper I).

Paper I: Fig. 4 shows the loss of acetyl during fungal exposure over time and the acetyl content in the non-inoculated control. Both inoculated and non-inoculated specimens had an apparent loss of acetyl of 3%. No significant difference between any of the samples could be detected. However, the non-inoculated control samples showed on average 3% loss of acetyl and had very large standard deviation.

Decay resistance as well as other properties gained by wood modification is dependent on the treatment level (Lande et al. 2004; Rowell et al. 2009; Verma et al. 2009). If wood degrading fungi are able to degrade the wood modification chemical itself, the modification level would locally be lower and may eventually allow degradation to occur, thereby affecting the service life of the product. In paper IV, we observed that furfurylated wood meal treated with Fenton's reagent turned the supernatant dark brown and analysis of the supernatant with DNS showed a high content of aldehydes (section 9.4). It was hypothesised that this may be due to the poly(furfuryl alcohol) having been degraded to furfurals. No further investigations of this peculiarity were made in this study and no equivalent studies on any other kind of modified wood, *in vitro* or *in vivo*, has been reported in the literature.

Measuring acetyl with gas chromatography is a destructive method and therefore initial acetyl content (before exposure to the fungus) was calculated from WPG using a standard curve of four samples with known WPG and acetyl content. The R^2-value was 0.98% and the equation of conversion was therefore considered reliable. However, the apparent loss of acetyl of 3% within the first week of exposure even though acetyl levels were maintained throughout the rest of the experiment indicates that the equation may have given values a little high. The fact that the calculation also led to an apparent loss of acetyl of 3% in average in the non-inoculated controls further strengthens this hypothesis.

The results in Paper I: Fig. 4 indicate that acetyl groups in acetylated wood were not degraded during the time course of the experiment. The absence of degradation of acetyl in the acetylated wood, may suggest that CMF degradation did not occur in these samples or that the hydroxyl radicals were not able to degrade the ester bonds connecting the acetyl groups to the wood polymers. On the other hand, the results show that the acetyl content of the acetylated wood was maintained during at least 120 days of exposure to *P. placenta*. Decrease in treatment level during prolonged exposure to *P. placenta* may therefore not be a problem in acetylated wood and may help to explain the long period of maintained decay resistance reported for this material when treated at high levels (Larsson Brelid et al. 2000).

In conclusion, the acetyl measurements in paper I indicate that *P. placenta* was not able to degrade acetyl in the acetylated specimens in this experiment. Furthermore, the results show that the acetyl content was maintained throughout the prolonged exposure to *P. placenta*. The *in vivo* ability of brown rot fungi to degrade modification chemicals in modified wood has not been previously reported.

9.7 Objective 7 - Ability of brown rot fungi to affect structural integrity in modified wood

The aim of this sub-objective was to investigate the effect of acetylated, furfurylated and thermally modified wood on the structural integrity during exposure to *P. placenta*. Structural integrity was measured using the HEMI-test as described in section 8.2.3.

In untreated wood, structural integrity decreased with exposure time throughout the experiment (Paper I: Fig. 3A). Loss of structural integrity was 10% after one week of exposure and at negative mass loss, 21% at 1% mass loss, 50% at 7% mass loss, and 98% at 34% mass loss, compared to the zero-time sample. The non-inoculated control sample, incubated under the same conditions as the inoculated samples for 14 and 29 days, had a loss of structural integrity of 6%. Structural integrity showed good correlation to mass loss (R^2=0.89) (Paper I: Fig. 3B).

Modification treatments caused loss of structural integrity in the samples. Acetylation decreased resistance to impact milling by approximately 15%, furfurylation by approximately 50% and thermal modification by 28% compared to untreated wood (Paper I: Fig. 4A, Fig . 4B; Paper III: Fig. 3). Average decrease in structural integrity over the whole decay test in paper I was 9.5% in the acetylated wood and 2.3% in the furfurylated wood compared to the zero-time samples (paper I). The structural integrity results for both materials showed correlation with neither time nor mass loss (R^2<10%) and had a slope between 0 and -1. Structural integrity in thermally modified wood during *P. placenta* decay had good correlation with both time and mass loss (R^2=0.77 and R^2=0.91 respectively) (paper III) and slopes of -0.2 and -1.8 respectively. Loss of structural integrity in thermally modified wood reached 14% after 56 days of exposure to *P. placenta* and 4.9% mass loss. The untreated and furfurylated non-inoculated control samples had on average 6.3% and 2.4% loss of structural integrity respectively.

In previous studies, furfurylation of pine sapwood decreased resistance to impact milling with 16%, but the WPG was only 15.6% (Brischke et al. 2012). Linear extrapolation of the results in Brischke et al. (2012) gives approximately 65% structural integrity at 70% WPG; however whether this correlation may not be linear. The decrease in structural integrity seen in the thermally modified wood was similar to previous results by Rapp et al. (2006).

Strength loss before mass loss is a well-known characteristic of brown rot degradation in untreated wood (Wilcox 1978; Winandy and Morrell 1993; Curling et al. 2002a; Brischke et al. 2008; Fackler et al. 2010; Maeda et al. 2014). Strength is lost when cellulose and hemicelluloses are depolymerised by e.g. CMF degradation (Kleman-Leyer et al. 1992; Winandy and Morrell 1993; Goodell 2003). Even though several studies show that no or very little mass loss occurs in modified wood materials with moisture exclusion efficiency above 40 % (Thybring 2013), strength loss due to fungal exposure in modified wood has not been investigated. Whether strength loss precedes mass loss also in modified wood materials is therefore not known.

All four specimens from the same time-point and treatment were run in a single test run; hence, the values are mean values and standard deviation and significance could not be calculated. Looking at the structural integrity data correlated to mass loss in Paper I: Fig. 3B one can see large standard deviation in mass loss in both acetylated and untreated wood. If the two untreated samples with largest standard deviation were excluded, remaining samples do not overlap and show a trend in which structural integrity decreases with mass loss. If the two samples were not removed, the mean values of all untreated samples show the same trend. In acetylated wood, more samples overlap each other. On the other hand, there is not considerable difference in strength loss either. In furfurylated wood, there is no considerable standard deviation in mass loss. In future studies, it is important to run samples with similar mass loss together when performing the HEMI test. Since four miniblock samples are needed for a single run of the HEMI test, more replicates would also allow for replicate runs and hence provide the possibility to calculate mean values and standard deviation.

For untreated wood, structural integrity of the samples with negative mass loss was lower than in the zero-time sample, which indicates changes in the chemical structure such as depolymerisation of cellulose caused by CMF degradation (Paper I: Fig. 3B). However, it is also possible that a smaller mass than the increase of fungal mass in the specimens was degraded. The large variation in both WPG and mass loss in the modified wood samples makes it impossible to find significant differences in structural integrity for these materials. However, the

specimens are randomly distributed and hence it is unlikely that a considerable loss of structural integrity over time would be masked by an increasing mean WPG. Therefore, we conclude that the acetylated and furfurylated specimens probably lost no or little structural integrity during exposure to *P. placenta*, which would suggest that CMF degradation may not have occurred. Furthermore, these results support the theory that the fungi in both acetylated and furfurylated wood were in the lag phase. The fact that the non-inoculated control specimens (incubated for 56 and 154 days) had similar structural integrity as the inoculated modified specimens further supports this theory. The thermally modified wood in paper III had similar loss of structural integrity up to 5% mass loss (where the degradation rate was low) as the untreated wood that had negative mass loss and thus were assumed to be degraded by CMF degradation.

In summary, these results indicate that structural integrity is not lost in acetylated and furfurylated wood while no or little mass was lost (paper I), while in thermally modified wood the structural integrity was similar to that in untreated wood once both materials started to lose mass (paper III). Structural integrity of acetylated, furfurylated and thermally modified wood materials during brown rot degradation has not previously been reported for as long a time period and with as frequent harvesting as in these experiments.

9.8 Objective 8 – Which brown rot degradation mechanism(s) is inhibited by wood modification?

The aim of this sub-objective was to discuss which mechanism(s) in the brown rot degradation machinery that might be inhibited in modified wood based on the findings of this thesis and in the literature.

Colonisation and transcription of genes involved in wood degradation have already been discussed in this thesis and they were found not to be inhibited by wood modification (Paper II: Fig 2; Paper III: Fig. 4). Translation of mRNAs into proteins involved in the enzymatic degradation and their secretion have been investigated by others (Elisashvili et al. 2012; Irbe et al. 2014) and also been found not to be inhibited. Translation and secretion of protein involved in CMF degradation has not been investigated so far.

If the wood modification does not inhibit colonisation or a mechanism inside the fungus as described above, it must inhibit a mechanism outside the fungus. Even though cellulases are known to be secreted in modified wood, they are not able to penetrate an intact wood cell wall (Cowling and Brown 1969; Koenigs 1974; Schmidt et al. 1981; Flournoy et al. 1991), and therefore the inhibition must in that case target the CMF degradation. The pores in wood cell walls are reduced in number and, in some cases, in size by wood modification but CMF metabolites have been estimated to be able to penetrate the wood cell wall of acetylated wood (Hill et al. 2005; Dieste et al. 2009a; Zauer et al. 2013; Kekkonen et al. 2014). It is however possible that a reduction in the size of pores in the wood cell wall may slow down the process.

To be able to reach the inner parts of the wood cell wall where the first signs of degradation are seen, the CMF metabolites need to diffuse through a liquid or at least gelatinous medium into the microvoids of the wood cell wall (Zelinka et al.; Fackler et al. 2010; Jakes et al. 2013). So far, the ability of CMF metabolites to diffuse through a modified wood cell wall has not been investigated. The reduced EMC in chemically modified wood may either directly impede the formation of an aqueous percolating network, or indirectly affect the formation of a network of plasticised hemicelluloses by changing the temperature and/or RH conditions at which the

hemicelluloses plasticise (see sections 2.2.1 and 4.2) (Zelinka et al. 2008; Jakes et al. 2013). In the case of thermally modified wood, it is also possible that a percolating network of plasticised hemicelluloses may not form due to that the majority of the hemicelluloses have been degraded (Hakkou et al. 2006; Paul et al. 2006; Boonstra et al. 2007). In acetylated wood and DMDHEU treated wood, the hemicelluloses are modified and may therefore have an altered plasticisation temperature and/or RH level and may not plasticise under normal conditions (Yasuda et al. 1994; Krause et al. 2003). However, the most likely cause for change in the plasticisation parameters for hemicelluloses in modified wood materials is that the relationship between the RH and the moisture content changed. Therefore, at a certain RH, the moisture content of the modified wood material is lower than that of untreated wood. Hence, a higher relative humidity might be required for the plasticisation of hemicelluloses in modified wood materials than in untreated wood.

However, acetylated wood in ground contact, whose cell walls in theory should be saturated with water, is still not degraded for a very long time (Larsson Brelid et al. 2000). The question is how much water is inside the wood cell wall in these samples. If the moisture content of the wood cell wall is still insufficient for diffusion, this would be a possible explanation for decay resistance. Furthermore, the moisture content of most saturated modified wood materials is 20-23%, which is similar to the moisture content at which untreated wood is usually said to be degraded (Cardias Williams and Hale 2003; Hill and Ormondroyd 2004; Thybring 2013). However, the moisture content threshold for chemical transport in the wood cell wall was concluded to be as low as 16%, maybe lower for some molecules (Zelinka et al. 2014). The moisture content threshold for the CMF metabolites has, on the other hand, not been investigated. It is theoretically possible that these have a higher threshold than the ions tested in Zelinka et al. (2014). The reason for the higher moisture content needed for decay in untreated wood may also be related to the moisture content in the lumen needed for colonisation. The results shown in Fig. 9 (see section 9.3) support this theory.

It is interesting to note that heartwood durability has been suggested to be due to both high density and extractives with iron chelating abilities, as described in section 2.2.2 (Eaton and Hale 1993; Chen et al. 2014). The iron chelators presumably binds iron ions and thereby inhibits the Fenton reaction, just as the Fenton reaction would be inhibited if the diffusion was inhibited by the wood modification.

As discussed above, *in vitro* studies have shown that if hydroxyl radicals would be formed inside the wood cell wall of modified wood, they would be able to degrade its polysaccharides. However, whether hydroxyl radicals are formed during the lag phase of the brown rot degradation has not been investigated. The reactions leading up to the Fenton reaction, i.e. when iron is sequestered by oxalic acid and subsequently reduced by fungal iron reductants, are pH sensitive (Arantes et al. 2009b). In theory it is possible that the CMF metabolites are able to diffuse through the wood cell wall, but are inhibited to react inside it due to altered pH. The pH is important for the reaction in which insoluble Fe^{3+}, located in the lumen or bound to the wood components, is sequestered by oxalic acid, transported into the wood cell wall and then reduced to Fe^{2+} by fungal iron reductants (Arantes et al. 2009). The pH was shown to be affected in acetylated wood (Larsson-Brelid 1998); however, recent unpublished results from our lab indicate that pH may not be altered very much at all in modified wood. As an alternative mechanism to inhibit the Fenton reaction, Xie et al. (2014) discussed the possibility that the modified lignin in modified wood does not support the subsequent and continuous reduction of

iron, which has been suggested to be an important part of brown rot degradation (Arantes et al. 2012). If the inhibition of brown rot degradation conferred by wood modification is not moisture dependent, the correlation between EMC and decay resistance in chemically modified wood may not be due to causality, but instead increased EMC and increased decay resistance may be caused by increased WPG through different mechanisms. Furthermore, it has not been established whether oxalic acid, hydrogen peroxide and/or the fungal iron reductants are found in the wood cell wall in modified wood during *P. placenta* decay or decay by any other brown rot.

When the wood cell wall has been sufficiently opened up by the CMF degradation, the enzymatic degradation will begin (Cowling and Brown 1969; Koenigs 1974; Schmidt et al. 1981; Flournoy et al. 1991). The results in this thesis have shown that acetylated wood can be degraded by fungal cellulases although less effective (Paper IV: Fig. 1). Together with previous results showing that also DMDHEU-treated and thermally modified wood can be hydrolysed by fungal cellulases (Venås 2008; Verma and Mai 2010), these results show that wood modification does not inhibit the enzymatic degradation but may make it less effective. Apart from experimental evidence, the brown rot wood degradation process indicates that the enzymatic degradation process can at least not be the only mechanism inhibited by wood modification, otherwise there would be substantial amounts of strength loss in the modified wood even though there is no or little mass loss. With the same line of reasoning, the loss or modification of accessible nutrients such as hemicelluloses cannot in itself confer decay resistance. Furthermore, hemicelluloses are degraded in the same manner as cellulose; CMF degradation followed by enzymatic hydrolysis (Arantes and Milagres 2006; Fackler et al. 2010; Arantes et al. 2011). Cellulases are constitutively expressed at a low level and are upregulated when the fungus starts to take up their metabolites, which shows that the degradation of hemicelluloses is not necessary for the expression of cellulases (Aro et al. 2005). Additionally, it seems unlikely that a microorganism would not degrade one particular nutrient in the absence of another one. Most microorganisms, including fungi, can degrade many different types of nutrient sources (Wainwright 1988).

An extension of the question of what mechanism in the brown rot decay process is inhibited by wood modification is for how long it is inhibited. Hill et al. (2005a) discussed the idea of a decay protection threshold for modified wood, and concluded that the longer the decay test is run the higher the threshold would be, i.e. if a modified wood material of a certain treatment level passes the 3% decay threshold in EN113 after 16 weeks of exposure (which is the standardised exposure time) it may not pass this threshold after 32 weeks but rather a higher treatment level would be needed to pass the threshold after this time. The fact that the mass loss of the samples in paper I flattened out after approximately 50 days and remained constant at a couple of percentages mass loss for the remaining 250 days of the experiment, indicates that there might still be a threshold in modified wood at which decay is inhibited (is below 3% for an extended period of time). However, at the end of the test, the variation began to increase which implies that this may have been the start of the logarithmic phase. If service life predictions are made on durability data from short-term laboratory decay tests, knowing whether the fungi eventually reach the logarithmic phase is crucial for a realistic estimation. Unfortunately, contaminating moulds forced the decay test in paper I to be terminated after 300 days. To be able to study what happens in modified wood specimens with the modification levels used in paper I beyond 300 days, the problem with contaminating moulds has to be overcome (discussed further in chapter 10).

In nature, brown rot wood degradation is usually preceded by bacterial infections and soft rot (Eaton and Hale 1993), which means that the wood cell wall may not be intact when the brown rot fungi colonise it. Since the conditions in the natural environment are different to the conditions in a lab test, it is possible that the brown rot degradation mechanisms that seem to be inhibited by wood modification in single species lab tests are not the mechanisms that provide an increased decay resistance in nature. Preceding wood degrading organisms may affect both the moisture content and the porosity of the wood cell wall. To be able to draw conclusions on the natural conditions for brown rot fungi in modified wood, the ability of wood degrading bacteria and soft rot fungi needs to be investigated.

To conclude, the research conducted for this thesis together with current research indicate that the enzymatic phase of the brown rot degradation process is not inhibited by wood modification but only decreased in rate. The initial inhibition of the brown rot degradation observed in modified wood materials must therefore be caused by the inhibition of the CMF degradation phase. Wood modification may inhibit an internal process in the fungus; for example by interfering with a regulatory process in the translation or post-translational modification of the enzymes involved in CMF degradation or in the formation and secretion of CMF metabolites. Alternatively, wood modification may inhibit a process that occurs outside of the fungus, such as the penetration and transportation of CMF metabolites through the wood cell wall, caused by pore blocking and moisture exclusion, respectively, or the sequestration and reduction of ferrous iron caused by inadequate pH levels. Out of the theories of mode of action of modified wood discussed in section 4.2, inhibition of diffusion of CMF metabolites through moisture exclusion seems to be the most probable theory out of the more established ones, while the other theories may explain a slower degradation rate once degradation has started. A comprehensive review and evaluation of experimental data and established theories in the literature based on the biochemical mechanism of the brown rot wood degradation process has not been previously reported.

9.9 Implication of the results

The results in this thesis show that modified wood is colonised by brown rot fungi already during the first week of exposure; nevertheless, at high treatment levels, only 3% mass loss was lost during 300 days of exposure to *P. placenta* and neither structural integrity nor acetyl content (in acetylated wood) was affected. The maintained acetyl content in acetylated wood indicates that mass loss would probably have remained low for some time even if the decay test had been run longer. This further strengthens the impression of heavily treated acetylated and furfurylated wood as being highly decay resistant for long periods of time and supports its use in outdoor construction.

However, results from thermally modified wood indicate that degradation may eventually increase to a rate that is somewhat lower but quite similar to that in untreated wood. This indicates that even though the material seems intact for a long period of time, it can suddenly become degraded. It is possible that the results would have been the same for acetylated and furfurylated wood if the treatment levels had been lower. These results stress the importance of sufficiently high treatment levels but may also indicate that some modified wood materials may perform better in a certain environment than others.

The indication that brown rot fungi oscillates between lag and logarithmic phase while alternating between degrading the most easily accessible nutrients and adapting to degrade

others, may make predictions of service life-time more difficult than previously believed. With a lag-logarithmic-stationary phase-like growth pattern, service life-time predictions would be similar to an estimation of the lag phase, while with an oscillating growth pattern the length and rate of each phase influence the total amount of degradation.

The fact that expression of genes involved in the CMF degradation process were up-regulated in the modified wood materials during the first two weeks of exposure, which is earlier than previously shown, despite no or little mass loss during a prolonged exposure of 300 days while Fenton derived hydroxyl radicals and fungal cellulases were shown to be able to degrade polysaccharides in modified wood materials, indicates that it is the CMF degradation that is inhibited by the wood modification and that it probably is inhibited outside of the fungi. The biochemical mechanisms of brown rot degradation, previous experimental results and the results in this thesis indicate that wood modification inhibits decay through inhibition of chemical transport of CMF metabolites through the wood cell wall. Even though more work needs to be done to confirm this hypothesis, this will eventually facilitate a more targeted development of new and existing modified wood materials. Knowing the underlying mechanisms may make it possible to specifically enhance properties related to e.g. moisture exclusion or diffusion inhibition. It may also be possible to develop new wood modifications that specifically target e.g. the plasticisation of hemicelluloses or even specific fungal enzymes or reactions needed for the degradation.

New and enhanced modified wood products that matches or exceeds preservative treated wood in cost and durability would make both a continued and a broadened use of wood in outdoor construction more attractive and thereby help decreasing the human environmental impact. Wood application in building and construction causes little environmental impacts both during production and during disposal compared to alternative construction materials. The wood in long-lived construction act as carbon sinks and the longer the service-life of wood materials, the more effective the carbon storage mitigates climate change effects. Furthermore, being able to replace preservative treated wood with non-toxic alternatives, would lead to less exposure to and emission of heavy metals and other toxic compounds in both developed areas and in nature. In context of strategies to enhance the material efficiency of wood, modification technologies are expected to support and facilitate wood cascading and final bioenergy conversion.

Increasing the durability of modified wood would make it an affordable alternative to preservative treated wood also for private house owners. Modified wood can be used in decking, façades, window constructions, outdoor furniture, etc. It can also be used in indoor applications such as flooring in bathrooms. Today, progress is being made within the area of wooden high-rise buildings with both comprehensive research and actual construction of such buildings. Modified wood may be an interesting façade material in these buildings, where weather loads are high and façade maintenance may be quite costly. Increasing competiveness of modified wood may also facilitate usage of these materials in developing countries, where low environment impact managing of preservative treated wood waste may be too costly. The absence of harmful chemicals in modified wood also facilitates alternative waste management methods at end-of-service. There is already at least one study in progress to find more valuable ways to manage the modified wood waste, for example by producing other products from the waste, such as food and feed based on fungi and insects.

10 Conclusion and outlook

The results in this thesis show that *P. placenta* was able to colonise modified wood materials already within the first week of exposure and up-regulate genes involved in wood degradation within the first two weeks of exposure. In spite of this, at the treatment levels used in this work, acetylated and furfurylated wood lost less than 3% mass and had maintained structural integrity and acetyl content (in acetylated wood) during a prolonged exposure to *P. placenta* of up to 300 days.

The results also show that Fenton derived hydroxyl radicals and fungal cellulases were able to degrade polysaccharides in acetylated wood, which suggests that if/when the fungi can initiate the formation of hydroxyl radicals inside the wood cell wall, the modified wood will be degraded. Furthermore, the results indicate that modified wood may be degraded at a lower moisture content than previously believed. While *P. placenta* in untreated wood was shown to go through similar growth phases as fungi in liquid culture, the growth pattern of the fungi growing in the acetylated, furfurylated and thermally modified wood may suggest that wood modification causes a prolonged lag phase, the length of which may depend on the treatment level. It is also possible that the fungi oscillate between the lag and logarithmic phases, thus depleting the most easily accessible nutrients first and then adapting to be able to degrade nutrients less accessible. During degradation of thermally modified wood, the patterns of expression of genes involved in wood degradation and structural integrity were similar to those in untreated wood but the rate of degradation was lower.

In summary, the results in this thesis lead to the conclusion that wood modification might inhibit *P. placenta* degradation either by i) interfering with regulatory mechanisms in the fungi resulting in that proteins needed for wood degradation are either not formed, secreted or activated or ii) by inhibiting CMF degradation in the wood cell wall, either through moisture exclusion which might inhibit diffusion of the CMF molecules or through inadequate pH-levels which might inhibit the chemical reactions leading up to the Fenton reaction. This conclusion was supported by current literature on the biochemical mechanisms of brown rot wood degradation as well as experimental data from previous studies on modified wood.

These results imply that the CMF degradation needs to be effectively and long term inhibited for modified wood to resist decay. In order to provide information for the industry that will facilitate efficient development and enhancement of modified wood materials, possible ways to inhibit CMF degradation need to be investigated. In the following section, ways to deepen, broaden and confirm the research in this thesis are discussed.

In the presented research, moisture in the wood samples after decay was only measured as total moisture content. Measuring the EMC instead would tell us how much water the wood cell wall can absorb and how this is changed during decay. The actual amount of water during decay in the wood cell walls would not be known, but it would show more accurately how and when the cell walls' moisture absorption capacity is affected by decay. Measuring EMC after decay could be done by putting dried harvested samples in high RH until equilibrium is achieved and subsequently weigh them. More advanced methods could be to measure the number of accessible water binding sites with nuclear magnetic resonance (NMR) or by exchanging hydrogen with deuterium and analyse with dynamic vapour sorption (DVS) (Engelund et al.

2010; Telkki et al. 2013; Popescu et al. 2013; Hill et al. 2014). To inoculate with mycelium mats would facilitate inoculation without the addition of water or nutrients.

Finding out the threshold for diffusion of CMF molecules and the major medium of transport (bound water, plasticised hemicelluloses etc.) in untreated wood would facilitate designing wood modifications that specifically inhibit this step. . It would also help further understanding the mode of action of existing modified wood materials and improving their efficacy. Subjecting modified wood to e.g. the diffusion test in Zelinka et al. (2014), would supply important information, in this case, on the moisture threshold for chemical transport in modified timber. Another way to study the ability of CMF molecules to diffuse in modified wood is to visually detect these molecules in modified wood exposed to fungi in the early stages of decay. Hydrogen peroxide can be precipitated with $CeCl_3$ and iron reductants can be detected immunochemically (Jellison et al. 1991; Kim et al. 2002) and both methods will make these CMF molecules visible in a transmission electron microscope (TEM). These analyses would show if these molecules can diffuse also *in vivo*, but the absence of CMF molecules inside the wood cell wall does not necessarily mean that diffusion was inhibited. It might just be an artefact from sample preparation. An indirect way of looking at diffusion of CMF molecules is to look at whether CMF degradation occurs; if CMF degradation occurs, diffusion is not inhibited. However, if CMF does not occur it might also be due to other reasons. The structural integrity results in this work may suggest that CMF does not occur in acetylated wood at the treatment level used in our experiments. The experiments should be repeated with other kinds of modified wood materials. However, analysis of structural integrity may not be the ideal method due to the proposed gluing effect of the fungal mycelium. Another way to look at whether CMF degradation occurs during incipient brown rot decay is to analyse the samples with spectroscopy or even micro-spectroscopy. These methods have been used in untreated wood and they were able to detect and distinguish between CMF induced and cellulase induced changes in structure and composition and even localise them to the different parts of the wood cell wall (Schwanninger et al. 2004; Schmutzer et al. 2008; Fackler et al. 2010; Fackler and Schwanninger 2012). Doing this with modified wood samples exposed to *P. placenta* for different amounts of time would provide important information of the dynamics of the degradation process, before wood polysaccharide degradation, during CMF degradation and during cellulase mediated degradation. It might also be possible to study CMF degradation in modified wood using ultraviolet microspectrophotometry (UMSP). Ehmcke et al. showed that after 21 days of incubation with *P. placenta*, changes in chemical distribution of modified lignin could be detected with the UMSP in the entire cell wall even though no structural cell wall damages were seen in light microscopy (Ehmcke et al. 2015).

To run longer decay tests than 300 days, a way to circumvent the mould problem needs be developed. A fungicide that exclusively eliminates mould but not brown rot would be ideal; however, it is unlikely that such a chemical will be discovered. In theory it would be possible to genetically modify the brown rot strain(s) used in the decay test in such a way that they are not affected by the fungicide used to kill the moulds. A more realistic approach is of course to improve the routines for the sterile work and the substrates and facilities used. Mould growth was usually discovered at about the same time in many but not all soil plates. If the soil plates were contaminated with mould from the surrounding air or inadequate sterile work during monthly watering, mould growth should have appeared at several time points. If the mould was in the soil (due to insufficient sterilisation) the growth might have been repressed by the

presence of *P. placenta*, which may explain the late outburst. Furthermore, if mould only survived in the inner parts of the soil bags during autoclaving, that might explain why not all soil plates were contaminated. Another way to avoid mould growth would be to shorten the time needed for the decay test by using a lower level of modification. However, at lower levels of modification, the effect of uneven distribution of the modification might be more pronounced since a larger part of the board or specimen might have insufficient treatment levels.

It would have been interesting to find out what the mass that the modified samples in paper I lost consisted of. Only very few samples lost more than the non-exposed acetylated and furfurylated controls (incubated on soil under the same conditions as the samples exposed to fungi for 8 and 23 weeks). However, the exposed samples also contained fungal biomass, and therefore must have lost more wood mass than the non-exposed samples. All samples had been leached, but perhaps it is possible that sugars and proteins in the lumen still accounted for the 1-3% mass lost in the acetylated and furfurylated wood samples. In the modified samples, residual acetyl and furfuryl alcohol could have been present in the lumen and been leached out during incubation, however this is highly unlikely at least for acetylated wood since the remaining acetyl reacts with water and is released as acetic acid during the treatment. Furthermore, our acetyl measurements suggested that acetyl was not degraded during incubation (Paper I: Fig. 4), which means that the mass lost was not the acetyl bound to the wood constituents either. This small change in mass could also be due to errors in the weighing of the samples. However, this should lead to just as many samples with negative mass loss and samples with positive and negative mass loss evenly distributed over the whole decay test, which is not the case here. Additionally, our results suggest that untreated wood started to lose structural integrity at a lower mass loss than acetylated, furfurylated and thermally modified wood did. The question is then if the same kind of mass is lost up to 3% mass loss in modified and untreated wood. Beside this, it would be interesting to complement the mass loss measurements with measurements of fungal biomass indicators, fungal DNA or fungal energy development to investigate whether the indirectly measured growth phases in this thesis can be seen when measuring fungal parameters as well.

The expression of only a few number of genes have so far been analysed in modified wood exposed to *P. placenta*. Looking at more genes would strengthen the conclusions of this thesis. It would be especially informative to look at genes related to alcohol oxidase Ppl118723, QRD Ppl124517 and laccase Ppl111314, such as: the acetylglucosaminyl transferase Ppl56703 (CRO1), the serine/threonine protein phosphatase Ppl130305 (CRO2) and the glucose oxidase Ppl108489 (involved in hydrogen peroxide formation); the hydroquinol 1,2-dioxygenase Ppl34850, the putative quinate transporter 44553 (involved in iron reductant formation, secretion and reduction); and the laccases Ppl89382, Ppl47589 and Ppl62097 (Martinez et al. 2009; Vanden Wymelenberg et al. 2010). Understanding the role of oxalic acid in the degradation process might also be informative, in which case the expression of the isocitrate lyase Ppl115235, the malate synthase Ppl119506, the oxaloacetase Ppl112832, a putative oxalate decarboxylase such as Ppl106821, the formate dehydrogenase Ppl98518 could be investigated (Munir et al. 2001b; Martinez et al. 2009; Vanden Wymelenberg et al. 2010). In this project, gene analysis was performed using qPCR which shows the gene expression of the selected genes. However, selecting genes limits the information gathered from the analysis. Another method possible to use in this respect is high-throughput sequencing, which shows the expression levels of all the genes transcribed at a certain time point. This method is more time-

consuming and more costly, but might be a good complement to qPCR and may provide information on which selection of genes for qPCR could be based. To be able to better correlate the gene expression levels with the state of decay in future gene analysis experiments, a method that in some way measures the decay or the amount of fungi in the same specimen as the one used for gene analysis should be used, such as DNA-measurements.

To further investigate what happens to the modification chemicals themselves during decay, a decay test could be run with samples treated to a much lower level than in this work. With an adequate level of modification, also Ac, furfurylated wood and DMDHEU treated samples should go into active degradation. Measurements of e.g. acetyl in wood as done in this thesis throughout the decay test would show both when and to what extent the modification chemicals are degraded and what WPG is needed for degradation in the logarithmic phase to occur. It may be possible to use FT-IR and/or microspectroscopy also here. But again, lower levels may lead to that the samples are more unevenly treated.

To be able to draw general conclusions of the mechanisms behind brown rot decay resistance in modified wood, wood modification needs to be investigated in more depth than is done in the current literature also for other species of brown rot fungi than *P .placenta*. Furthermore, modified wood has also an increased resistance to white rot decay, why investigating the mode of action of modified wood against these fungi is also important. Additionally, the effect of preceding bacterial infections and soft rot should be investigated. Our studies have so far mainly been performed on pine species, so to be able to draw conclusions for other wood species, especially hardwood species, these species need to be examined too. Research on decay resistance in modified hardwood species should include white rot fungi since hardwood species are the most susceptible to these fungi (Eaton and Hale 1993; Schmidt 2006).

In this work, the focus has been on the four most investigated wood modifications at the time when this project started. In future work on the decay resistance mechanisms of modified wood, novel wood modifications that inhibit degradation without inhibiting colonisation may be included.

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Paper I

Incipient brown rot decay in modified wood: patterns of mass loss, structural integrity, moisture and acetyl content in high resolution.

Paper II

Effect of wood modification on gene expression during incipient *Postia placenta* decay.

Paper III

Effects of thermal modification on *Postia placenta* wood degradation dynamics: measurements of mass loss, structural integrity and gene expression.

Paper IV

***In vitro* oxidative and enzymatic degradation of modified wood.**

Paper V

Mode of action of brown rot decay resistance in modified wood: a review.

I

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Incipient brown rot decay in modified wood: patterns of mass loss, structural integrity, moisture and acetyl content in high resolution

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ABSTRACT
The study of degradation and growth patterns of fungi in modified wood may increase the understanding of their mode of action and may lead to more accurate service-life predictions. The aim of this paper was to study the degradation and growth patterns of brown rot fungi in modified wood and to measure moisture content (MC), structural integrity and the acetyl content by frequent monitoring over 300 days. Mass loss (ML) in the modified wood materials increased slowly up to 3% for 50–100 days after which it flattened out and remained constant during the remainder of the test. Structural integrity and acetyl content were maintained in the modified wood materials and MC was lower compared to untreated wood throughout the decay test. ML results of untreated wood indicate that fungi in solid wood go through distinct phases; the degradation patterns in the modified wood materials were more difficult to interpret.

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Introduction

Brown rot attack is a particular challenge in wooden constructions because it causes more loss of strength at low mass loss (ML) than white rot, resulting in great damage within a short period of time (Eaton and Hale 1993; Witomski et al. 2016). Brown rot fungi preferably attack coniferous wood, which is used in the majority of constructions in the Northern hemisphere (Schmidt 2006). New alternatives to traditional, toxic wood preservation methods are being developed, such as modified wood (Hill 2006). To efficiently develop and improve modified wood materials, understanding the mode of action of wood modification is utterly important. The degradation and growth patterns of fungi growing in modified wood may increase the understanding of the mode of action of modified wood and may be of help in service-life predictions.

Modified wood is defined as chemically or physically altered wood materials with increased decay resistance, which are non-toxic under service conditions and at the end of service life (Hill 2006). Acetylation of wood is commonly achieved by reacting wood with acetic anhydride, which causes acetyl groups to bind to the OH-groups of the wood constituents (Rowell et al. 1994; Larsson Brelid et al. 2000; Hill et al. 2005). Decrease in equilibrium moisture content (EMC) and increase in decay resistance in acetylated wood have previously been primarily ascribed to the volume of added modification agent, i.e. bulking (Papadopoulos and Hill 2003; Hill et al. 2004). However, a recent study addressing the moisture content (MC) of modified wood materials during drying below the fibre saturation point indicated that acetylation changes the interaction between water and the wood cell wall (Thygesen and Elder 2009).

Furfurylation of wood involves impregnation of the wood with furfuryl alcohol and subsequent curing during which polymerised furfuryl alcohol (poly(furfuryl alcohol)) is formed (Goldstein 1960). Based on that furfuryl alcohol binds to a lignin model compound, it has been suggested that furfuryl alcohol binds to lignin; however, this has not been shown *in situ* (Nordstierna et al. 2008). The mechanisms behind the EMC reduction in furfurylated wood are less clear than for acetylated wood. Binding of furfuryl alcohol to lignin would not directly block the sorption sites in the wood carbohydrates and does, therefore, not fully explain the reduction in EMC (Thygesen et al. 2010). Thygesen and Elder (2009) indicated that furfurylation mostly affects the amount of water present within the cell wall before drying, which may suggest that bulking is the main mechanism for EMC reduction in furfurylated wood.

Microorganisms in a liquid culture go through three distinct stages: (i) the lag phase in which the microorganisms adapt to the new environment, (ii) the logarithmic phase where the microorganisms are actively degrading the nutrient in the medium and grow exponentially and (iii) the stationary phase where the

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growth is impaired by nutrient deficiency, a change in pH or an accumulation of toxic compounds (Baranyi and Roberts 2000; Madigan et al. 2000; Rolfe et al. 2012). *Penicillium chrysosporium* was shown to go through lag, logarithmic and stationary phase like phases when grown on a solid food substrate, while *Physisporinus vitreus* has been modelled to go through similar phases when growing in solid wood (Fuhr et al. 2011; Arquiza and Hunter 2014). If the growth phases of microorganisms in liquid culture are applicable on filamentous fungi growing in wood, the lag phase might be equivalent to the time it takes for the fungi to adapt to the environment provided by the wood material. For example, the absence of glucose will lead to an up-regulation of genes involved in wood degradation (Aro et al. 2005; Martinez et al. 2009). The fungi may start the chelator-mediated Fenton (CMF) degradation at the end of the lag phase. No change in composition is noted, but a change in structure (Fackler et al. 2010). The fungi depolymerise cellulose chains and hemicelluloses and modify lignin through induction of the Fenton reaction in which hydroxyl radicals are formed (Fenton 1894; Goodell et al. 1997; Arantes et al. 2012). The depolymerisation of the wood cell wall polysaccharides leads to a loss in strength, which is notable before ML can be detected (Wilcox 1978; Winandy and Morrell 1993; Curling et al. 2002; Brischke et al. 2006; Fackler et al. 2010; Maeda et al. 2015). Once the CMF degradation has opened up the wood structure sufficiently, the enzymatic degradation machinery starts degrading the wood constituents, which presumably puts the fungi in the logarithmic phase. Enzymatic degradation further reduces the strength of the wood while also causing ML (Curling et al. 2002; Brischke et al. 2008; Fackler et al. 2010). The stationary phase in liquid culture may be equivalent to the stage when the fungi have degraded the wood material to such an extent that all available nutrients are depleted and the degradation rate flattens out. In a recent review, Alfredsen et al. (2014) stated that the expression of genes involved in CMF degradation seems to be higher in modified wood than in untreated wood and it tends to increase with time of exposure, while genes involved in enzymatic degradation are expressed at similar levels in both modified and untreated wood materials.

In a liquid culture, fungal growth is measured as the change in fungal biomass. In wood, where the mycelium cannot be extracted, fungal biomass is determined by assays of cell constituents such as ergosterol, total extractable liquid phosphates, nucleic acids and chitin as well as indicators of biological activity such as GTP, enzyme and respiratory activities (Lena et al. 1994). To be reliable, an indicator of fungal biomass must correlate with mycelium increase and be independent of growth conditions. In a solid substrate, measuring the loss of mass of the substrate is an indirect measurement of the growth of the fungi (Mohebby et al. 2003; Verma et al. 2008). Since ML only occurs once the enzymatic degradation activities have begun, measuring ML will not show the incipient degradation in which only CMF degradation occurs (Fackler et al. 2010). CMF degradation instead causes structural changes in the wood cell wall, such as modification of lignin and depolymerisation of cellulose (Fackler et al. 2010). Measurements of CMF degradation may, therefore, include strength loss analyses, especially of specimens exposed to fungi for such a short time that ML cannot be detected. In recent years, an alternative to measuring strength loss in decayed wood has been developed, called the high-energy multiple impact (HEMI) test, which instead addresses the structural integrity of the wood (Brischke et al. 2006; Rapp et al. 2006). This method detects both changes in fibre strength (in the fibre direction) and strength between fibres (perpendicular to the fibre direction). In the early stages of decay, both should mainly be affected by CMF degradation because radicals randomly attack the wood polymers. The advantages of HEMI tests are small variances, high reproducibility of results, short time for specimen preparation and a small number of specimens needed (Rapp et al. 2006). Furthermore, in this study the possibility to measure structural integrity in miniblock samples was important. In modified wood materials, CMF degradation may also affect the amount of modification chemicals in the wood material, depending on whether the modification chemical can be degraded by hydroxyl radicals.

In the research on decay resistance and mode of action of chemically modified wood, the majority of studies have measured the ML after a fixed time of exposure, similarly to durability standards such as EN 113 (1996) and AWPA E 10 (1991), or only at a few different time points (Papadopoulos and Hill 2002; Rapp et al. 2008; Verma et al. 2009; Esteves et al. 2011). Therefore, from the current literature, it is difficult to determine the effect of wood modification on the growth pattern of wood degrading fungi (Papadopoulos and Hill 2002; Rapp et al. 2008; Verma et al. 2009; Esteves et al. 2011). Looking at multiple samples harvested continuously over a long period of time would possibly provide better insights into the dynamics of the decay of modified wood and potentially reveal whether the growth phases of microorganisms in liquid culture can be applied also on wood degrading fungi growing in solid untreated and modified wood. As for ML, MC and structural integrity during brown rot decay of modified wood materials have not previously been measured over a prolonged time of exposure. Measuring the loss of strength or structural integrity during fungal exposure of modified wood may show if there is a depolymerisation or loss of components that influence the strength of wood, such

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as cellulose. Whether the modification chemicals are degraded during brown rot exposure of modified wood has not previously been investigated.

The aim of this study was (1) to compare the degradation and growth patterns of the brown rot fungi *P. placenta* in acetylated and furfurylated wood with those in untreated wood, and (2) to investigate whether these show if fungi growing on treated or untreated solid wood undergo the same growth phases as are seen for fungi in liquid cultures. This was done through frequent monitoring of ML, MC and structural integrity during the exposure of acetylated and furfurylated wood to *P. placenta* for more than 300 days. Acetyl content was measured in acetylated wood under the same conditions.

Materials and methods

Wood material and sample preparation

Miniblock samples ($10 \times 5 \times 30$ (ax.) mm^3) (Bravery 1979) of Southern yellow pine sapwood were acetylated or furfurylated, as previously described (Larsson Brelid 1998; Lande et al. 2004; Ringman et al. 2015). The samples were selected based on weight per cent gain (WPG) and EMC (20°C, 65% relative humidity (RH)), resulting in (i) for acetylated wood samples, an average WPG of 22.6% (ranging from 19.0% to 25.9%) and an average EMC of 3.54% (ranging from 2.80% to 7.23%), and (ii) for furfurylated wood samples, an average WPG of 69% (ranging from 45.2% to 98.3%) and an average EMC of 4.53% (ranging from 3.38% to 5.56%). The selection was made based on the decay protection threshold levels proposed by Thybring (2013). The samples were leached according to EN 84 (1996) and conditioned in 20°C and 65% RH for two weeks, after which the samples were weighed and the EMC was calculated based on the weight of oven-dried samples after treatment. The samples were sterilised with gamma radiation (>30 kGy, all samples sterilised at the same time) and placed two by two in Petri dishes containing sterile soil and subsequently inoculated with 1 ml *P. placenta* (strain FPRL 280) liquid culture. In total, 576 acetylated, 596 furfurylated and 216 untreated specimens were inoculated and 24 samples of each treatment were placed in Petri dishes without fungi (non-inoculated controls).

Mass loss and moisture content

Samples ($n = 8$) were harvested at frequent intervals during the decay test, approximately every week for untreated and every four weeks for treated samples. At harvest, mycelium covering the samples was removed and each sample was weighed wet and dried (103°C, 18 h). The decay test was terminated after 60 days (untreated), 357 days (acetylated) and 396 days (furfurylated).

Structural integrity

Four dried samples from each set of ML samples were selected for HEMI tests. From each sample, three specimens of $10 \times 5 \times 10$ (ax.) mm^3 were cut out with a clipper. The development and optimisation of the HEMI test have been described by Rapp et al. (2006). The following procedure was used: 12 oven-dried specimens were placed in the bowl of a heavy-impact ball mill, together with one steel ball of 40 mm, three of 12 mm and three of 6 mm diameter. The bowl was shaken for 60 s at a rotary frequency of 23.3 s^{-1} and a stroke of 12 mm. The fragments of the 12 specimens were fractionated on a slit screen according to ISO 5223 (1996), slit width of 1 mm). The following values were calculated: (i) the degree of integrity (I), which is the ratio of the mass of the 12 biggest fragments to the mass of all fragments after crushing, (ii) the fine fraction (F), which is the ratio of the mass of fragments under 1 mm to the mass of all fragments, multiplied by 100 and (iii) the resistance to impact milling (RIM), which is calculated from I and F as follows:

$$\mathrm{RIM} = (I - 3 \cdot F + 300)/4 \ [\%]$$

The threefold weighing of the fine fraction was performed according to earlier studies (Rapp et al. 2006) and can finely distinguish between different intensities of fungal decay. To ensure that RIM varies between 0 and 100%, the constant, 300, was added.

Acetyl content

Before chemical analyses, dried samples were cut into small pieces and then ground with a vibration mill (MM 400, Retsch) under cooling with liquid nitrogen and finally air-dried overnight. The MC of the air-dried wood samples was determined separately by drying at 105°C. The determination of acetyl groups was carried out according to Månsson and Samuelsson (1981) by means of an aminolysis with pyrrolidine and subsequent GC analysis on a GC 2010 (Shimadzu) equipped with a BP5 (SGE) or a HP-5 (Agilent-Technologies) capillary column. Temperatures: Inj.: 300°C; Det.: 310°C; column 115°C. Columns: BP5 (30 m, 0.25 µm film, 0.25 mm ID) and HP-5 (30 m, 0.25 µm film, 0.32 mm ID).

Results

Mass loss

Untreated wood had 2% ML after 14 days, 19% ML after 28 days and reached 41% ML after 55 days (Figure 1(a)). During the first 7 days, the ML was negative.

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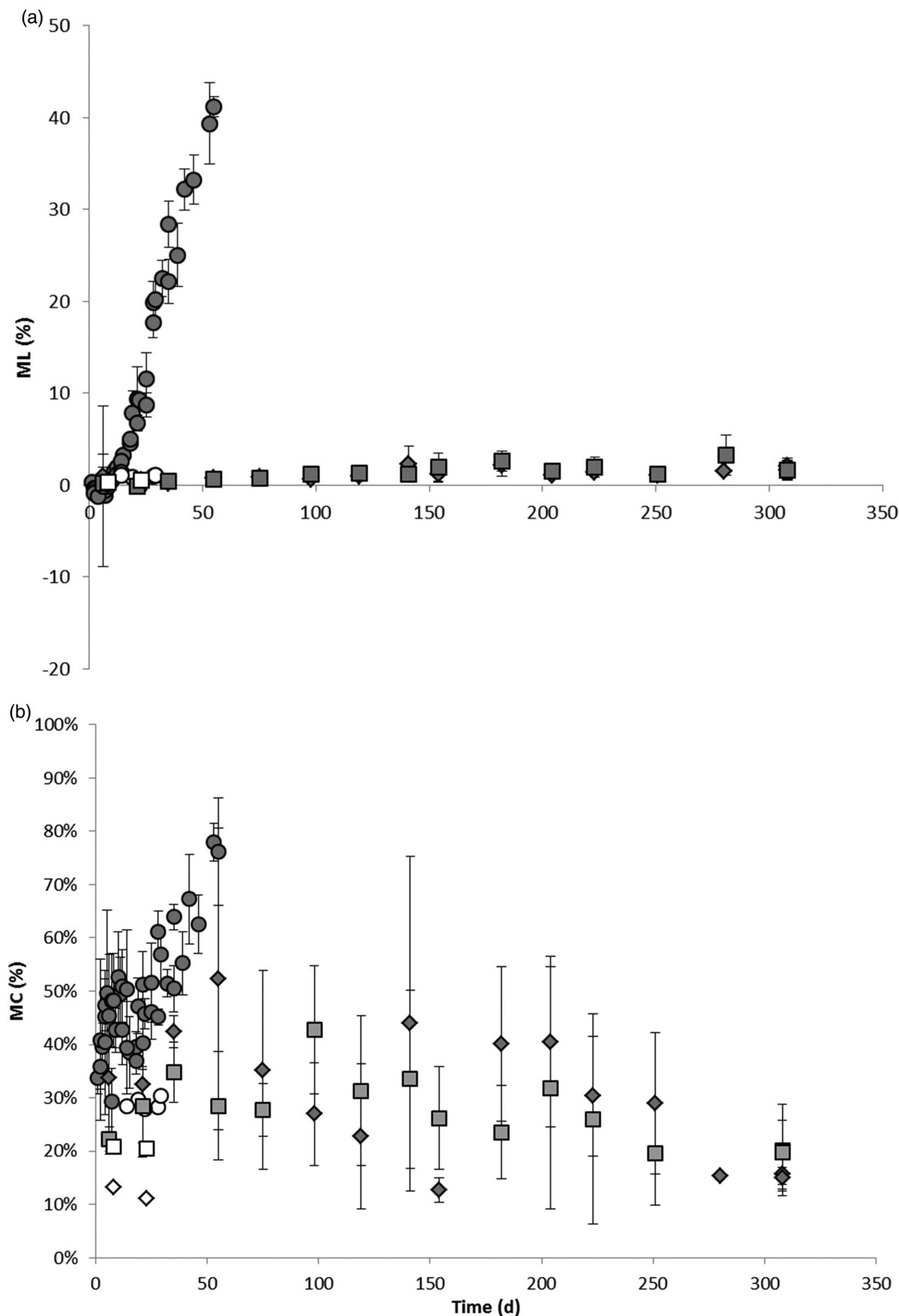

Figure 1. Mass loss (a) and moisture content (b) during the decay test in untreated (circles), acetylated (diamonds) and furfurylated (squares) wood. White symbols are non-inoculated controls.

The acetylated and furfurylated wood materials reached a maximum of 4% and 7% ML, respectively (single samples) during the 300 days of the test period (Figure 1(a)). Just as for untreated wood, the ML was negative one week after exposure for both modified wood materials. The ML increased for 55 days in acetylated wood and 98 days in furfurylated wood with a rate that was 100 times lower than in the logarithmic phase of the untreated wood. After these time points, the decay rate flattened out and the average ML for acetylated wood was 1.44% and for furfurylated wood 1.95% during the remaining part of the decay test. Even though the WPG of the furfurylated wood samples varied considerably, ML did not vary accordingly. This is probably because all samples were treated to a level above the decay threshold of 35% WPG proposed by Thybring (2013). The acetylated samples were, on average, above the proposed decay threshold

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of 20% WPG, but a few single samples had a WPG slightly lower.

Moisture content

In untreated wood, MC was ranging from 22 to 72% below 5% ML (one sample had a MC below 25% and less than 3% ML, which EN 113 (1996) states as abnormal, after which MC increased with ML (Figure 1(b)). The MC of the acetylated and furfurylated wood samples varied between 13–52% and 20–43%, respectively, with no clear trend except a possible decrease during the last 100 days (Figure 1(b)). However, the MC of the modified wood samples never decreased below the MC of that of the acclimatised (20°C, 65% RH) samples before the decay test.

Growth phases

The ML data of the present study were plotted logarithmically (Figure 2). For untreated wood, it was possible to detect three different stages in the ML curve similar to the phases seen in liquid fungal cultures; the lag phase where the fungi adapt to the new environment, the logarithmic phase where the growth rate of the fungi increases logarithmically and the stationary phase where the growth rate of the fungi flattens out. Unfortunately, many of the samples in the lag phase had negative ML and did, therefore, not show in the logarithmic graph. For acetylated and furfurylated wood, there seems to be an increase in degradation rate up to approximately 150 days; however, the rate is 100 times lower than in the logarithmic phase of fungi in the untreated wood.

Structural integrity

In untreated wood, structural integrity decreased with exposure time throughout the experiment (Figure 3(a)). Loss of structural integrity was 10% after one week of exposure and negative ML, 21% at 1% ML, 50% at 7% ML and 98% at 34% ML, compared to the zero-time sample. The non-inoculated control sample incubated under the same conditions as the inoculated samples for 14 and 29 days had a loss of structural integrity of 6%. Structural integrity showed good correlation to ML ($R^2 = 0.89$) (Figure 3(b)).

The modification treatments caused loss of structural integrity in the samples (Figure 3(a)). Acetylation decreased structural integrity by approximately 15% and furfurylation with approximately 50% compared to untreated wood. However, it has to be noted that the variation in WPG for the furfurylated samples was large. Average loss of structural integrity over the whole decay test in acetylated wood was 9% compared to the zero-sample (Figure 3(a)). In acetylated wood, samples with negative ML had 4% loss of structural integrity (Figure 3(b)). In furfurylated wood, samples with negative ML had higher structural integrity than the zero-time sample (Figure 3(b)). The average loss of structural integrity over the whole decay test was 0.5% in furfurylated wood (2% without the two samples with increased structural integrity). Looking at the time frame when mass is lost in the modified wood materials, there is no correlation between loss

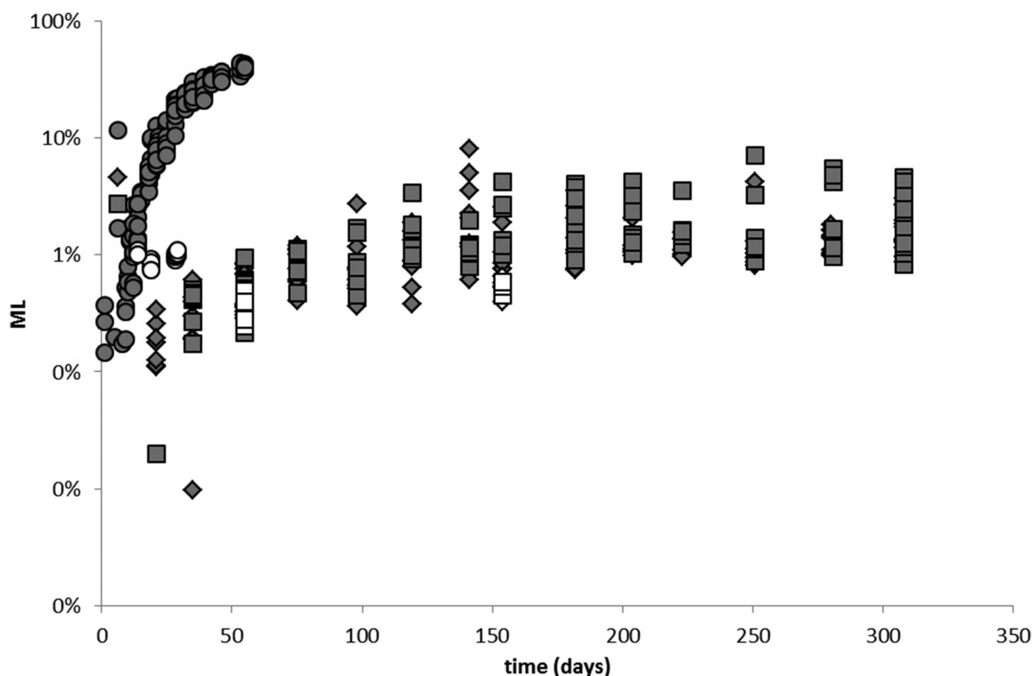

Figure 2. Logarithmic representation of ML during the decay test in untreated wood. Untreated (circles), acetylated (diamonds) and furfurylated (squares) wood. White symbols are non-inoculated controls.

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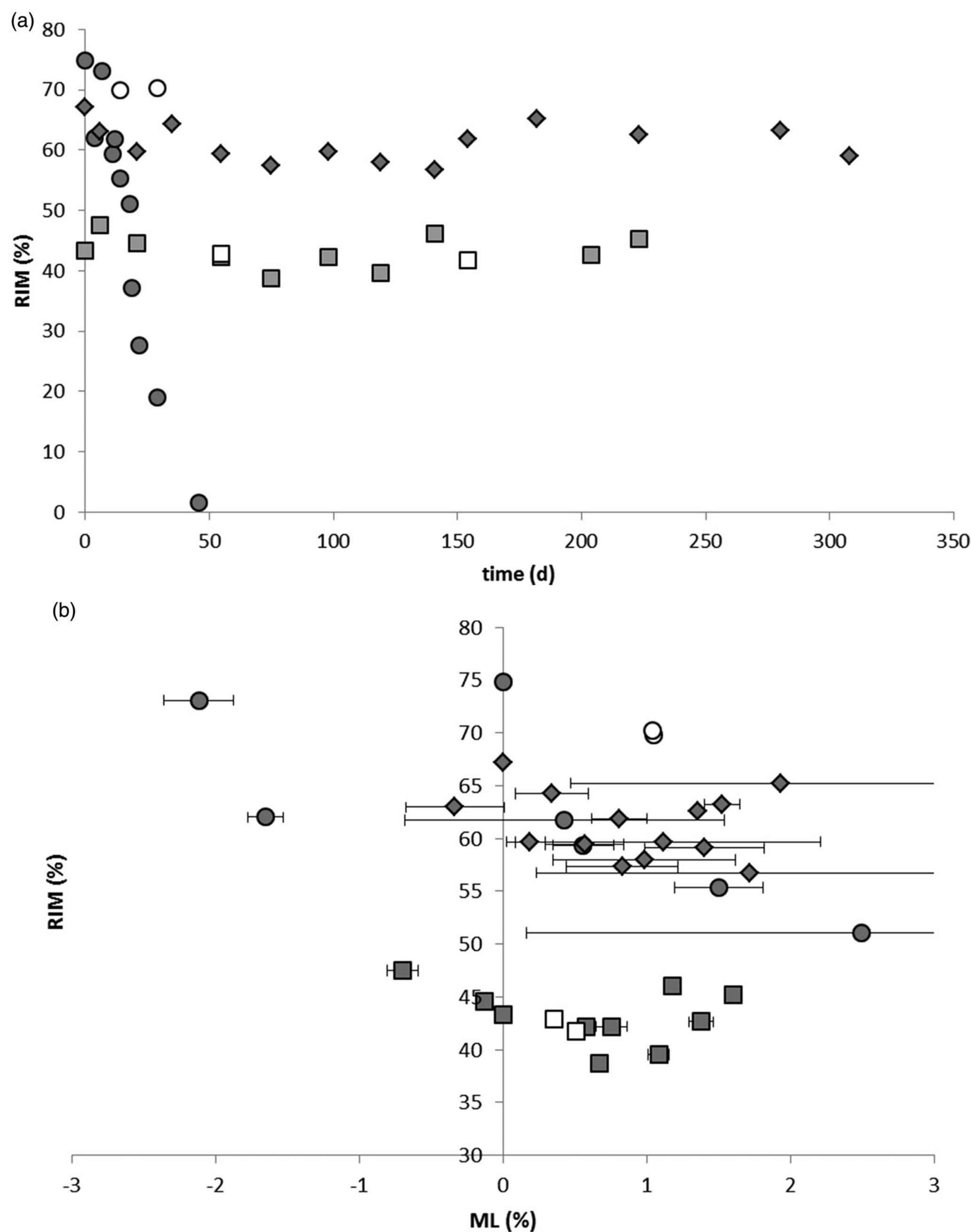

Figure 3. Structural integrity expressed as resistance of impact milling (RIM) over time (a) and against ML up to 3% (b) in untreated (circles), acetylated (diamonds) and furfurylated (squares) wood. White symbols are non-inoculated controls. Note the scale of the Y-axis in (b).

of structural integrity and loss of mass ($R^2 = 0.056$ and 0.098 for acetylated and furfurylated wood, respectively), while untreated wood in the same range of ML had a high positive correlation between loss of structural integrity and ML ($R^2 = 0.859$) (Figure 3(b)).

Acetyl content

Measurements of acetyl content indicated that acetyl in acetylated wood was not degraded during the time course of this experiment (Figure 4). Since measuring acetyl is a destructive method, acetyl before exposure to fungi was calculated from WPG using a standard curve of five samples. The R^2-value was 0.98% and the equation of conversion was, therefore, considered reliable. Figure 4 shows the loss of acetyl during fungal exposure over time and the non-inoculated control ($n = 3$). No significant difference between any of the samples could be detected at a significance level of $p = 0.05$ in Student's t-tests. Since the non-inoculated control samples showed on average 3% loss of acetyl, it is possible that the calculation of original acetyl resulted in values a little low, in which case the loss of acetyl in all samples would be even less. However, the lack of a significant difference between exposed samples over time indicates that during the 120 days when bound acetyl was measured, no fungal degradation of acetyl occurred.

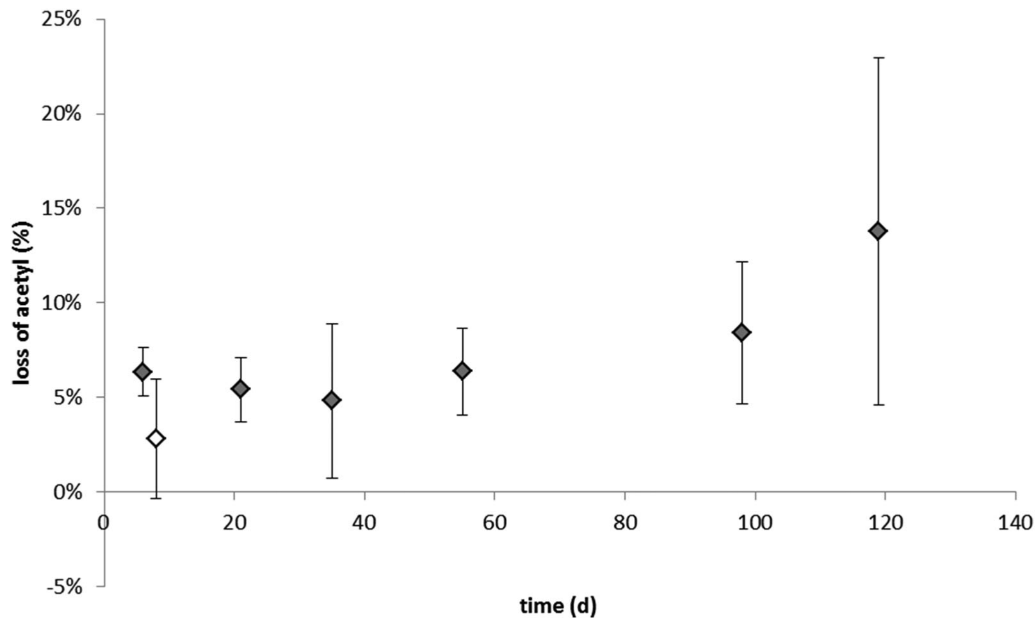

Figure 4. Loss of acetyl in per cent of original acetyl content over time in acetylated wood. White symbol is the non-inoculated control.

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Discussion

Mass loss

The ML data shown in Figure 1(a) give a detailed picture of the degradation pattern in acetylated and furfurylated wood with the treatment levels used in this study. The ML of the modified wood samples is in accordance with previous studies, in which ML in furfurylated wood after 16 weeks of exposure to *P. placenta* was reported to be 1.1–2.4% at >120% WPG, 4.3% at 75% WPG and 1.11% at 38.9% WPG, although the samples' dimensions were bigger than in the present study and dimensions as well as treatment methods varied between the previously reported studies (Lande et al. 2004; Esteves et al. 2011). In previous durability studies on acetylated wood, no or little ML was seen in acetylated wood with approx. 20% WPG up to 36 weeks (Hill 2009; Pilgård et al. 2012; Alfredsen and Pilgård 2014).

Negative ML, as seen in Figure 1(a), could be due to increasing fungal mass inside the samples. It is possible that while the net weight increases, the weight of the wood sample decreases, but this is masked by the increasing fungal mass. Negative ML has been previously reported during brown rot decay, e.g. in Brischke et al. (2006, 2008) and Meyer and Brischke (2015), who suggested that, besides ingrown mycelium, nutrients could have been transported from the agar to the wood samples by the fungus. In the present study, soil plates were used, where ingrown mycelium probably constituted the major part of the negative ML. The fact that the mass of the ingrown mycelia cannot be distinguished from the wood mass leads to that it is impossible to see from ML data when the wood starts to be degraded.

Moisture content

In a previous study, MC in acetylated wood with approximately 20% WPG was 10–50% after four weeks and 5–70% after 28 weeks (Alfredsen and Pilgård 2014). Although the variation was higher, these results are in general in accordance with the results reported here. Schmöllerl et al. (2011) reported the MC in acetylated wood (23% WPG) to be approximately 45% after 2 weeks, 20% after 14 weeks and 15% after 26 weeks. During the same time period, the MC in furfurylated wood (37% WPG) varied between 25 35%. However, in Schmöllerl et al. (2011) no water was added to the soil which may have caused the samples to dry out during the test.

In this study, fungi were added as liquid culture, which may explain the difference in MC between acclimatised samples before the decay test and the inoculated samples harvested in the beginning of the decay test. This is supported by the comparatively low MC values of the non-inoculated control samples, which were not inoculated with any liquid (Figure 1(a)). The variation in initial MC of the samples may be because the liquid culture droplet sometimes stayed on top of the samples but sometimes ran into the soil.

Growth phases

The ML results from the untreated wood supports the proposed model which depicts that filamentous fungi growing in wood go through a lag, logarithmic and a stationary phase (Figure 2) (Fuhr et al. 2011). It is, however, important to note that all measurements were done on the substrate and not on the fungi and

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therefore rather show the degradation phases than the growth phases.

The frequent monitoring of ML over a prolonged time of exposure in this study enables the evaluation of the degradation pattern based on the lag, logarithmic and stationary phases also in the modified wood materials. Since the fungi seem to undergo these phases in solid untreated wood, they probably undergo them also in modified wood. The question is in which phase the fungi in the modified wood materials might have been in this experiment. The degradation rates in the modified wood materials were 100 times lower during the time period when ML increased than during logarithmic phase in untreated wood, which may suggest that the fungi in the modified samples were in the lag phase (Figure 2). However, if the fungi in the modified wood samples in this experiment were indeed in the lag phase throughout the experiment, the question remains why the increase in ML eventually flattens out and what kind of mass is lost. The samples are leached before inoculation and therefore there should be only little nutrients and non-polymerised modification chemicals in the lumen that may leach out during the experiment. The untreated as well as the modified non-inoculated control samples had a ML of 0.5–1% up to 23 weeks of incubation (Figure 2). It is possible that this is at least partly due to the evaporation of volatile substances. The ML seen in the modified samples is however 2–3 times higher than that in the non-inoculated controls.

If the fungi in the modified wood samples are in the logarithmic phase instead, the flattening out of the ML curve may be because the fungus is beginning to die, due to e.g. starvation. Furthermore, if the fungi are in the logarithmic phase, the ML seen is probably wood cell wall hemicelluloses and celluloses degraded by CMF and enzymatic degradation. If the degradation takes place mainly in areas where the modification level is locally very low, the fungi may go into starvation when the low treatment areas have been mainly degraded. As mentioned above, the MC is lower in the modified wood samples than in the untreated samples in which the fungi are in the logarithmic phase. However, if degradation occurs in areas with locally lower treatment levels, it is possible that the MC is also higher in these areas. It is also possible that the sample has dried a little between when the degradation occurred and when the sample was harvested.

In a complex solid material, such as wood, it is not unlikely that the fungi alternate between restricted and unrestricted growth. This may be due to stepwise invasion in the longitudinal direction (Fuhr et al. 2011). In modified wood, it may also be because the fungi will degrade areas with locally lower treatment levels faster than ones with higher treatment levels. Maybe, *P. placenta* in the acetylated and furfurylated samples in this experiment degraded the low treated areas exponentially during the first part of the decay experiment, but then went into a new lag phase. In that case, it may be possible that the fungi would have started exponential degradation of areas with higher treatment levels once it had overcome the inhibition by the modification and if the test had been run longer. If this is true, using samples with lower treatment levels should lead to several consecutive cycles of lag and logarithmic phase.

Structural integrity

In a previous study by Brischke et al. (2006), the correlation between structural integrity and ML during *Coniophora puteana* degradation of untreated wood was estimated to $R^2 = 0.99$, compared to $R^2 = 0.9$ in the present study (Figure 3(b)). Brischke et al. (2012) showed that furfurylation of pine sapwood decreased structural integrity with 16%, but the WPG was only 15.6%. Assuming linear relationships, extrapolation of the results also with lower WPG in Brischke et al. (2012) gives approximately 75% loss of RIM at 70% WPG, which is in accordance with the results in Figure 3(a).

In untreated wood subjected to brown rot, it is widely accepted that considerable strength loss occurs before ML can be detected (Winandy and Morrell 1993; Curling et al. 2002). The authors have not found any previous recordings of structural integrity in acetylated and furfurylated wood after brown rot exposure. The results in this study indicate that the correlation between strength loss and ML may be different in modified wood materials than in untreated wood.

For untreated wood, structural integrity of the samples with negative ML was lower than in the zero-time sample, which indicates changes in the chemical structure such as depolymerisation of cellulose caused by CMF degradation (Figure 3(b)). Additionally, it is possible that a smaller mass than the fungal mass in the samples was degraded. Unfortunately, because all four samples were run in a single test run, the structural integrity values are mean values and standard deviation and significance could not be calculated. The large variation in both WPG and ML in the modified wood samples makes it impossible to find significant differences in structural integrity for these materials. However, the samples are randomly distributed and hence it is unlikely that a considerable loss of structural integrity over time would be masked by an increasing mean WPG. Therefore, we conclude that the acetylated and furfurylated samples probably lose no or little structural integrity during exposure to *P. placenta*, which suggests that CMF degradation may not have occurred. Furthermore, these results support the theory that the fungi in both acetylated and furfurylated wood were in the lag phase. The fact that the non-inoculated control samples (incubated for 56

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and 154 days) had similar structural integrity, as the inoculated samples further support this theory.

In future studies, it is important to run samples with similar ML simultaneously when performing the HEMI test. Replicates with more uniform ML may be achieved using samples with similar WPG. Since four miniblock samples are needed for a single run of the HEMI test, more replicates would also allow for replicate runs and hence provide the possibility to calculate mean values and standard deviation.

Acetyl content

Figure 4 shows the loss of acetyl in per cent of original acetyl content over time in acetylated wood. No significant change in acetyl content is seen between 6 and 120 days of exposure, which indicates that no or little acetyl was degraded in the acetylated samples in this experiment. All of the samples show approximately 3% lower levels of acetyl after exposure to fungi. It is unlikely that this is because 3% of the acetyl was degraded by the fungi during the first week, while no further degradation of acetyl occurred in the following weeks. The apparent loss of 3% acetyl in all samples is probably rather connected to the conversion of WPG to acetyl content. The absence of degradation of acetyl in the acetylated wood shows that the acetyl content of the acetylated wood remained intact during the 300-day decay test which means that the treatment level was maintained.

Conclusions

In this study, we frequently monitored the degradation of *P. placenta* in acetylated and furfurylated wood over 300 days, in terms of ML, MC, structural integrity and acetyl content. Our results show that degradation in acetylated and furfurylated pine sapwood miniblock samples with the treatment levels used in this study was inhibited or kept at a slow rate for more than 300 days of exposure to *P. placenta*. Structural integrity in the modified samples was maintained and acetyl in the acetylated wood material was not degraded. Additionally, our results indicate that *P. placenta* growing on untreated solid pine undergoes the same growth phases as fungi in a liquid culture and thus support the model in which the filamentous fungi *P. vitreus* was predicted to go through a lag, logarithmic and stationary phase while growing on solid wood (Fuhr et al. 2011). More research of growth phases in modified wood is needed to increase the understanding of mode of action of wood modification.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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Effect of wood modification on gene expression during incipient *Postia placenta* decay

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ABSTRACT

The mode of action of nontoxic wood modifications and the fungal response to modified wood are not fully elucidated. The aim of this study was to investigate the reaction of *Postia placenta* in terms of gene expression of selected genes upon the encounter of acetylated, DMDHEU-treated and thermally modified wood. Quantitative PCR (qPCR) showed that the investigated *P. placenta* genes involved in oxidative degradation were expressed at higher levels in modified wood than in untreated wood, while expression levels in modified wood for the investigated genes involved in enzymatic degradation were slightly lower than those in untreated wood. The results indicate that the response of *P. placenta* upon the encounter of modified wood is to up-regulate the expression of the oxidative degradation machinery. In addition, our results support the theory that the decay resistance of the herein studied modified woods is due to inhibition of fungal molecules, needed for oxidative degradation of wood polymers, to penetrate the wood cell wall.

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1. Introduction

Modified wood materials have an improved resistance against wood decaying fungi (Hill, 2006). In contrast to traditional wood preservation, where the durability is achieved from the toxicity of the added chemicals and possibly non-recognition of substrate, very little is known about the mode of action of the nontoxic wood modifications and even less about fungal response to modified wood. Elucidating the response of the fungi towards modified wood may indicate the mechanisms behind the decay resistance and in what way wood modification can be improved.

Wood modification involves the action of a chemical, biological or physical agent upon the material, resulting in increased durability and/or other desired enhancements. Acetylation of wood is commonly done by reacting wood with acetic anhydride, which results in esterification of the accessible hydroxyl groups in the wood cell wall (Rowell et al., 1994). 1,3-dimethylol-4,5-dihydroxy ethylene urea (DMDHEU) modification is a wood modification with a water solution of DMDHEU (Militz, 1993). Indications of cross linking within the cell wall are found but the mechanisms of the reaction between DMDHEU and the wood cell wall are unknown (Yasuda et al., 1994; Krause et al., 2003). Thermal modification is a heat treatment of wood using mild pyrolysis, (Militz, 2002; Hakkou et al., 2006). During the process, hemicelluloses are degraded and autocondensation of lignin and lignin cross-linking with polysaccharides occur (Hakkou et al., 2006; Mohareb et al., 2011; Tjeerdsma et al., 1998).

Today, four established theories of mode of action of modified wood are prevalent: fungal enzyme inefficiency due to non-recognition (Rowell, 2005); fungal enzyme inefficiency due to lack of water at glycosidic bonds (Rowell, 2005; Rowell et al., 2009); reduced flow of fungal molecules into the wood cell wall due to micropore blocking (Hill et al., 2005); and inhibition of diffusion of fungal molecules due to insufficient amounts of moisture in the wood cell wall (Papadopoulos and Hill, 2002; Boonstra and Tjeerdsma, 2006). It has been shown that fungal enzymes are less efficient at degrading modified wood than untreated wood (Lekounougou et al., 2008; Venås, 2008; Verma and Mai, 2010), that acetylation and DMDHEU-treatment decreases micropore size (Hill et al., 2004; Dieste et al. 2009), that the size of the anhydride used in acetylation is more important than the number of OH-groups

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blocked (Papadopoulos and Hill, 2002; Hill, 2009) and that cross-linking in thermally modified wood is important for decay resistance (Rapp et al., 2008). To what extent the different modes of action contribute to decay resistance is unknown; however, the biochemical mechanisms of brown rot decay support the theory of inhibition of diffusion due to moisture exclusion (Ringman et al., 2013).

Among wood decaying fungi, brown-rotting basidiomycetes contribute to the most destructive type of decay in wooden buildings (Gilbertson and Ryvarden, 1986; Zabel and Morell, 1992). Brown rot fungi degrade wood through oxidative and enzymatic action (Goodell et al., 1997; Arantes et al., 2012). First, the fungus induces production of hydroxyl radicals inside the wood cell wall through secreting reductants and hydrogen peroxide (Goodell et al., 1997). The reductants reduce ferric iron (Fe^{3+}) in the wood cell wall to ferrous iron (Fe^{2+}) (Goodell et al., 1997, 2006), which react with the hydrogen peroxide and produce hydroxyl radicals in the Fenton reaction (Fenton, 1894). The hydroxyl radicals depolymerise hemicellulose and cellulose, modify lignin and generate sufficient rearrangements so that hydrolysing enzymes, which are too big to penetrate the intact wood cell wall, can diffuse into the cell wall and continue the degradation of the polysaccharides (Goodell et al., 1997; Baldrian and Valaskova, 2008; Arantes et al., 2012).

The most studied type of fungal reductants is hydroquinones (Baldrian and Valaskova, 2008; Arantes et al., 2012). After reduction of Fe^{3+}, the hydroquinones are recovered by a quinone reductase (Paszczynski et al., 1999; Jensen et al., 2002; Qi and Jellison, 2004). A protein model in *Postia placenta* (Ppl124517) is identified as a putative quinone reductase (Martinez et al., 2009) and transcription of this gene is significantly up-regulated in cellulose and lignocellulose medium (Martinez et al., 2009; Vanden Wymelenberg et al., 2010). Alcohol oxidase may contribute to extracellular production of hydrogen peroxide, since it has a preference for methanol which potentially is available from demethylation of lignin. A putative alcohol oxidase (Ppl118723) in *P. placenta* is transcribed at high levels and is highly up-regulated in cellulose medium compared to glucose medium (Martinez et al., 2009).

Following oxidative degradation, wood polysaccharides are further degraded enzymatically (Goodell et al., 1997; Fackler et al., 2007; Arantes et al., 2012). Enzymatic degradation by cellulose degrading fungi generally happens as follows: Endoglucanases cut the cellulose chains internally, providing ends for cellobiohydrolases to bind to and cleave off cellobiose units; and β-glucosidases subsequently hydrolyse cellobiose to glucose (Aro et al., 2005). However, brown rot fungi lack cellobiohydrolases (Baldrian and Valaskova, 2008). Processive endoglucanases, which both cleave cellulose internally and release oligosaccharides before detaching from the polysaccharide, could potentially substitute for their absence (Baldrian and Valaskova, 2008). Brown rot β-glucosidases are either intracellular, membrane bound or extracellular (Baldrian and Valaskova, 2008). They are relatively nonspecific and cleave xylose, mannose and galactose in addition to cellobiose (Baldrian and Valaskova, 2008). Sequencing of the genome of *P. placenta*, showed that this fungus possesses only two potential endoglucanases (Ppl115648 and Ppl103675) and several β-glucosidases (Martinez et al., 2009).

Previous studies have shown that genes involved in oxidative degradation of holocellulose in general are up-regulated during brown rot decay in modified wood, while genes involved in enzymatic degradation are not (Alfredsen and Fossdal, 2010; Schmöllerl et al., 2011; Pilgård et al., 2012).

To the knowledge of the authors, it is not known which regulatory mechanisms of the fungal wood degradation machinery are prevalent during brown rot degradation in modified wood. In order to improve the decay resistance of modified wood, it is of great importance to understand the decay mechanisms of incipient decay; however, fungal gene expression during this time-frame has not been extensively studied. Furthermore, the exact mechanisms for decay resistance in modified wood have still to be elucidated.

The aim of this study was to investigate the reaction of *P. placenta*, in terms of gene expression of alcohol oxidase Ppl124517, quinone reductase Ppl124517, endoglucanase Ppl103675 and β-glucosidase Ppl112501, upon the encounter of acetylated, DMDHEU-treated and thermally modified wood and to use these results for discussion of possible modes of action of modified wood.

Table 1
Primer sequences of target genes and endogenous control (previously used by Alfredsen and Fossdal (2010)).

Gene	JGI number	Primer sequences
β-tubulin	113,871	CAGGATCTTGTCGCCGAGTAC/ CCTCATACTCGCCCTCCTCTT
Alcohol oxidase	118723	CATCAAGAGCGCCAATCCAT/ GGCGCAAAGTCAGCCTTGT
Quinone oxidoreductase	124517	CGACGACAAGCCCAACAAG/ GATGACGATGGCGATTTTAGG
Endo-β-1,4-glucanases	103675	GTTCAGGCCGCATTGTCCT/ TTCCACCTGGCGTAATTGTG
Putative β-glucosidase	112501	TGCGCACGAATGAGTTGATAG/ CGCCTGCACACACACAACA

2. Experimental methods

2.1. Wood material and sample preparation

Boards of *Pinus sylvestris* sapwood were acetylated to 21 weight per cent gain, treated with 1.3 M DMDHEU using 5% MgCl2 as catalyst, or thermally modified according to the ThermoWood D scheme (212 °C). Miniblock samples (10 × 5 × 30 mm^3) (Bravery, 1979) were prepared from untreated and modified wood and leached according to EN 84 (1996). The samples were acclimatised in 20 °C and 65% RH for two weeks and subsequently sterilised with gamma radiation. To ensure similar conditions for fungal colonisation of all wood samples within each treatment (Junga and Militz, 2005), the samples were placed in pairs, one for each analysis, in Petri dishes containing 4% malt agar medium inoculated with *P. placenta* (strain FPRL 280). The exposed samples ($n = 4$) were harvested after 2, 6, 10, 14 and 56 days of incubation at 22 °C and 70% relative humidity. After harvesting, the mycelia covering the wood samples was manually removed and the samples were either dried and weighed for mass loss or frozen at −80 °C. Wood powder from the frozen samples was provided using a drill (3 mm bit diameter) (Jasalavich et al., 2000) followed by grinding with mortar and pestle.

2.2. RNA analysis

Total RNA was isolated from the wood samples and DNA was removed using MasterPure™ RNA Purification Kit (Epicenter), according to the manufacturer's instructions. RNA from each sample was converted into cDNA using TaqMan Reverse Transcription Reagents (using Oligo d (T)16) (Applied Biosystems) with 10 times the standard dNTP concentration. The samples were subsequently amplified with PCR (Rotor-gene, Qiagen). qPCR (Rotor-gene, Qiagen) was performed on cDNA samples ($n = 3$) using Rotor-gene SYBRGreen PCR kit (Qiagen). Primer sequences for target genes and endogenous control are listed in Table 1 (previously used by Alfredsen and Fossdal (2010)). As endogenous control, the house keeping gene β-tubulin was used. No PCR product was formed

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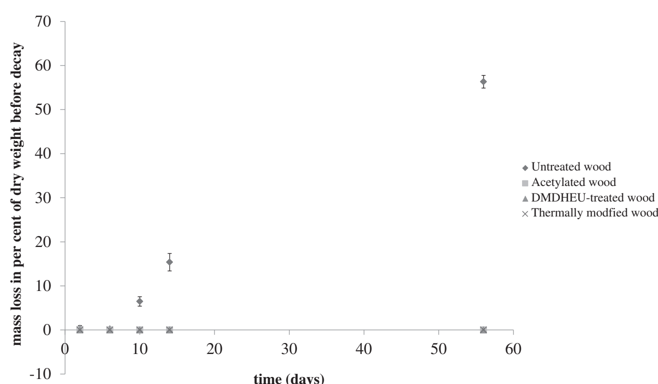

Fig. 1. Mass loss in per cent of dry weight before decay, mean values ($n = 4$) with error bars representing standard deviation. Circles – untreated wood; squares – acetylated wood; triangles – DMDHEU-treated wood; crosses – thermally modified wood.

using *P. sylvestris*. Only runs with reaction efficiency and R^2-values 0.8–1.2 were used for analysis. Standard curves were produced and the original concentration of each gene was calculated. Mean concentration of the target gene was calculated for each wood sample, and was normalised through division by the corresponding amount of endogenous control. Median values of the normalised target gene concentrations were calculated for each treatment and time point, and were plotted with error bars showing maximum and minimum values. Significance ($p < 0.5$) was calculated using independent 2-sample *t*-tests assuming unequal variances.

3. Results

3.1. Mass loss

During the time-course of the experiment, no mass loss was detected in the modified wood materials (Fig. 1). Mass loss in untreated wood was first detected at ten days and was >50% at 56 days.

3.2. Gene expression

Gene expression levels were analysed with qPCR (Fig. 2). The house-keeping gene β-tubulin, which is expected to be expressed at a constant level, was used as an endogenous control. The gene expression values were therefore not influenced by the amount of fungi in the sample.

3.2.1. Genes involved in oxidative degradation of holocellulose

The trends and levels of expression of quinone reductase Ppl124517 in modified wood differed from those in untreated wood and also between modifications (Fig. 2a). In untreated wood, the up-regulation of the expression of quinone reductase Ppl124517 between two and six days was significant. Even though there were no other significant changes in gene expression during the time course of the experiment, the results indicate that expression of this gene in untreated wood peaked during the first ten days of exposure. In acetylated wood, both the up-regulation between ten and 14 days and the subsequent down-regulation were significant. In DMDHEU-treated wood, expression of this gene seemed to be somewhat higher than in untreated wood already after two days of exposure and the down-regulation between six and ten days was significant. In thermally modified wood, there were no significant changes in expression of this gene throughout the time course of the experiment. However, the results indicate that the levels might be somewhat higher than in untreated wood. After 56 days, expression was down to levels similar to those at two days for all woods materials.

The expression of alcohol oxidase Ppl118723 was more similar between wood modifications, but still differed from that of untreated wood (Fig. 2b). The results indicate that there might be a small up-regulation of this gene in untreated wood between six and ten days of exposure; however no significant differences could be detected. All modified woods showed higher levels at 56 days than at two days of exposure; however, the difference was only significant in thermally modified wood. In acetylated and thermally modified wood, the up-regulation of expression of alcohol oxidase Ppl118723 between 10 and 14 days of exposure was significant. In DMDHEU-treated wood, the up-regulation of expression was somewhat delayed than in the other modified woods (between 14 and 56 days of exposure) but no significant difference between the timepoints could be detected.

3.2.2. Genes involved in enzymatic degradation of holocellulose

Expression levels and trends of endoglucanase Ppl103675 in the modified woods were similar to those in untreated wood (Fig. 2c). There were no significant differences between the levels of expression in any of the modified woods compared with the untreated wood at any given time-point, but the results indicate that expression levels in modified woods were slightly lower than in untreated wood. In untreated wood, the present study found no significant changes in expression of endoglucanase Ppl103675 during the time-course of the experiment. However, the results indicate that the expression was up-regulated between six and ten days of exposure, and subsequently down-regulated between ten and 14 days. In DMDHEU-treated wood, the up-regulation between six and ten days of exposure and the subsequent down-regulation were significant. The results from acetylated and thermally modified wood indicate the same trend as for DMDHEU-treated wood, but no significant changes could be detected.

The levels and trends of expression β-glucosidase Ppl112501 in the modified woods were similar to those in untreated wood (Fig. 2d). The up-regulation of this gene in untreated wood between six and ten days of exposure and the subsequent down-regulation were significant. In acetylated and thermally modified wood materials, the up-regulations of the expression of β-glucosidase Ppl112501 between six and ten days of exposure are significant and the results indicate the same trend in DMDHEU-treated wood. In spite of this up-regulation, the levels at ten days seemed to be lower in the modified woods than in the untreated wood. In thermally modified wood, the down-regulation between ten and 14 days of exposure was also significant. For acetylated and thermally modified wood, no significant down-regulation could be detected at this time-point; however, the results indicate the same trend. In DMDHEU-treated wood, the subsequent up-regulation of expression between 14 and 56 days of exposure was significant.

4. Discussion

The results of this study indicates that *P. placenta* up-regulates the genes involved in oxidative degradation upon the encounter of modified wood. These results are in accordance with previous results by Pilgård et al. (2012) and Schmöllerl et al. (2011). The results by Alfredsen and Fossdal (2010) on furfurylated wood is also in accordance with the results on acetylated, DMDHEU-treated and thermally modified wood in this study, which indicates a similar response by *P. placenta* to furfurylated wood as to the herein studied modified wood materials.

It is possible that the *P. placenta* quinone reductase Ppl124517 is not mainly involved in oxidative degradation. The brown rot *Gloeophyllum trabeum* possesses two quinone reductase genes with

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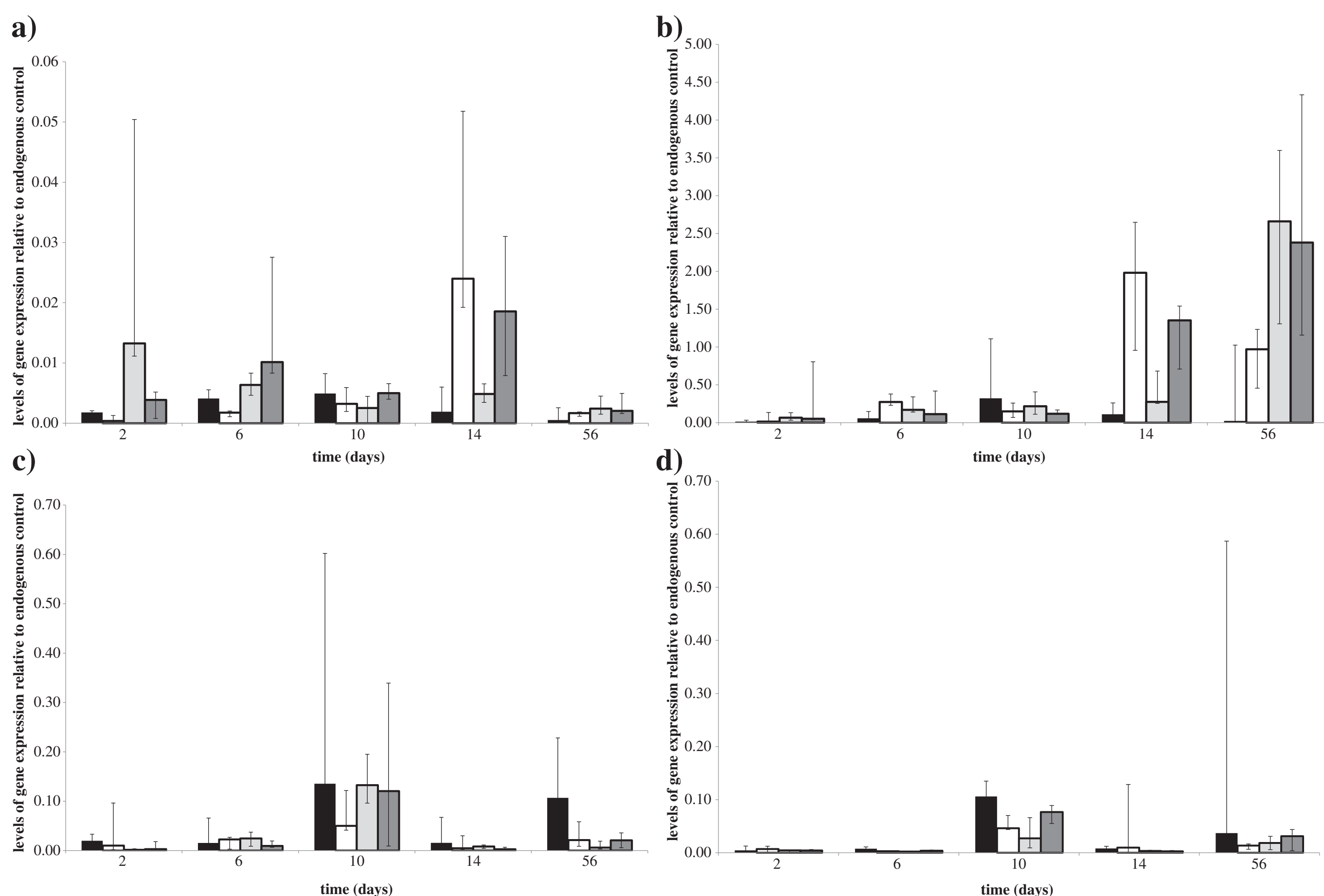

Fig. 2. Levels of gene expression relative to endogenous control of a) quinone reductase Ppl124517; b) alcohol oxidase Ppl118723; c) endoglucanase Ppl103675; d) β-glucosidase Ppl112501, median values ($n = 4$) with error bars representing maximum and minimum values. Black bars: untreated wood; white bars: acetylated wood; light grey bars: DMDHEU-treated wood; dark grey bars: thermally modified wood.

differential regulation (Cohen et al., 2004). One is shown to be involved in oxidative degradation of wood while the other is shown to be involved in the defence towards different kinds of stress (Cohen et al., 2004). *P. placenta* possesses only one gene encoding quinone reductase and it is currently unknown whether this is more important for oxidative degradation or for stress defence. In this study, the expression pattern of quinone reductase Ppl124517 differed from the expression pattern of alcohol oxidase Ppl118723 in the modified woods and it was also very different between wood modifications. It is possible that wood modification induces a stress response in fungi, since wood modification changes the moisture content, pH and other parameters (Militz 1993, 2002; Rowell et al., 1994; Hakkou et al., 2006).

Genes involved in enzymatic degradation, on the other hand, were, not generally up-regulated in *P. placenta* during degradation of modified wood. These results are in accordance with previous results by Pilgård et al. (2012) and Schmöllerl et al. (2011). Pilgård et al. (2012) saw higher levels in modified wood than in untreated wood at 20 and 36 weeks of exposure. It is possible that this up-regulation occurs after the eight weeks investigated in this study. The results by Alfredsen and Fossdal (2010) on furfurylated wood are also in accordance with the results on acetylated, DMDHEU-treated and thermally modified wood in this study.

The up-regulation of endoglucanase Ppl103675 and β-glucosidase Ppl112501 between six and ten days of exposure can be due to an unknown regulatory mechanism. While oxidative degradation causes depolymerisation and modification of the wood polymers, enzymatic degradation leads to that the polysaccharides are degraded into di- and mono-saccharides which are subsequently mineralized by the fungus (Baldrian and Valaskova, 2008; Arantes et al., 2012). Genes encoding enzymes involved in enzymatic degradation of holocellulose are constitutively expressed at low levels and induced by the presence of by-products from polysaccharide degradation (Aro et al., 2005). An up-regulation of these genes around the time where mass loss is first detected is therefore expected. We saw such an up-regulation in untreated wood; however, both endoglucanase Ppl103675 and β-glucosidase Ppl112501 are subsequently down-regulated. The up-regulation in the modified woods could not be due to induction by by-products from polysaccharide degradation, since there is no mass loss in these samples.

In this study, at the time-points after six days, the modified woods and the untreated wood were not in the same stage of decay. We saw no mass loss at all for any of the modified woods during the time course of the experiment, but for untreated wood mass loss was detectable at ten days. Therefore, differences in trends and levels of fungal gene expression in the modified woods compared to the untreated wood are expected.

As seen in Fig. 2, the range of variation in this experiment was large. This could be due to multiple factors. Firstly, since both wood and fungi have biological origin, it is possible that not all samples harvested at the same time-point were in the same stage of decay. The weight loss was measured on one set of samples and gene expression was measured on another set, due to that RNA is

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degraded by heat. This means that it is possible that the weight loss of the gene expression samples was different from that in the weight loss samples and they may have varied within each set. It would be possible to determine the stage of decay in each of the gene expression samples by measuring the fungal DNA content. Secondly, the treatment levels may have differed between samples with the same treatment. Thirdly, different amounts of cDNA may have been loaded for the different technical replicates. If different amounts of cDNA were loaded for the endogenous control and the gene of interest, the ratio would be misleading. However, differences in moisture content did not correlate with differences in gene expression.

The high levels of alcohol oxidase Ppl118723 in all modified woods, despite no detectable mass loss, indicate that the fungal molecules needed for the formation of radicals are not able to penetrate the wood cell wall. It is possible that this is due to that the moisture content in the wood cell wall is too low to allow for diffusion (Papadopoulos and Hill, 2002; Boonstra and Tjeerdsma, 2006). Verma and Mai (2010) showed that oxidative degradation enhances the efficiency of fungal enzymes to degrade DMDHEU-treated wood. Presumably, if oxidative degradation occurs, fungal enzymes would eventually be able to degrade the modified wood. Taken together, this indicates that wood modification protects wood rather from oxidative degradation than from enzymatic degradation. Further research on whether oxidative degradation occurs in modified wood is needed to be able to draw any conclusions on the mode of action of modified woods. In future studies, measuring the fungal DNA content in the RNA samples may help circumvent the issue of not knowing the mass loss of these specimens. More genes need to be investigated to be able to draw conclusions on how the oxidative and enzymatic degradations are regulated during degradation of modified wood. It would also be interesting to see what the gene expression looks like when the modified samples start to lose mass.

5. Conclusions

In this study, the investigated *P. placenta* genes presumed to be involved in oxidative degradation (quinone reductase Ppl124517 and alcohol oxidase Ppl118723) were expressed at higher levels in acetylated, DMDHEU-treated and thermally modified wood than in untreated wood. For the investigated genes involved in enzymatic degradation (endoglucanase Ppl103675 and β-glucosidase Ppl112501), the levels of expression in modified woods were equal to or lower than those in untreated wood. The results indicate that the response of *P. placenta* upon the encounter of modified wood is to up-regulate the expression of the oxidative degradation machinery. Furthermore, the results suggests that the *P. placenta* quinone reductase Ppl124517 may not be mainly involved in oxidative degradation of wood but could instead be involved in stress defence and that endoglucanase Ppl103675 and β-glucosidase Ppl112501 may be induced in modified wood through an unknown regulatory mechanism. In addition, the results support the theory that the decay resistance of the herein studied modified woods is due to inhibition of cell wall penetration of fungal molecules needed for oxidative degradation of wood polymers through, for example, insufficient cell wall moisture content to allow for diffusion.

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ORIGINAL

Effects of thermal modification on *Postia placenta* wood degradation dynamics: measurements of mass loss, structural integrity and gene expression

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Abstract The mechanism by which modified wood resists decay has long been discussed, but is still not fully understood. A better understanding of decay resistance mechanisms could improve wood protection processes and product properties. In this study, the dynamics of brown rot decay in thermally modified wood was examined through measurements of mass loss, strength loss and gene expression. Close monitoring of mass loss over 120 days in thermally modified wood exposed to *Postia placenta* showed a delay in the onset of degradation compared to untreated wood, and once the degradation had started, the rate was lower. Thermally modified wood did not inhibit expression of wood degradation-related genes before mass loss and was similar to that in untreated wood once mass loss could be detected. Comparing gene expression as well as strength loss at the same stage of decay rather than at the same time after exposure showed smaller differences in decay patterns between thermally modified and untreated wood than previous results indicate. It is concluded that the key to understanding degradation resistance in thermally modified wood is to compare the decay patterns in thermally modified wood and untreated wood before mass loss occurs.

Introduction

Biodegradation, or the decomposition of wood by microbes, is a natural process that is essential for nutrient cycling in forest ecosystems (Eriksson et al. 1990). When decomposition happens in wooden structures, it can lead to great economic losses.

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The fungal decay of wood products has traditionally been combated with broad-spectrum chemical preservatives. Because of restrictions and a growing environmental concern over toxic wood preservatives, new alternative wood protection systems are being developed that are based on technologies with low environmental impact (Hill 2006).

One of the most promising alternatives is wood modification, which is based on a non-toxic mode of action through a chemical and/or physical alteration of the wood (Hill 2006). Thermal modification is a heat treatment of wood using mild pyrolysis (>180 °C) (Militz 2002; Hakkou et al. 2006). During the process, hemicelluloses are degraded, lignin auto-condenses, and crosslinks with polysaccharides are created (Tjeerdsma et al. 1998; Hakkou et al. 2006; Mohareb et al. 2011). It is still not experimentally shown which mechanism(s) in the *Postia placenta* degradation process is affected by thermal modification. Mechanisms which might be affected include internal fungal processes such as gene regulation and expression as well as external processes such as the formation of radicals in the wood cell wall. With a better understanding of the fungal decay mechanisms in thermally modified wood, it can be revealed how fungal decay is inhibited or delayed, and subsequently, protection processes and product properties improved.

Brown rot fungi have been argued to be the most common and most destructive organisms involved in the degradation of softwood products (Goodell 2003; Vanden Wymelenberg et al. 2010). Brown rot fungi have traditionally been discriminated from white rot based on that white rot is able to degrade lignin, while brown rot is not. However, recent results show that there is rather a continuum between the two types of rot than a clear line (Riley et al. 2014). Furthermore, since brown rot fungi have evolved from white rot at least four times, the decay mechanisms may differ extensively between brown rot species (Eastwood et al. 2011). The brown rot wood degradation process has mainly been studied in *Gloeophyllum trabeum*, but certain aspects have been verified in, for example, *P. placenta, Coniophora puteana* and *Serpula lacrymans* (Hyde and Wood 1997; Cohen et al. 2002; Steenkjær Hastrup et al. 2013). However, certain differences have also been demonstrated as, for example, the amount of oxalic acid secreted and the role of laccase (Espejo and Agosin 1991; Green and Clausen 2005; Wei et al. 2010; Hastrup et al. 2012). *P. placenta* (Fr.) M.J. Larsen & Lombard is a very useful test organism because it has more capacity than most brown rot fungi to degrade thermally modified wood, but its ability to decay wood is adversely affected by thermal modification (Lekounougou and Kocaefe 2013, 2014a, b).

It has been hypothesised that *P. placenta*, as well as some other species of the orders Gloeophyllales, Polyporales and Boletales, secretes iron reductants, oxalic acid and hydrogen peroxide to initiate the Fenton reaction inside the wood cell wall and therefore form hydroxyl radicals that will depolymerise the polysaccharides and make them accessible for the cellulases to degrade (Goodell et al. 1997; Kerem et al. 1999; Paszczynski et al. 1999; Jensen Jr. et al. 2001). Extracellular hydrogen peroxide may be produced by alcohol oxidase since it has a preference for methanol, a product that is potentially available from the demethylation of lignin during degradation (Martinez et al. 2009). The *P. placenta* alcohol oxidase has been shown to be up-regulated in the presence of cellulose and wood compared to glucose

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(Martinez et al. 2009; Vanden Wymelenberg et al. 2010). Iron reductants and oxalic acid are formed as by-products in the fungus metabolism, but exactly which genes are responsible for the increased formation and/or secretion of these molecules during wood degradation is not known (Martinez et al. 2009; Vanden Wymelenberg et al. 2010). However, after reduction in ferrous iron, some iron reductants (hydroquinones) may be recovered by a quinone reductase (Paszczynski et al. 1999; Jensen et al. 2002; Qi and Jellison 2004). A protein model in *P. placenta* has been identified as a putative quinone reductase (Martinez et al. 2009). Methoxyhydroquinones may be oxidised by laccase into semiquinones which are expected to be better reductants of ferrous iron (Wardman 1989). However, it has also been suggested that the main role of the *P. placenta* methoxyhydroquinone/laccase system is to produce hydrogen peroxide (Wei et al. 2010). Four putative laccases have been found in *P. placenta* (Martinez et al. 2009; Vanden Wymelenberg et al. 2010). The regulation of cellulases is not known in *P. placenta*, but in other filamentous fungi, genes encoding cellulases are induced by by-products from the cellulose catabolism, such as sophorose, and in *P. placenta*, *these* genes are generally up-regulated during mass loss (Ilmén et al. 1997; Margolles-clark et al. 1997; Ringman et al. 2014b).

Genes encoding alcohol oxidase (Ppl118723), quinone reductase (Ppl124517) and laccase (Ppl111314) were highly expressed in *P. placenta* growing on acetylated, furfurylated, DMDHEU-treated and thermally modified wood while little or no mass loss was recorded (Alfredsen and Fossdal 2010; Schmöllerl et al. 2011; Pilgård et al. 2012; Ringman et al. 2014a; Alfredsen and Pilgård 2014). Even though durability tests of thermally modified wood have been reported, the dynamics of brown rot degradation has not previously been studied through close monitoring of relevant parameters in thermally modified wood, and therefore, it is not known to what extent decay is initially inhibited, for how long it is inhibited and at which rate degradation occurs once it has started. When comparing expression of genes involved in CMF degradation over time, previous studies have shown higher levels of expression of CMF-related genes in modified wood compared to untreated wood (Alfredsen and Fossdal 2009; Schmöllerl et al. 2011; Pilgård et al. 2012; Ringman et al. 2014b). Furthermore, these measurements were done on samples with little or no mass loss. From the current literature, it is therefore not possible to draw any conclusions on the effect of modification on the gene expression once degradation has started (Alfredsen and Fossdal 2009; Schmöllerl et al. 2011; Pilgård et al. 2012; Ringman et al. 2014b). An obstacle in the research on the brown rot decay resistance of modified wood is the difficulty to measure the amount of CMF degradation.

The aim of this study was to examine the dynamics of *P. placenta* wood degradation, in terms of mass loss, structural integrity and gene expression, in thermally modified wood and compare it to untreated wood. In particular, incipient decay was studied and the following questions were addressed: (1) Are *P. placenta* fungi degrading thermally modified wood already from the beginning of exposure but slowly, or is the degradation inhibited for some time? (2) If degradation is inhibited in the beginning of exposure, is it the CMF, the enzymatic degradation or both that are inhibited? (3) Once degradation has started, is it as rapid as in untreated wood?

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Materials and methods

Wood material and sample preparation

Miniblock specimens (10 × 5 × 25 mm^3) (Bravery 1979) were prepared from untreated and thermally modified (tightly wrapped in aluminium foil and heated to 220 °C for 4 h, with a median mass loss of 6.1 % due to the heat treatment) southern yellow pine (*Pinus elliotti*, *Pinus palustris*, *Pinus echinata*, *Pinus taeda*, *Pinus rigida* and *Pinus serotina*) sapwood samples and leached according to EN 84 (1996). The samples were sterilised with gamma radiation and placed two by two with other samples subjected to the same treatment (Junga and Militz 2005) in Petri dishes (90 mm) containing sterile soil and inoculated with *P. placenta* (strain FPRL 280) liquid culture. Samples were incubated at 22 °C and 70 % RH. Samples exposed to fungi were harvested regularly for a period of up to 126 days or until a mass loss of approximately 25 %. Untreated samples were harvested more frequently than thermally modified samples due to their higher growth rate. At harvest, the mycelia covering the wood samples were manually removed, and the samples were either (1) weighed for moisture content, dried and weighed for mass loss before being subjected to high-energy multiple impact (HEMI) tests or (2) dipped in liquid nitrogen and then frozen at −80 °C, awaiting molecular analysis.

RNA analysis

Samples for RNA analysis were chosen in the range of −0.9 % to 3.2 % mass loss. Wood powder from the frozen samples was produced using a Mixer Mill MM 400 (Retsch GmbH, Haan, Germany) using 1.5-cm steel balls and 30 Hz for 2 min. Total RNA was isolated from the wood samples (four biological replicates), and DNA was removed using the MasterPure™ RNA Purification Kit (Epicenter). RNA from each sample was converted into cDNA using TaqMan Reverse Transcription Reagents (using Oligo d(T)16) (Applied Biosystems) with 10 times the standard dNTP concentration. The samples were subsequently amplified with PCR (Rotor-gene, Qiagen). Real-time PCR (Rotor-gene, Qiagen) was performed on cDNA samples (three technical replicates) using the Rotor-gene SYBRGreen PCR Kit (Qiagen). Primer sequences for target genes and endogenous controls were prepared based on the genomic sequence of *P. placenta* MAD 698-R (Martinez et al. 2009 and available at http://genome.jgi-psf.org/Pospl1/Pospl1.home.html) and are listed in Table 1. Standard curves were produced, and the original concentration of each gene was calculated. All target genes were normalised to the endogenous control, β-tubulin. Significant differences ($p < 0.05$) in gene expression were calculated using independent two-sample t tests assuming unequal variances.

High-energy multiple impact (HEMI) tests

Samples were chosen in the range of −0.9 to 10 % mass loss. Three specimens of 10 (axial) × 5 × 10 mm^3 were cut from each of the four miniblock samples from

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Table 1 Primer sequences of target genes based on the genomic sequence of *P. placenta* MAD 698-R (Martinez et al. 2009, available at http://genome.jgi-psf.org/Pospl1/Pospl1.home.html)

Gene	JGI no.	Primer sequences
β-Tubulin	113871	CAGGATCTTGTCGCCGAGTAC/ CCTCATACTCGCCCTCCTCTT
Alcohol oxidase	118723	CATCAAGAGCGCCAATCCAT/GGCGCAAAGTCAGCCTTGT
Laccase	111314	CGGTGCTCTTGGCCACTTAG/CCATTGGTTATGGGCAGCTC
Quinone oxidoreductase	124517	CGACGACAAGCCCAACAAG/ GATGACGATGGCGATTTTAGG
Putative β-glucosidase	112501	CGGGTAACGCCATTGTTGAT/GCGCTTCGCAATGGTGTAC
Endo-β-1,4-glucanase	103675	GTTCAGGCCGCATTGTCCT/TTCCACCTGGCGTAATTGTG

untreated and thermally modified wood from each selected time point. The development and optimisation of the HEMI test have been described by Rapp et al. (2006). The following procedure was used: twelve oven-dried HEMI specimens were placed in the bowl of a heavy-impact ball mill, together with one steel ball of 40 mm, three of 12 mm and three of 6 mm diameter. The bowl was shaken for 60 s at a rotary frequency of 23.3 s^{-1} and a stroke of 12 mm. The fragments of the 12 specimens were fractionated on a slit screen (ISO 5223, 1996, slit width of 1 mm).

The following values were calculated: (1) the degree of integrity (I), which is the ratio of the mass of the 12 biggest fragments to the mass of all fragments after crushing, (2) the fine fraction (F), which is the ratio of the mass of fragments under 1 mm to the mass of all fragments, multiplied by 100, and (3) the resistance to impact milling (RIM), which is calculated from I and F as follows:

$$\mathrm{RIM} = (I - 3 \cdot F + 300)/4\ (\%)$$

The threefold weighting of the fine fraction was according to earlier studies (Rapp et al. 2006) and can finely distinguish between different intensities of fungal decay. To ensure that RIM varies between 0 and 100 %, the constant 300 was added. The calculated value is a mean value of the structural integrity of the four samples that run together (harvested at the same time point).

Results and discussion

Mass loss

To study the dynamics of degradation in thermally modified wood exposed to *P. placenta*, mass loss was closely monitored over 126 days. In this paper, any negative deviation in mass compared to the dry weight before fungal exposure is described as mass loss. Mass loss was first detected after 9 days of incubation in untreated wood and after 28 days in thermally modified wood (Fig. 1). Untreated wood reached a mass loss of 20 % after 28 days, whereas thermally modified wood reached 27 % after 126 days. For untreated wood, this is in accordance with

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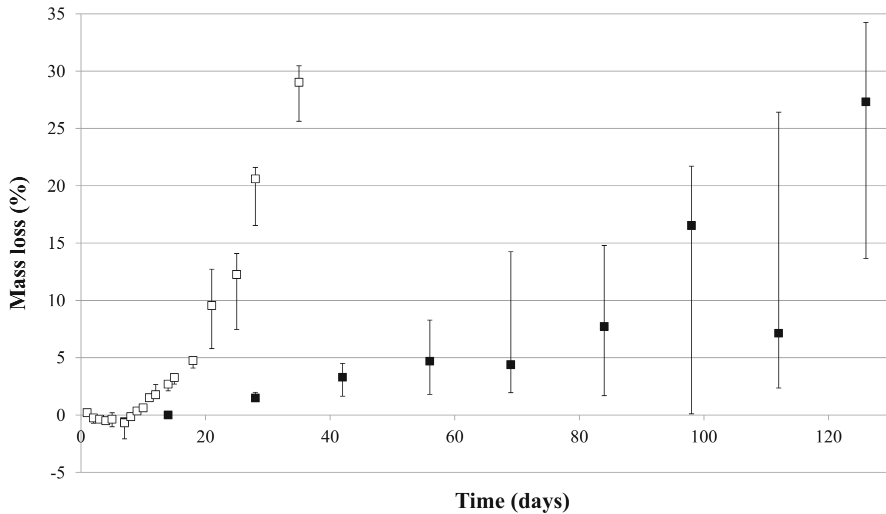

Fig. 1 Mass loss expressed as the per cent of dry weight before decay. *Black squares* thermally modified wood, *white squares* untreated wood. Median values ($n = 4$) with *error bars* representing maximum and minimum values

previous studies, but for thermally modified wood, the mass loss is dependent on several properties such as wood species, treatment temperature and time and therefore difficult to compare with results in the literature (Esteves and Pereira 2009; Ringman et al. 2014b). Mass loss was negative during the first week of exposure in both materials. This could be due to the fact that a mass loss will not show until the decrease in mass due to degradation becomes larger than the increase in mass due to the fungal colonisation, as shown earlier by Brischke et al. (2008). The standard deviation of mass loss in the thermally modified samples increased with time, which might be due to uneven treatment which becomes more visible the more mass is lost.

If a mass loss curve is plotted logarithmically, it is possible to detect three different stages similar to the phases seen in liquid fungal cultures; in the lag phase, the fungi adapt to the new environment, in the logarithmic phase, the growth rate of the fungi increases logarithmically, and in the stationary phase, the growth rate of the fungi decreases. In this experiment, untreated wood never reached the stationary phase. The mass loss curve for thermally modified wood resembled that of untreated wood, although thermally modified wood had a longer lag phase and a smaller gradient in the logarithmic phase. The results indicate that decay is initially inhibited by thermal modification, and, after degradation started, it has a reduced rate. In a recent review of established theories on mode of action of acetylated, furfurylated, DMDHEU-treated and thermally modified wood, it is suggested that moisture exclusion may inhibit decay for a certain amount of time, while, for example, micropore blocking and enzyme non-recognition might slow down degradation after its initiation (Ringman et al. 2014a).

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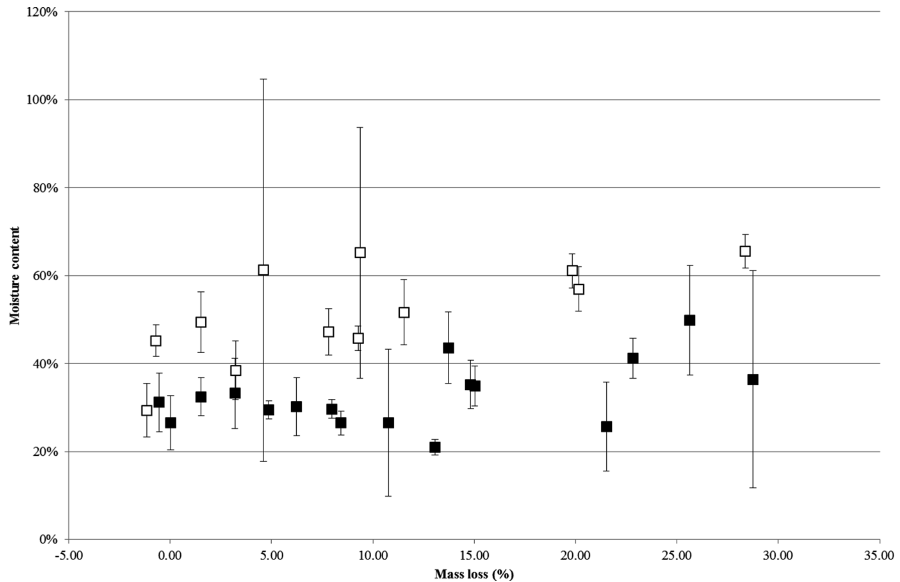

Fig. 2 Moisture content expressed as the per cent of dry weight before decay. *Black squares* thermally modified wood, *white squares* untreated wood. Mean values ($n = 4$)

Moisture content

Moisture content in thermally modified wood remained lower than that in untreated wood throughout the course of the experiment, with moisture contents of 20–40 % up to 15 % mass loss compared to 40–60 % in untreated wood with few exceptions (Fig. 2). The moisture content in untreated wood was significantly higher at 30 % mass loss than at negative mass loss due to an apparent increase in moisture content after approximately 20 % mass loss. Moisture content in thermally modified wood showed a similar trend, but due to large standard deviation there was no significant difference.

Structural integrity

Since CMF degradation causes loss of structural integrity even when mass loss cannot be detected, measurements of structural integrity were taken on exposed samples continuously throughout the experiment. The heat treatment itself decreased RIM by 28 % (Fig. 3), which is similar to previous results by Rapp et al. (2006). In untreated wood, HEMI results in samples with negative mass loss suggest a decrease in structural integrity compared to the zero-time sample (not inoculated with *P. placenta*, shown as large squares in Fig. 3). Since the presence of fungi was demonstrated by gene expression data (Fig. 4), the detected decrease in RIM could indicate CMF degradation. In thermally modified wood, there may be a slight increase in RIM for the negative mass loss samples, which might be explained

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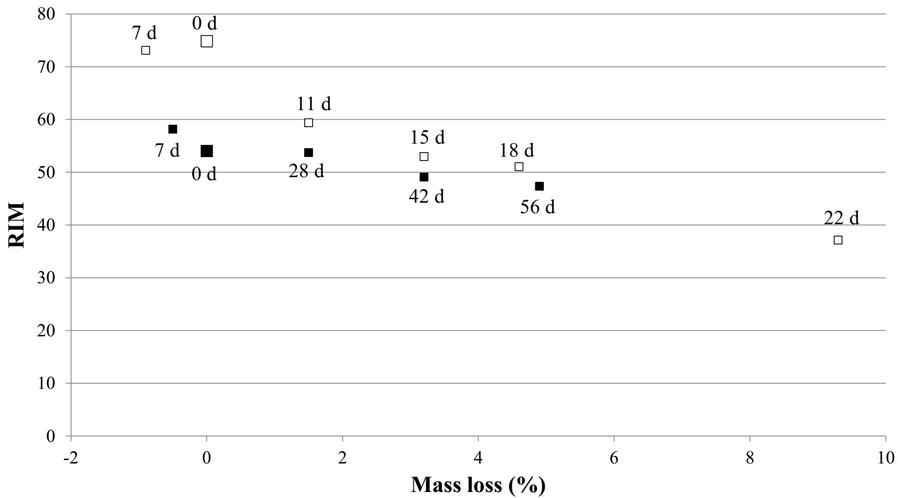

Fig. 3 Resistance to impact milling (RIM). *Black squares* thermally modified wood, *white squares* untreated wood. *Large squares* indicate zero-time samples (not inoculated with *P. placenta*). Exposure time is marked in the figure for each set of samples

by the gluing effect of ingrown mycelium (Brischke et al. 2008). Hence, no conclusion on whether CMF degradation was inhibited or not during incipient exposure of thermally modified wood could be drawn. When using tests of structural integrity, the problem with the gluing effect of the fungi must be considered and has to be overcome in order to use this method to determine the occurrence of CMF degradation. However, the structural integrity measurements of samples with positive mass loss support the findings in the mass loss measurements, since also the structural integrity shows similar patterns in thermally modified and untreated wood when compared at the same mass loss.

Gene expression

Gene expression studies were carried out to (1) see whether degradation is inhibited on gene expression level and (2) compare gene expression levels in samples at the same degradation state, measured as mass loss. In general, there was little significance between samples both over time and between treatments at the same mass loss. However, looking at the median values, some trends could be seen (Fig. 4).

In samples with negative mass loss and 3.25 % mass loss, no significant difference in alcohol oxidase (Ppl118723) gene expression could be seen between thermally modified wood and untreated wood. At 1.5 % mass loss, expression of this gene was approximately 10 times lower in two out of three untreated samples than in the thermally modified wood samples, but the third one was as high as the thermally modified wood samples and there was no significance. Looking at the data over time, the trend indicated that alcohol oxidase (Ppl118723) gene expression in

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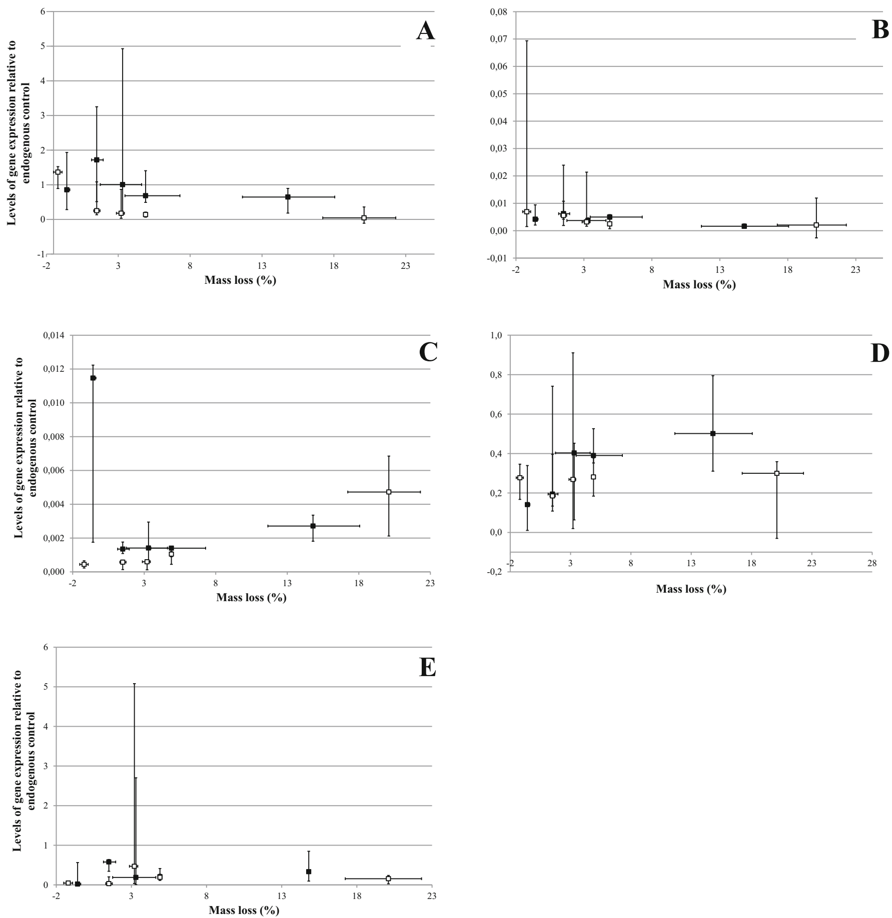

Fig. 4 Levels of gene expression relative to the endogenous control for **a** alcohol oxidase Ppl 118723, **b** laccase Ppl 111314, **c** quinone oxidoreductase Ppl 124517, **d** β-glucosidase Ppl 112501, **e** endo-β-1,4-glucanases Ppl 103675. *Black squares* thermally modified wood, *white squares* untreated wood. Median values ($n = 4$) with *error bars* representing maximum and minimum values

thermally modified wood went up while going down in untreated samples, but again there was no significant difference. The level of expression of this gene at negative mass loss was similar to the results for thermally modified wood after 1 week of exposure in Ringman et al. (2014b).

The gene expression of laccase (Ppl111314) was similar both over time and between treatments, no matter if the expression was plotted against mass loss or time, all through the experiment, and no significant differences were found. Expression of laccase (Ppl111314) has not been studied in thermally modified wood at this time of exposure and/or mass loss before.

The median value of expression of quinone reductase (Ppl124517) was 10–100 times higher in thermally modified wood samples than in untreated samples with

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negative mass loss, but there was no significant difference due to large variation. At 1.5 % mass loss, expression of quinone reductase was only three times as high in thermally modified wood samples as in untreated ones, but this difference was significant. The levels were similar at 3.25 % mass loss, but then there was no significant difference between treatments. The results indicate that the gene expression levels of quinone reductase (Ppl124517) were somewhat higher in thermally modified wood than in untreated wood all through the experiment, but when mass loss started, they were quite similar. The gene expression levels in this experiment were similar to the results in Ringman et al. (2014b), in which elevated quinone reductase (Ppl124517) gene expression could be seen in thermally modified wood after six days of exposure to *P. placenta*. In Ringman et al. (2014b), the gene expression pattern of quinone reductase (Ppl124517) differed in the differently modified wood materials and from the expression pattern of alcohol oxidase. It was therefore suggested that quinone reductase (Ppl124517) may not be involved in wood degradation. Cohen et al. (2004) showed that the two quinone reductases in *G. trabeum* are induced by different factors, and therefore, one was suggested to play a role in wood degradation, while the other might be involved in stress response. In the *P. placenta* genome, only one quinone reductase gene has been found (Martinez et al. 2009). It is therefore possible that the *P. placenta* quinone reductase is rather involved in stress defence, perhaps caused by the modification. When looking at the gene expression values over time, similar differences in expression patterns can be detected in this study. However, in Ringman et al. (2014b) none of the thermally modified samples showed any mass loss. The role of quinone reductase (Ppl124517) therefore remains to be found out.

β-Glucosidase (Ppl112501) and endoglucanase (Ppl103675) had similar gene expression levels all through the experiment. There were neither significant differences between time points or treatments nor signs of trends. Compared to the results in Ringman et al. (2014b), the expression levels for these genes were approximately 10 times higher in this study.

As shown in Fig. 4, the range of variation in this experiment was large. This could be due to multiple factors. The weight loss was measured on one set of samples and gene expression was measured on another set, because RNA is degraded by heat. This means that it is possible that the weight loss of the gene expression samples was different from that in the weight loss samples and they may have varied within each set. Furthermore, the treatment levels may have differed between samples with the same treatment.

Expression levels of genes involved in wood degradation could also be detected in thermally modified wood, which supports previous studies showing that expression of genes involved in wood degradation is not inhibited by wood modification. However, results from previous studies that defined degradation as a function of time instead indicated that expression of genes involved in CMF degradation was induced by acetylated, furfurylated, DMDHEU-treated and thermally modified wood (Alfredsen and Fossdal 2010; Schmöllerl et al. 2011; Pilgård et al. 2012; Ringman et al. 2014a; Alfredsen and Pilgård 2014). When looking at the gene expression levels in this study as a function of mass loss, there was no significant difference in *P. placenta* gene expression between

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thermally modified wood and untreated wood over the mass loss range of −0.9 to 20 %. Therefore, it is suggested that differences seen in previous studies could have been because samples in different degradation stages were compared. In this study, samples with the same mass loss were compared, and the results could indicate that once degradation has started, the fungi degrade thermally modified wood and untreated wood in a similar manner, a finding that is also supported by the analysis of structural integrity. Before mass loss can be detected, there are results that indicate that quinone reductase and laccase may be expressed at higher levels in modified wood than in untreated wood, but in this experiment there were no significant differences at negative mass loss. However, it should be noted that gene expression levels are related to the endogenous control, β-tubulin, that is supposed to but may not be expressed at similar levels in all cells at all times.

In this experiment, only one set of exposed replicates with negative mass loss from each treatment was analysed for gene expression. However, previous studies have shown that *P. placenta* expresses genes involved in CMF degradation at high levels in modified wood up to 36 weeks without being able to degrade the wood (Alfredsen and Fossdal 2010; Schmöllerl et al. 2011; Pilgård et al. 2012; Ringman et al. 2014b; Alfredsen and Pilgård 2014). This indicates that CMF degradation may hold the answer as to why modified wood resists brown rot fungi.

Conclusion

In this paper, the dynamics of degradation in thermally modified wood was addressed and compared to untreated wood. Mass loss results from this study showed that *P. placenta* degradation in thermally modified wood is initially inhibited, causing a longer lag phase than in untreated wood. Measurements of structural integrity using HEMI tests could not detect CMF degradation before mass loss was detected in thermally modified wood, probably due to a gluing effect of the fungal mycelia, but there were indications of CMF in untreated wood using this technique. However, more research is needed to confirm the finding and to verify the potential of HEMI as a reliable tool for CMF degradation analysis. Expression of neither genes involved in CMF degradation nor genes involved in enzymatic degradation was inhibited by thermal modification. Once the degradation started, the rate was lower than in untreated wood, but both structural integrity and gene expression showed similar patterns in both materials. This indicates that, once degradation has started, *P. placenta* uses the same mechanisms for degrading thermally modified wood as for degrading untreated wood. It is concluded that the key to understanding the decay resistance mechanism in thermally modified wood lies in the time from inoculation to detectable mass loss.

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IV

In vitro oxidative and enzymatic degradation of modified wood

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Fungal cellulases have been shown to be less efficient in modified wood than in untreated wood (Lekounogou *et al.* 2008; Venås 2008). However, Verma and Mai (2010) showed that cellulase efficacy is partly restored in 1,3-dimethylol-4,5-dihydroxyethyleneurea (DMDHEU) treated wood by pre-treatment with Fenton's reagent, simulating the oxidative degradation phase in initial brown rot decay. In this study, we examined whether Fenton derived hydroxyl radicals (·OH) and cellulases are able to cleave polysaccharides in furfurylated and acetylated wood and to what extent enzyme efficacy is increased by oxidative pre-treatment of these materials. The results show that fungal cellulases were able to degrade acetylated wood and that the cellulase efficacy was increased by oxidative pre-treatment by 20%, which is half of the increase in untreated wood. Furthermore, the results indicate that poly(furfuryl alcohol) is degraded by Fenton derived ·OH. This indicates a possible route for the eventual degradation of modified wood.

Keywords: Acetylated wood, Chelator mediated Fenton (CMF) degradation, Fenton's reagent, Furfurylated wood, Mode of action

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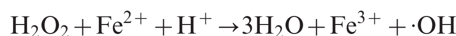
Introduction

Modified wood is an alternative to preservative treated wood and involves the action of a chemical, biological or physical agent upon the material, resulting in increased durability and/or other desired enhancements. During furfurylation of wood, furfuryl alcohol polymerises inside the wood and may bind to lignin (Nordstierna *et al.* 2008). Acetylation of wood is commonly achieved by reacting wood with acetic anhydride, which results in esterification of the accessible OH-groups in the wood cell wall (Rowell *et al.* 1994). Furfurylation and acetylation confers decay resistance and has been shown to be non-toxic (Pilgård *et al.* 2010; Lande *et al.* 2004). The mechanism behind the increased decay resistance in modified wood is not fully understood (Hill 2006; Ringman *et al.* 2013).

Brown rot fungi degrade wood through oxidative and enzymatic action (Goodell *et al.* 1997; Arantes *et al.* 2012). The fungus, growing in the lumen, induces production of ·OH inside the wood cell wall through secreting Fe^{3+}-reductants and H_2O_2 (Goodell *et al.* 1997). In the wood cell wall, the reductants reduce Fe^{3+} to Fe^{2+}, which react with the H_2O_2 and produce ·OH in the Fenton reaction (equation (1)) (Goodell *et al.* 1997; Goodell *et al.* 2006; Fenton 1894)



$$H_2O_2 + Fe^{2+} + H^+ \rightarrow 3H_2O + Fe^{3+} + \cdot OH$$

The ·OH cleaves hemicelluloses and cellulose internally, modify lignin and thereby generate sufficient rearrangements so that hydrolysing enzymes, which are too big to penetrate the intact wood cell wall, can diffuse into the wood cell wall and continue the degradation of the polysaccharides (Goodell *et al.* 1997; Baldrian and Valaskova 2008; Arantes *et al.* 2012).

Verma and Mai (2010) suggested that Fenton derived ·OH is able to cleave DMDHEU treated wood polysaccharides in a similar manner as in brown rot, based on that brown rot cellulase efficacy is enhanced after oxidative pre-treatment. Their study also shows that fungal cellulases are able to degrade polysaccharides in DMDHEU treated wood to almost the same extent as in untreated wood when using oxidative pre-treatment, even though enzyme efficacy in modified wood materials that has not been oxidatively pre-treated is substantially lower than in untreated wood (Lekounougou *et al.* 2008; Venås 2008; Verma and Mai 2010). It is not known whether ·OH is able to cleave polysaccharides in other kinds of modified wood. Furthermore, it is not known whether fungal cellulase efficacy can be restored in these wood materials as it is in DMDHEU treated wood.

The aim of this study was to examine whether ·OH is able to cleave polysaccharides in furfurylated wood and to what extent enzyme efficacy is restored by oxidative pre-treatment in these materials.

Experimental methods

Wood material

Southern yellow pine sapwood was cut to $30 \times 10 \times 5$ mm samples, leached according to EN84 (EN

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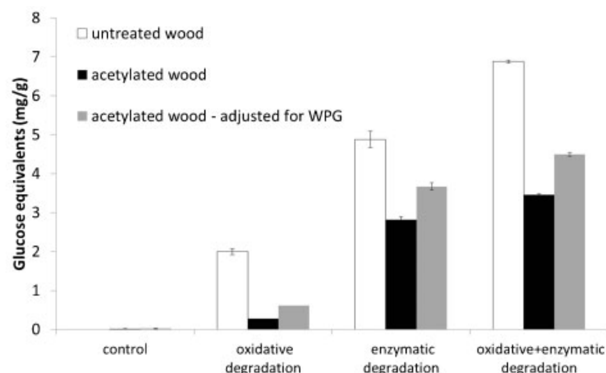
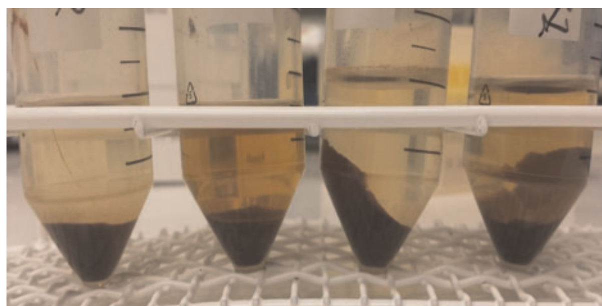


1 Efficacy of *in vitro* simulated oxidative, enzymatic and oxidative+enzymatic degradation



2 Furfurylated wood meal (from left to right): untreated, treated with Fenton's reagent, treated with cellulases, treated with Fenton's reagent followed by cellulases

84 1996) and dried. A portion of the samples was furfurylated in the laboratory at NFLI (Ås, Norway) according to Lande *et al.* (2004) with the exception that the samples were soaked in the modification mixture overnight to ensure full penetration, to an average weight per cent gain (WPG) of 91·5%. Another portion was acetylated at the laboratory at SP Wood Science (Borås, Sweden) according to Larsson-Brelid (1998), to an average WPG of 22%.

Oxidative and enzymatic degradation of wood powder

The degradation tests were carried out in triplicates and performed according to Verma and Mai (2010) with minor changes. For *in vitro* simulation of oxidative degradation followed by enzymatic degradation, 0·5 g of wood powder was incubated in 12·5 mL 100 mM sodium acetate (NaAc) buffer (pH 5·0) with 0·5 mM $(NH_4)Fe(SO_4)_2$ and 1% H_2O_2 in 23°C on a shaker (150 rev min^{-1}) for 24 h. The tubes were centrifuged at 7850 rev min^{-1} for 5–10 min and the supernatant was removed. The wood powder was washed three times with sterile deionised water. 10 mL sterile deionised water and 0·125 mL Kieralon antiperox (BASF, Limburgerhof, Germany) was added and the tubes were incubated for 3 h on a shaker (250 rev min^{-1}) in 23°C. The tubes were again centrifuged at 7850 rev min^{-1} for 5–10 min and the supernatant was removed. The wood powder was washed three times with sterile deionised water. 10 mL NaAc buffer and 28 U of Celluclast 1·5 L (Sigma-Aldrich Sweden, Stockholm, Sweden) were added and the tubes were incubated on a shaker (250 rev min^{-1}) in 23°C for ~160 h.

In order to ensure the same experimental conditions, all samples were washed according to the routine above. Treatment with Kieralon antiperox was shown not to affect cellulase activity (data not shown), therefore this step was left out in the other samples. For *in vitro* simulation of oxidative degradation, 0·5 g of washed wood powder was incubated in 12·5 mL NaAc buffer in 50 mL tubes with 0·5 mM $(NH_4)Fe(SO_4)_2$ and 1%H_2O_2 in 23°C on a shaker (250 rev min^{-1}) for ~160 h. For *in vitro* simulation of enzymatic degradation, 0·5 g of washed wood powder was incubated in 12·5 mL NaAc buffer with 28 U of Celluclast 1·5 L in 23°C on a shaker (250 rev min^{-1}) for ~160 h.

The pH of NaAc buffer inoculated with wood meal was 5·05±0·05 for all wood treatments.

Reducing sugar assay

Reducing sugar content was assayed according to Miller (1959) and Verma and Mai (2010). Each tube was assayed in triplicates. The tubes were centrifuged at 7850 rev min^{-1} for 5–10 min. 1 mL supernatant was transferred to a 2 mL tube. When necessary, the samples were diluted in NaAc buffer. 1 mL of DNS solution (0·63% 3,5-dinitrosalicylic acid, 0·57% phenol, 0·5%Na HSO_3, and 2·14%NaOH) was added and the tubes were incubated at 100°C for 10 min. The tubes were subsequently cooled down to room temperature and the absorbance was measured at 540 nm. A standard curve was prepared from a glucose solution.

Results and discussion

The results from the untreated wood are in accordance with previously published results by Verma and Mai (2010), except for the results after treatment with Fenton's reagent (Fig. 1). In our study, almost 2 mg reducing sugars per g wood meal were released after treatment with Fenton's reagent while in the study by Verma and Mai's levels of released reducing sugars are lower than 1 mg g^{-1} which was also almost the same as the control. However, in both studies, the efficacy of the cellulases was increased after pre-treatment with Fenton's reagent. In this study the increase was approximately 40%.

The results from the acetylated wood meal show that brown rot cellulases were able to degrade acetylated wood (Fig. 1). Furthermore, the efficacy of the cellulases was increased after pre-treatment with Fenton's reagent by approximately 20%. The levels were overall lower in the acetylated wood. Also after adjustment for the average WPG of the acetylated wood, the levels of released reducing sugars were lower in the acetylated wood than in untreated wood (Fig. 1).

This is in accordance with the study by Verma and Mai (2010), where the levels of released reducing sugars were shown to be lower in DMDHEU treated wood compared to untreated wood. When treating modified wood meal with Fenton's reagent, lower levels of released reducing sugars may be due to that ·OH is reacting also with the modification chemical. When treating modified wood meal with cellulases, lower levels of released reducing sugars may be due to that the acetyl groups are physically hindering the cellulases to gain access to the polysaccharides. The increased efficacy of the cellulases seen after pre-treatment with Fenton's

reagent may in that case be due to that the ·OH has cleaved off the acetyl groups.

The increase in cellulase efficacy after pre-treatment with Fenton's reagent is higher in DMDHEU treated wood than in untreated wood (Verma and Mai 2010), while the increase in acetylated wood was lower than the untreated wood. This may be due to that the WPG of the DMDHEU treated wood meal used was 7%, while the WPG of the acetylated wood meal in this study was 22%. If this is true, a more effective treatment with Fenton's reagent would increase cellulase efficacy in acetylated wood even further.

When treating furfurylated wood meal with Fenton's reagent, the supernatant turned brown, while the supernatant in the control was uncoloured (Fig. 2). Even after washing with water and treatment with cellulases the brown colour remained, although somewhat lighter. This is an indication that the Fenton derived ·OH was able to degrade poly(furfuryl alcohol).

However, the degraded poly(furfuryl alcohol), possibly in the form of furfurals or as sugar contaminations from the production process, reacted with the DNS reagent and thus made it impossible to separate the reducing sugars released from the holocellulose from the poly(furfuryl alcohol) remnants. We were, however, able to confirm previous results by Venås (2008) that brown rot cellulases are able to degrade furfurylated wood.

Today, four established theories of mode of action of modified wood are prevalent: fungal enzyme inefficacy due to non-recognition (Rowell 2005); fungal enzyme inefficacy due to lack of water at glycosidic bonds (Rowell 2005; Rowell *et al.* 2009); reduced flow of fungal molecules into the wood cell wall due to micropore blocking (Hill *et al.* 2005); and inhibition of diffusion of fungal molecules due to insufficient amounts of moisture in the wood cell wall (Papadopoulos and Hill 2002; Boonstra and Tjeerdsma 2006). To what extent the different modes of action contribute to decay resistance is unknown; however, the biochemical mechanisms of brown rot decay support the theory of inhibition of diffusion due to moisture exclusion (Ringman *et al.* 2013).

The results from this study suggest that poly(furfuryl alcohol) can be degraded by Fenton derived ·OH. This may explain why furfurylated wood is eventually degraded. At a place in the wood cell wall where the WPG is locally low enough to allow diffusion of the fungal molecules needed for degradation, ·OH might form. The radicals might attack the modification chemicals inside the wood cell wall and thereby the area with low WPG will expand and even more ·OH might form. Alternatively, very low amounts of ·OH may be formed in the lumen and attack the wood cell wall from the outside.

Conclusion

Brown rot cellulases were able to degrade acetylated wood. Cellulase efficacy was increased after treatment with Fenton's reagent, but not to the same extent as in untreated wood. This shows that Fenton derived ·OH facilitates cellulase degradation, possibly by depolymerisation of polysaccharides and/or removal of acetyl groups from wood polymers. Furthermore, the results from this study indicate that Fenton derived ·OH is able to degrade poly(furfuryl alcohol). These results makes it tempting to speculate that the eventual degradation of furfurylated wood might be due to low levels of ·OH formation in the lumen or at places inside the wood cell wall where the WPG is low enough to allow for diffusion. Once ·OH has been formed, poly(furfuryl alcohol) may start to be degraded.

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Review

Rebecka Ringman*, Annica Pilgård, Christian Brischke and Klaus Richter

Mode of action of brown rot decay resistance in modified wood: a review

Abstract: Chemically or physically modified wood materials have enhanced resistance to wood decay fungi. In contrast to treatments with traditional wood preservatives, where the resistance is caused mainly by the toxicity of the chemicals added, little is known about the mode of action of nontoxic wood modification methods. This study reviews established theories related to resistance in acetylated, furfurylated, dimethylol dihydroxyethyleneurea-treated, and thermally modified wood. The main conclusion is that only one theory provides a consistent explanation for the initial inhibition of brown rot degradation in modified wood, that is, moisture exclusion via the reduction of cell wall voids. Other proposed mechanisms, such as enzyme nonrecognition, micropore blocking, and reducing the number of free hydroxyl groups, may reduce the degradation rate when cell wall water uptake is no longer impeded.

Keywords: acetylated wood, basidiomycetes fungi, DMDHEU-treated wood, furfurylated wood, mode of action, thermally modified wood

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Introduction

At present, the use of traditional toxic wood preservatives is being questioned and their application is restricted (Hingston et al. 2001; Ibach 2005; Townsend et al. 2005). As alternatives to these preservatives, wood can be modified by nontoxic chemicals and/or by physical treatments (Hill 2006). The optimization of processes and product properties could be further improved by a better understanding of the underlying mechanisms of inhibition or delay of biological degradation after nontoxic modifications. Decay resistance has been reviewed independently for acetylated wood (Papadopoulos 2010) and thermally modified wood (Esteves and Pereira 2009) as well as in a combined review (Rowell et al. 2009). Previous research concerning the degradation of cellulose by brown rot fungi was summarized by Baldrian and Valaskova (2008). Aro et al. (2005) reviewed the transcriptional regulation of enzymes involved in the breakdown of cell wall biopolymers by filamentous fungi. The biochemical mechanisms that underlie nonenzymatic degradation of wood were recently summarized in a comprehensive review (Arantes et al. 2012). However, general comparative reviews are not available in terms of the modes of action of different wood modification methods. In particular, only a few studies are dealing with the biochemical mechanisms of decay resistance in modified woods.

Thus, the present review attempts a comparative evaluation of established theories related to the mode of action of brown rot fungi resistance in modified woods with biochemical mechanisms in focus. The following modifications are considered: acetylated wood (Militz 1991; Larsson Brelid et al. 2000), furfurylated wood (Schneider 1995; Westin 1996; Lande et al. 2008; Bryne and Wålinder 2010; Thygesen et al. 2010), dimethylol dihydroxyethyleneurea (DMDHEU)-treated wood (Militz 1993; Dieste et al. 2009b), and thermally modified wood (Tjeerdsma et al. 1998; Welzbacher 2007; Windeisen et al. 2009; Pfriem et al. 2010).

Degradation of wood by brown rot

Brown rot wood degradation has been studied mainly based on *Gloeophyllum trabeum*, although some mechanisms have also been confirmed in *Postia placenta*, *Serpula lacrymans*, and others (Goodell et al. 1997; Shimokawa et al. 2004; Martinez et al. 2009). Differences between species have also been reported, which support the theory of the repeated evolution of brown rot fungi from white rot fungi (Green and Highley 1996; Hibbett and Donoghue 2001; Niemenmaa et al. 2008; Kang et al. 2009).

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Degradation of polysaccharides

Details of polysaccharide degradation by brown rot are summarized in Figure 1, which is based mainly on studies by Goodell et al. (1997), Baldrian and Valaskova (2008), and Arantes et al. (2012). Brown rot fungi degrade polysaccharides via oxidative and enzymatic activities (Eriksson et al. 1990; Eaton and Hale 1993; Goodell 2003; Aro et al. 2005; Fackler et al. 2010). The fungus induces the production of hydroxyl radicals within the polymer matrix by the secretion of reductants and hydrogen peroxide (Goodell et al. 1997). The reductants reduce ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}), which reacts with hydrogen peroxide to produce hydroxyl radicals via the Fenton reaction (Fenton 1894). The hydroxyl radicals then depolymerize hemicelluloses and cellulose and modify lignin (Halliwell 1965; Cohen et al. 2002; Kaneko et al. 2005). During these reactions, microcapillary pathways are generated so that hydrolyzing enzymes, which are too large to penetrate the intact wood cell wall, can diffuse into the previously damaged matrix. In this way, the degradation of polysaccharides is initiated, in the course of which oligomers, dimers, and monomers via enzymatic hydrolysis arise. Enzymatic degradation, which becomes noticeable by compositional changes, can be detected only in the advanced state of the decay process (Fackler et al. 2010).

The first signs of brown rot decay are visible in the outer regions of the cell wall, that is, in the middle lamella, S1, and the outer parts of S2 (Irbe et al. 2006; Fackler et al. 2010). The S3 layer remains intact throughout the early stages of decay, which contributes to the limited permeability of enzymes into the S2 layer (Highley et al. 1985; Eriksson et al. 1990; Kleman-Leyer et al. 1992). The formation of hydroxyl radicals only inside the wood cell wall and the delayed degradation of the S3 layer may protect the hyphae from oxidative injuries.

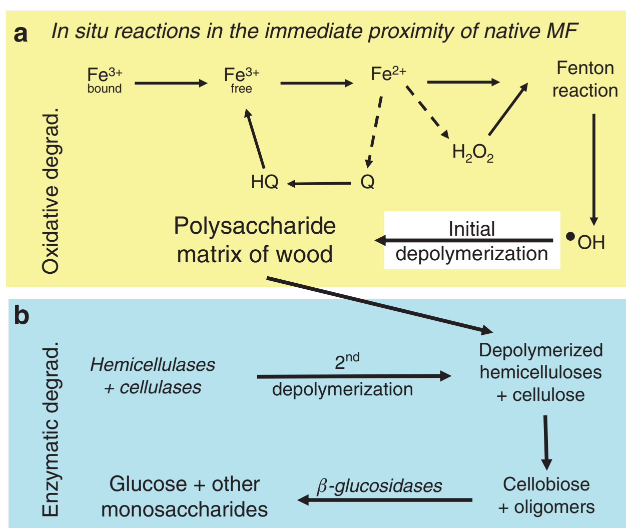

Figure 1 Polysaccharide degradation in untreated wood. Based on Goodell et al. (1997), Arantes et al. (2012), and Baldrian and Valaskova (2008). HQ, hydroquinone; MF, microfibrils; Q, quinone.

Hemicelluloses surround the cellulose fibrils (Zabel and Morell 1992) and they are amorphous compared with to the mainly crystalline cellulose. Their higher accessibility is the reason why hemicelluloses are degraded before cellulose (Curling et al. 2002; Irbe et al. 2006; Fackler et al. 2010).

Oxidation of lignin

Brown rot fungi circumvent lignin mineralization and degrade the polysaccharides in a straightforward manner (Koenig et al. 2010; Martinez et al. 2011). Lignins maintain their macromolecular nature throughout brown rot decay (Kirk 1975; Niemenmaa et al. 2008; Yelle et al. 2008), but their structure is modified substantially by hydroxyl radicals (Yelle et al. 2008, 2011; Arantes et al. 2009, 2011; Martinez et al. 2011). This can be detected in the outer parts of the cell wall based on increases in the carbonyl group content and demethylation (Eriksson et al. 1990; Filley et al. 2002; Fackler et al. 2010). Lignin modification contributes to the formation of microcapillary pathways that allow enzymes to penetrate the wood cell wall (Arantes et al. 2012). It has been suggested that fragments of oxidatively remodeled lignin in brown-rotted wood may contribute to the formation of hydroxyl radicals (Xu and Goodell 2001; Filley et al. 2002; Goodell et al. 2006) as an integral part of the redox processes. The strong relationship between lignin demethylation and polysaccharide degradation suggests that these processes are mechanistically interlinked (Filley et al. 2002). However, no water-soluble lignin products with Fe^{3+}-reducing activities have been detected in brown rotted wood (Aguiar et al. 2013).

Mode of action theories from a biochemical perspective

Table 1 provides an overview of established theories related to the protective effects of wood modification. Despite the higher decay resistance of modified wood, these modifications do not inhibit the expression of the fungal genes required for degradation. Alfredsen and Fossdal (2010) showed that genes encoding enzymes involved in oxidative degradation are expressed at higher

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Table 1 Theoretical modes of action of brown rot decay resistance in modified wood.

Mode of action	Principle of action	Proposed mechanism	References
Biochemical	Unavailability of easily accessible nutrients	Lack of easily accessible nutrients, such as hemicelluloses, and this prevents cellulose from fungal decay.	Boonstra et al. 2007; Rowell et al. 2009
	Enzyme nonrecognition	Wood polymers are modified in such a way that fungal enzymes no longer recognize them; therefore, enzymatic hydrolysis is not possible.	Rowell 2005
Physical	Micropore blocking	Micropores in the cell wall are blocked, so that fungal reductants and enzymes penetrate the cell wall at a slower rate.	Hill et al. 2005
	Moisture exclusion		
	OH-group blocking/ reduction	OH-groups in the cell wall are blocked or hindered, so that less water binding sites are available, which leads to a less efficient hydrolysis of polysaccharides.	Rowell et al. 2009
	Reduction in void volume	The amount of free water in the cell wall is diminished through bulking of voids by chemicals or by reduction of void sizes, so that diffusion of fungal reductants and enzymes is impeded.	Papadopoulos and Hill 2002; Boonstra and Tjeerdsma 2006

levels in furfurylated wood than in untreated one. Pilgård et al. (2012) reported similar findings for acetylated wood. However, it is not known whether these genes are translated into proteins that can induce the formation of hydroxyl radicals in the wood cell wall.

Role of accessible nutrients

As pointed out above, brown rot fungi degrade hemicelluloses before cellulose (Militz 2002; Weiland and Guyonnet 2003; Rowell et al. 2009) and this order of degradation is considered by many investigators to be a key step (Boonstra et al. 2007; Rowell et al. 2009). Rowell et al. (2009) proposed that arabinose, which is the only L-pentose sugar found in wood, is a key compound that triggers the chain reactions of degradation and that its absence would prevent the initiation of degradation. Indeed, most hemicelluloses are degraded in thermally modified wood (Boonstra et al. 2007) and hemicelluloses are considerably modified by acetyl groups in acetylated wood (Rowell et al. 2009).

However, the biochemical mechanism of brown rot does not support this theory. Hemicelluloses are degraded in the same manner as cellulose; oxidative degradation occurs, which is mediated by hydroxyl radicals followed by enzymatic hydrolysis (Arantes and Milagres 2006; Fackler et al. 2010; Arantes et al. 2011). Cellulases are constitutively expressed at a low level and are up-regulated when the fungus starts to metabolize any type of sugar (Aro et al. 2005), which shows that the degradation of hemicelluloses is not necessary for the expression of cellulases. It seems unlikely that a microorganism would not degrade one particular nutrient in the absence of another one. Most microorganisms, including fungi, can degrade many different types of nutrient sources (Wainwright 1988).

Nonrecognition of substrates by enzymes

Rowell (2005) proposed that fungal enzymes are unable to recognize their substrates in modified wood (Figure 2A). This theory may be supported by the fact that hemicellulases and cellulases are less efficient in thermally modified, DMDHEU-treated, and furfurylated wood (Lekounougou et al. 2008; Venås 2008; Verma and Mai 2010). Their action is, however, not inhibited. Furthermore, the nonspecific oxidative degradation that precedes enzymatic degradation is highly effective even without enzymatic hydrolysis (Verma and Mai 2010). Thus, this theory does not provide an explanation for the initial inhibition of oxidative degradation.

Blocking of micropores

Hill et al. (2004) suggested that a decrease in the micropore size might reduce the rate of diffusion into the wood cell wall by fungal reductants. The bulking effect caused by acetylation and DMDHEU treatment has been shown to reduce the size of micropores (Hill et al. 2004; Dieste et al. 2009b).

However, this theory does not explain the initial inhibition of decay, because micropore blocking can only reduce rate of diffusion of fungal reductants rather than inhibit their diffusion (Hill et al. 2005). Thus, micropore

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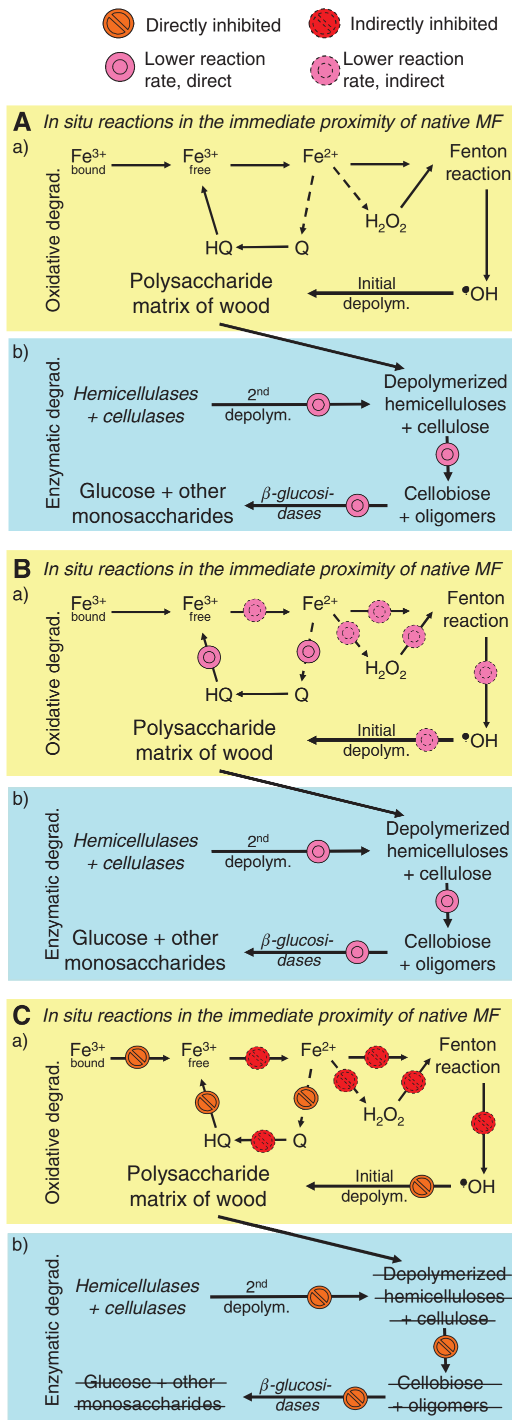

Figure 2 Illustration of the effects of different mechanisms of brown rot decay resistance wood polysaccharide degradation as described in Table 1.
(A) Enzyme nonrecognition and moisture exclusion due to the reduced availability of free OH-groups. (B) Micropore blocking. (C) Moisture exclusion by reducing of the cell wall void volume.

blocking will only slow down oxidative degradation and the formation of the microcapillary pathways required for enzyme diffusion and subsequent hydrolysis (Figure 2B).

Exclusion of moisture via the reduction of accessible OH-groups

It has been suggested that the reduced availability of water binding sites may lead to a lower equilibrium moisture content (EMC) observed in modified wood (Rowell et al. 2009). The same authors have also proposed that a lack of water bound to OH-groups may impede the hydrolysis of glycosidic bonds and the subsequent formation of cellobiose and monomeric sugars.

OH-groups are blocked by acetylation and reduced in number by thermal modification because of the loss of hemicelluloses (Phuong et al. 2007; Rowell et al. 2009). However, Hill et al. (2005, 2009) and Papadopoulos and Hill (2002) showed that the number of OH-groups blocked in acetylated wood do not correlate with decay resistance. Accordingly, the loss of hemicelluloses from thermally modified wood does not correlate with decay resistance (Hakkou et al. 2006; Welzbacher 2007). The number of available (free) OH-groups is increased in DMDHEU-treated wood, which supports the subordinate role of free OH-groups in decay resistance (Dieste et al. 2009a). The loss of water from glycosidic bonds may reduce the efficiency of fungal hydrolases but not completely inhibit it (Lekounougou et al. 2008; Venås 2008; Verma and Mai 2010). Furthermore, oxidative degradation would not be affected.

Exclusion of moisture through decrease in cell wall void volume

Reduction in the wood cell wall void volume may mean that there is insufficient water inside the wood cell wall to allow diffusion (Papadopoulos and Hill 2002; Rapp et al. 2008). If fungal reductants, hydrogen peroxide, and oxalic acid cannot diffuse into the wood cell wall, hydroxyl radicals will not be formed and oxidative degradation will not occur.

The bulking effect of acetylation leads to a reduced EMC (Ibach and Rowell 2000; Papadopoulos and Hill 2003). The correlation between weight percent gain and decay resistance was unambiguous in Corsican pine (*Pinus nigra*), whereas the results were less clear or even contradictory in a number of other wood species (Hill et al. 2004, 2009; Hill 2009). The more even distribution of acetyl groups in Corsican pine may explain the effectiveness of decay prevention (Habu et al. 2006) compared with Scots pine investigated by Hill et al. (2004, 2009). Crosslinking in thermally treated wood reduces the swelling capacity and lowers EMC, which correlates

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well with decay resistance (Hakkou et al. 2006; Rapp et al. 2008). After the heat-induced cross-linking was disrupted by treatment with KOH, EMC and decay resistance declined to the levels found in untreated wood (Rapp et al. 2008). The bulking effect and the subsequent exclusion of moisture from the smaller voids are most important for the decay resistance caused by DMDHEU modification (Dieste et al. 2009a,b). Reducing of the void volume via bulking is a possible explanation for the increased decay resistance in furfurylated wood (Venås 2008).

The theory of moisture exclusion by reducing the void volume may explain the inhibition of oxidative and enzymatic degradation (Figure 2C). Furthermore, it explains the delayed but eventual onset of degradation, because the EMC will increase with time due to moisture uptake from the surrounding air via mall cracks and voids in the wood cell wall (Welzbacher and Rapp 2004; Thygesen et al. 2010).

Proposed mechanism

No degradation occurs during the first contact between brown rot fungi and modified wood. Brown rot fungi colonize modified wood only when sufficient water has accumulated in the lumen. The fungi secrete reductants, hydrogen peroxide, and oxalic acid, but they are unable to diffuse into the cell wall matrix due to lack of water for diffusion. Thus, hydroxyl radicals cannot be formed and oxidative degradation does not occur (Figure 2Ca). The reductants can diffuse only if the cell walls lose their ability to resist water uptake. This process may be slowed down because of micropore blocking (Figure 2Ba). Hydroxyl radicals are formed that react with hemicelluloses and lignin. After the hemicelluloses have been degraded sufficiently to expose the cellulose, the hydroxyl radicals start to depolymerize the cellulose, beginning with its paracrystalline moieties. Lignin modification by hydroxyl radicals facilitates the penetration of hydrolyzing enzymes.

The rate of the enzymatic hydrolysis of hemicelluloses and cellulose may be lower than that in untreated wood because of three reasons: (1) micropore blocking leads to a slower rate of enzyme diffusion into the wood cell wall (Figure 2Bb); (2) the lack of water bound to OH-groups decreases the enzyme efficiency (Figure 2Ab); and (3) the recognition of modified polysaccharides by enzymes is hindered (Figure 2Ab). In summary, moisture exclusion delays the onset of degradation, and micropore blocking, insufficient amounts of water bound to OH-groups, and enzyme nonrecognition slow down degradation after its initiation.

Discussion and conclusions

This review considers only brown rot fungi and four types of modification. Different mechanisms may apply to white rot fungi. The mechanisms of fungal degradation are known to be species specific (Green and Highley 1996; Niemenmaa et al. 2008; Kang et al. 2009) as well as dependent on the growth medium and wood species attacked (Tomberg and Olsson 2002; Vanden Wymelenberg et al. 2011). The multitude of wood modification methods and the different methods for evaluating fungal decay also aggravates a comparative evaluation and general conclusions. However, focusing on EMC and mass loss (ML) during decay may facilitate comparisons of different mechanisms. Standardized decay tests, which are designed to assess the durability of wood products, may also pose problems. First, they give an illusion of a threshold level that will prevent decay, but the “threshold” will change if the decay test is run for a longer time (Hill et al. 2009). Second, decay test standards (the European standards EN 113 (1996) and EN 252 (1989) and the American standard ASTM E10) accept a level of treatment that results in a ML of 3%, but this can be misleading. A ML of 3% might be a critical limit for determining the durability of a certain type of wood material, but it is not helpful for evaluating the protective mechanism (Brischke et al. 2008). Third, standardized decay tests for assessing the durability of modified wood were originally designed for testing preservative treated wood. Environmental conditions, such as wetness, are less important for the durability of preservative treated woods than in case of modified woods (Junga and Militz 2005; Meyer et al. 2012).

The present evaluation of theories related to the mode of action of brown rot decay resistance with four types of wood modification suggests that moisture exclusion caused by reductions in the wood cell wall void volume is the most essential parameter that delays the onset of wood decay. Other mechanisms, such as enzyme nonrecognition, micropore blocking, and reduced number of free OH-groups, probably affect the degradation speed after water uptake is initiated.

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Peer-reviewed publications

Ringman, R., Pilgård, A., Richter, K. (2014) Effect of wood modification on gene expression during incipient *Postia placenta* decay. International Biodeterioration & Biodegradation, 86 Part B: 86-91.

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