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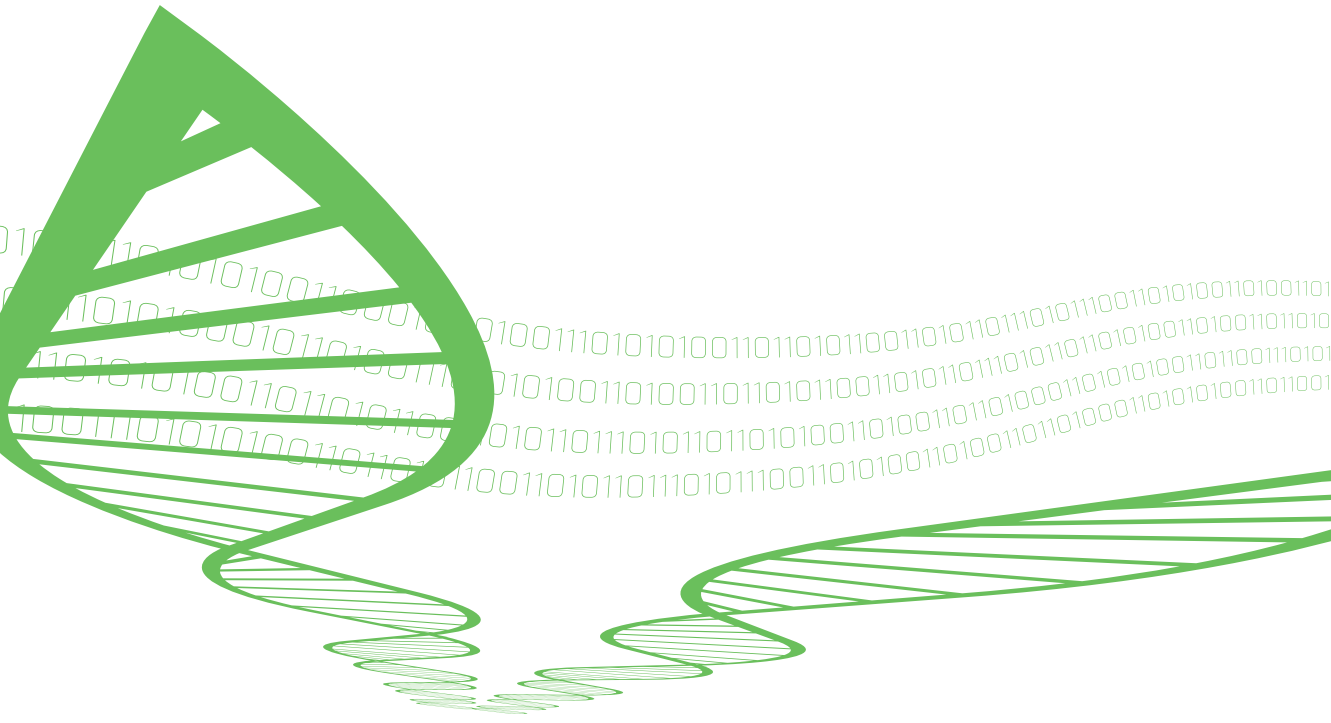
Action of laccase on mechanical softwood pulps

Stina Grönqvist

VTT



VTT SCIENCE 60

Action of laccase on mechanical softwood pulps

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Thesis for the degree of Doctor of Science to be presented with due permission of the Department of Chemical Engineering at Åbo Akademi University for public examination and criticism in Auditorium Salin, Axelia II, at Åbo Akademi University, on the 21th of August, 2014 at 12 p.m.

ISBN 978-951-38-8269-3 (Soft back ed.)
ISBN 978-951-38-8270-9 (URL: http://www.vtt.fi/publications/index.jsp)

VTT Science 60

ISSN-L 2242-119X
ISSN 2242-119X (Print)
ISSN 2242-1203 (Online)

JULKAISIJA – UTGIVARE – PUBLISHER

VTT
PL 1000 (Tekniikantie 4 A, Espoo)
02044 VTT
Puh. 020 722 111, faksi 020 722 7001

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Grano Oy, Kuopio 2014

Action of laccase on mechanical softwood pulps

Lakkaasin vaikutukset mekaanisiin havupuumassoihin. Lackasens inverkan på mekaniska massor framställda av barrträd. **Stina Grönqvist.** Espoo 2014. VTT Science 60. 94 p. + app. 53 p.

Abstract

During recent years, the traditional pulp and papermaking business in Europe has been striving to find new viable applications for wood fibres. The target has been to improve the value and properties of traditional fibres and fibre products and to find new applications for wood fibres that would support much-needed growth in the industry. At the same time, interest in using renewable materials in new applications has increased. However, the natural properties of the fibres limit their use in many applications. Fibre functionalization, i.e. bonding of new compounds to the fibres, is a method to produce fibres with altered properties.

An interesting option is targeted modification of fibre surface lignin via enzymatic radical formation with oxidative enzymes. The highly reactive radicals generated on the fibre surface can be utilised in the bonding of new compounds. In order to exploit the laccase-based functionalization method, deep understanding of factors affecting the formation of phenoxy radicals in fibres is needed. Furthermore, factors affecting the degree of bonding need to be clarified. The main aim of this thesis was to elucidate the effects of laccase treatments on softwood TMPs and their fractions. Furthermore, potential utilisation of the radicals formed by laccase-catalysed oxidation in fibre functionalization was assessed.

The studied laccases were found to be reactive with the studied TMPs and their fractions. The degree of oxidation of TMP was found to be influenced by the presence of dissolved and colloidal substances (DCS). However, the results did not confirm the previously suggested role of DCS in the laccase-catalysed oxidation of fibre-bound lignin.

Laccase appeared to be able to catalyse the oxidation of free fatty and resin acids. The type of chemical linkages present in fatty and resin acids was found to define the effect of laccase. It seems that laccases can be used to oxidise fatty acids with several double bonds and resin acids with conjugated double bonds.

Laccase treatment of milled wood lignin (MWL) was not found to decrease the amount of total phenols in lignin, whereas the amount of conjugated phenols in lignin was found to increase. It was concluded that the effects of laccase on low-molecular mass substrates, such as lignans, are different to those on the more complex lignin. Apparently, in larger lignin structures, the formed radicals can delocalise into the structure.

Two types of radicals can be detected after laccase treatments in wood fibres, i.e. “short-living” radicals that can only be detected immediately after the laccase treatment and stable, “long-living” radicals that can be detected in dried samples even days after the treatment. The stable radicals detected in dry samples

represent only a small part of the originally generated radicals. The formed radicals should be utilised in bonding of the new compounds within an appropriate short time after activation, before the radicals are delocalised in the structure.

Bleaching of TMP affects the amount and the stability of radicals formed in the laccase-catalysed oxidation. More radicals were generated in the laccase-catalysed oxidation on bleached TMP than on unbleached TMP. Peroxide bleaching was found to cause changes in surface chemistry so that “long-living” radicals could only be detected in the fines fraction. This might indicate that the possible levels of modification of unbleached and bleached fines and fibres are different.

Bonding of 3-hydroxytyramine hydrochloride to TMP could be demonstrated, which suggests that compounds containing functional groups can be bonded to wood fibres via laccase-catalysed oxidation of surface lignin. Even though the laccase-aided fibre functionalization method is limited to lignin-rich pulps, its potential is remarkable. It has been shown that the method can be used to create completely new properties in lignin-containing fibres.

Keywords fibre activation, fibre functionalization, surface modification, oxidative enzymes, laccase, lignin, TMP

Lakkaasin vaikutukset mekaanisiin havupuumassoihin

Action of laccase on mechanical softwood pulps. Lackasens inverkan på mekaniska massor framställda av barrträd. **Stina Grönqvist.** Espoo 2014. VTT Science 60. 94 s. + liitt. 53 s.

Tiivistelmä

Eurooppalainen massa- ja paperiteollisuus on viime vuosien aikana etsinyt uusia kannattavia sovelluksia puukuiduille. Tavoitteena on ollut parantaa nykyisten kuitujen ja kuitutuotteiden ominaisuuksia sekä löytää kuiduille uusia sovelluskohteita, jotka tarjoaisivat alalle toivottua kasvua. Samaan aikaan kiinnostus hyödyntää uusiutuvia raaka-aineita erilaisissa sovelluksissa on kasvanut. Puukuitujen luontaiset ominaisuudet rajoittavat kuitenkin niiden hyödyntämistä monissa sovelluksissa. Kuidun funktionalisonnilla, eli liittämällä kuidun pintaan uusia yhdisteitä, voidaan parantaa puukuitujen ominaisuuksia, nostaa niiden arvoa ja siten parantaa massa- ja paperiteollisuuden kilpailukykyä.

Kun puukuidun pinnan ligniiniä muokataan hapettavilla entsyymeillä, muodostuu kuidun pintaan reaktiivisia radikaaleja. Syntyneiden radikaalien avulla kuituihin voidaan liittää yhdisteitä, jotka antavat kuidulle uusia ominaisuuksia. Menetelmän tarjoamien mahdollisuuksien hyödyntämiseksi tarvitaan tietoa kuidun radikalisointiin ja yhdisteiden liittämiseen vaikuttavista tekijöistä. Tämän väitöskirjan tavoitteena oli selvittää lakkaasin vaikutuksia kuusen TMP-massoihin ja niiden fraktioihin. Lisäksi työssä arvioitiin lakkaasin avulla hapetuksessa syntyneiden radikaalien hyödyntämistä kuidun funktionalisoinnissa.

Tutkitut lakkaasit hapettivat tutkittuja TMP-massoja ja niiden fraktioita. Hapetuksen todettiin olevan riippuvainen liuenneiden ja kolloidaalisten aineiden määrästä. Tulokset eivät todentaneet aiemmin esitettyjä väitteitä liuenneiden ja kolloidaalisten aineiden roolista ligniinin lakkaasiavusteissa hapetuksessa. Saatujen tulosten perusteella voidaan olettaa, että lakkaasin avulla vapaiden rasvahappojen sekä hartsihappojen konjugoituneiden kaksoissidoksien hapettaminen on mahdollista.

Puusta eristetyn ligniinin lakkaasiavusteisessa hapetuksessa fenolien kokonaismäärän ei todettu vähenevän, mutta konjugoituneen ligniinin määrän havaittiin kasvavan. Tässä työssä ja kirjallisuudessa esitettyjen tulosten perusteella voitiin todeta, että lakkasi vaikuttaa eri tavalla substraatteihin, joilla on korkea moolimassa (ligniini) ja alhainen moolimassa (lignaanit). Korkean moolimassan rakenteissa, kuten ligniinissä, hapetuksessa muodostuneet radikaalit voivat stabiloitua siirtymällä rakenteessa.

Saatujen tulosten perusteella voitiin päätellä, että puukuituihin syntyy sekä lyhytkestoisia että pitkäkestoisia radikaaleja. Lyhytkestoiset radikaalit voidaan havaita kuidussa vain hetki hapetuksen jälkeen, kun taas pitkäkestoiset radikaalit voidaan havaita vielä useamman päivän säilytyksen jälkeen. Kuivatuista näytteistä mitatut pitkäkestoiset radikaalit edustavat vain pientä osaa alkuperäisestä radikaalien kokonaismäärästä. Lakkaasiavusteisessa hapetuksessa syntyneet

radikaalit tulisikin hyödyntää uusien komponenttien liittämiseen suhteellisen nopeasti radikaalien muodostumisen jälkeen.

TMP:n valkaisun todettiin vaikuttavan lakkaasiavusteisessa hapetuksessa syntyvien radikaalien määrään ja niiden stabiilisuuteen. Valkaistusta TMP:stä voitiin mitata suurempia määriä radikaaleja kuin valkaisemattomasta TMP:stä. Valkaisun vaikutuksesta, säilytyksen jälkeen, ainoastaan hienoaineesta voitiin mitata radikaaleja. Saatujen tulosten perusteella on syytä epäillä, että valkaistujen kuitujen ja hienoaineen hapettumisessa on suuria eroja. Näin ollen on myös mahdollista, että kuidut ja hienoaines ovat eri tavoin muokattavissa.

Työssä voitiin osoittaa 3-hydroksityramiinihydrokloridin sitoutuminen TMP:hen lakkaasiavusteisesti. Tulos osoittaa, että uusia funktionaalisia ryhmiä voidaan sitoa ligniinipitoisiin puukuituihin aktivoimalla kuitujen pinnan ligniiniä lakkaasilla. Vaikka menetelmä soveltuu ainoastaan ligniinipitoisten puukuitujen muokkaukseen, avaa menetelmä täysin uudenlaisia mahdollisuuksia puukuitujen hyödyntämiselle.

Avainsanat fibre activation, fibre functionalization, surface modification, oxidative enzymes, laccase, lignin, TMP

Lackasens inverkan på mekaniska massor framställda av barrträd

Action of laccase on mechanical softwood pulps. Lakkaasin vaikutukset mekaanisiin havupuumassoihin. **Stina Grönqvist.** Espoo 2014. VTT Science 60. 94 s. + bil. 53 s.

Svensk sammanfattning

Under de senaste åren har den europeiska massa- och pappersindustrin sökt nya lönsamma tillämpningar för träfibrer. Målet har varit att förbättra de traditionella fibrernas och fiberprodukternas egenskaper, samt att hitta nya tillämpningar för träfibrerna. Nya fiberegenskaper och -tillämpningar skulle kunna ge sektorn den tillväxt som behövs. Samtidigt har intresset för att använda förnyelsebara råvaror i en mängd olika tillämpningar ökat. Träfibrernas naturliga egenskaper begränsar dock deras användning i många tillämpningar. Modifiering av träfibrerna skulle kunna vidga fibrernas andvändbarhet, öka fibrernas värde och därmed förbättra massa- och pappersindustrins konkurrenskraft.

Träfibrernas egenskaper kan modifieras genom att binda nya komponenter med önskade egenskaper till fiberns yta. Ett sätt att utföra modifieringen är att med hjälp av oxiderande enzymer, såsom lackas, bilda reaktiva radikaler i ligninen på fibrernas ytor och vidare utnyttja de bildade radikalerna till att binda komponenter med nya egenskaper till fiberytan.

För att kunna utnyttja den fulla potentialen av den lackasbaserade modifieringsmetoden, behövs mera information om både de faktorer som påverkar bildningen av radikaler samt om mekanismerna hur nya komponenter binds till fibrerna. Syftet med denna avhandling var att undersöka effekterna av lackas på TMP av gran och olika fraktioner av TMP. Därtill undersöktes modifiering av fibrerna genom bindning av nya komponenter via radikalerna som uppstått under lackasbehandlingen.

De undersökta lackaserna kunde oxidera TMP-massor och deras fraktioner. Lösta och kolloidala substanser hade en klar inverkan på oxidationen. På basen av resultaten i detta arbete kan man anta att lackas kan oxidera fria fettsyror och hartssyror med konjugerade dubbelbindningar. Efter lackasbehandling av isolerat lignin förblev den totala mängden fenoler oförändrad, medan andelen konjugerade strukturer i lignin ökade. På basen av resultaten som presenterats i detta arbete och de resultat som hittats i litteraturen, kunde man konstatera att lackas har olika effekt på substrat som har en hög molmassa (t.ex. lignin) och tydligt lägre molmassa (t.ex. lignaner). I de högmolekylära strukturerna, såsom lignin, stabiliseras radikaler in i strukturen.

På basen av resultaten kunde man dra slutsatsen att oxideringen av fibrer med lackas resulterar i att både kortvariga och långvariga radikaler bildas. Kortvariga radikaler kan upptäckas i fibern bara en kort tid efter oxideringen, medan de långvariga radikalerna kan observeras ännu efter flera dagars förvaring. Långvariga radikaler, som kunde mätas i proverna efter förvaring, utgjorde endast en liten del av det ursprungliga antalet radikaler.

På grund av att en stor andel av de bildade radikaler snabbt stabiliseras in i ligninens struktur, bör bindning av nya komponenter ske relativt snabbt efter att radikalerna bildats. Blekning av TMP visade sig påverka både mängden och stabiliteten av radikaler som bildas i de lackas katalyserade reaktionerna. Mängden radikaler var högre i blekt massa. Peroxid blekningen påverkade ytkemin så att efter lagring kunde radikaler mätas endast i finmaterialet. Enligt resultaten finns det anledning att tro att möjligheterna att modifiera blekta fibrer och finmaterial är olika.

I detta arbete kunde det bevisas att bindning av 3-hydroxythyramineklorid till fibrer är möjligt. Resultatet kan ses som ett bevis att nya funktionella grupper kan bindas till träfibrerna med hjälp av lackas. Även om denna metod är endast lämplig för ligninhaltiga träfibrer, öppnar metoden helt nya möjligheter för utnyttjande av träfibrer.

Nyckelord fibre activation, fibre functionalization, surface modification, oxidative enzymes, laccase, lignin, TMP

Preface

This thesis work was carried out during the years 2000–2014 at the VTT Technical Research Centre of Finland. VTT has very long experience in enzyme-aided modification of lignocellulosics and thus it has been a privilege to carry out this work at VTT. VTT is acknowledged for providing excellent working facilities and funding for this study. The work has also been funded by the Tekes-funded UUTE and Fibrefun projects.

I warmly thank my supervisors at VTT, Dr. Anna Suurnäkki and Dr. Terhi K. Hakala. Anna has given me invaluable scientific support both when carrying out the experimental work and during the writing process. Terhi has patiently supervised and encouraged me through the writing process.

My supervisor and Custos from Åbo Akademi University, University lecturer Anna Sundberg, is acknowledged for her very positive attitude and for her help during the final preparation of the thesis.

My guide to the world of science, Professor Liisa Viikari, is warmly acknowledged for guidance in science as well as in the most effective sightseeing methods. I express my sincere thanks to Dr. Johanna Buchert for her never-ending enthusiasm for this thesis, even though the “delivery time” was “somewhat” prolonged due to the other important chapters in my life.

I am grateful to Professor Claus Felby and Professor Tapani Vuorinen for taking the time to review the manuscript of my thesis and for their constructive feedback and valuable suggestions.

I also wish to thank my co-authors Professor Raimo Alén, Dr Johanna Buchert, Carmen Canevali, Professor Bjarne Holmbom, Dr Kristiina Kruus, Dr Maija Mattinen, Dr Annika Mustranta, Dr Marja-Leena Niku-Paavola, Professor Marco Orlandi, Kari Rantanen, Peter Spetz, Dr. Anna Suurnäkki and Professor Liisa Viikari.

I warmly thank Dr Kristiina Kruus, Dr Tarja Tamminen, Dr Martina Andberg, Jaakko Pere and Matti Siika-aho, who have patiently tried to answer my more or less scientific questions during the past years. Michael Bailey is thanked for reviewing the language of this thesis. Päivi Vahala and VTT publication services are thanked for helping with technical editing. VTT library services are thanked for finding “missing” articles in no time.

I would like to acknowledge the current and former Ladies of the “big lab” and the “fibre lab”. It has been a privilege to be able to work with you all. In particular I express my warmest thanks to Teija Jokila, who carried out most of the laboratory

work for this thesis. I cannot even remember all my roommates and next door roommates over the years, who have provided answers to pressing questions in science and life in general.

My friends, even though we are all in the middle of a very hectic period, I do appreciate you in my life and thank you for your support.

In the never-ending battle between a sister and her brothers, Erik you are most appreciated. Sadly the battle with Magnus ended so early. My sister-in-law Katri is thanked for helping with IT problems during the course of the work.

Mamma och Pappa, I have always known, even though I rather do it by myself and my way, that when I really need it – I can count on you. Tack Mamma och Pappa!

Dear Kajsa and Wilmer – you most certainly have given me a real perspective in life. You willingness to play at Hoplop when I needed to finalise my thesis (at Hoplop) is much appreciated. I also highly and warmly appreciate our scientific discussions; hopefully you will be willing to continue them over the coming years.

Most of all, I am grateful to Jucki for his love and support. Finally I would like to point out: Jucki, as agreed, you never missed Akilles home games due to the writing process of this thesis.

Stina

Academic dissertation

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Professor Tapani Vuorinen
Department of Forest Products Technology, Aalto University

Opponent Professor Art Ragauskas
School of Chemistry and Biochemistry
Institute of Paper Science and Technology at Georgia Institute of Technology

List of publications

This thesis is based on the following original research papers, which are referred to in the text as I–V. The publications are reproduced with kind permission from the publishers. Some unpublished material is also presented.

I. **Grönqvist, Stina**; Suurnäkki, Anna; Niku-Paavola, Marja-Leena; Kruus, Kristiina; Buchert, Johanna; Viikari, Liisa (2003). Lignocellulose processing with oxidative enzymes. Applications of Enzymes to Lignocellulosics. Mansfield, S. D. & Saddler, J. N. (Eds.). ACS Symp. Ser. 855. American Chemical Society. Washington, DC, 46–65.

II. **Grönqvist, Stina**; Buchert, Johanna; Rantanen, Kari; Viikari, Liisa; Suurnäkki, Anna (2003). Activity of laccase on unbleached and bleached thermomechanical pulp. Enzyme and Microbial Technology 32(3–4), 439–445.

III. **Karlsson, Stina**; Holmbom, Bjarne; Spetz, Peter; Mustranta, Annikka; Buchert, Johanna (2001). Reactivity of *Trametes* laccases with fatty and resin acids. Applied Microbiology & Biotechnology 55, 317–320.

IV. **Grönqvist**, **Stina**; Viikari, Liisa; Niku-Paavola, Marja-Leena; Orlandi, Marco; Canevali, Carmen; Buchert, Johanna (2005). Oxidation of milled wood lignin with laccase, tyrosinase and horseradish peroxidase. Applied Microbiology and Biotechnology 67(4), 489–494.

V. **Grönqvist**, **Stina**; Rantanen, Kari; Alén, Raimo; Mattinen, Maija-Liisa; Buchert, Johanna; Viikari, Liisa (2006). Laccase-catalysed functionalisation of TMP with tyramine. Holzforschung 60(5), 503–508.

Author’s contributions

I. The author planned and wrote the publication together with the co-workers. The author had the main responsibility for writing the publication under the supervision of Liisa Viikari.

II. The author planned the work together with the co-authors. The author carried out part of the experimental work. The EPR spectroscopy measurements were carried out by Kari Rantanen. The author had the main responsibility for interpreting the results and writing the publication.

III. The author (Karlsson at the time) planned the work together with the co-authors. The author carried out the experimental work. The analyses were carried out under the supervision of Peter Spetz. The author had the main responsibility for writing the publication under the supervision of Johanna Buchert.

IV. The author had the main responsibility for experimental design, evaluating the results and writing the first draft and finalising the manuscript. The EPR spectroscopy measurements were carried out by Carmen Canevali.

V. The author had the main responsibility for experimental design, evaluating the results and writing the first draft and finalising the manuscript. The ESCA, EPR, and FTIR spectroscopy analyses were carried out by experts.

The author had the main responsibility of planning the work related to the unpublished material. The ERP measurements in that work were carried out by Kari Rantanen.

The enzymes used in this work were obtained from the collection available at the VTT Technical Research Centre of Finland.

Supporting publications

1. Gustafsson, Piia; **Grönqvist**, **Stina**; Toivakka, Martti; Smolander, Maria; Erho, Tomi; Peltonen, Jouko (2011). Incorporation of laccase in pigment coating for bioactive paper applications. Nordic Pulp and Paper Research Journal 26(1), 118–127.

2. Viikari, Liisa; **Grönqvist**, **Stina**; Kruus, Kristiina; Pere, Jaakko; Siika-aho, Matti; Suurnäkki, Anna (2010). Industrial biotechnology in the paper and pulp sector. Industrial Biotechnology. Sustainable Growthand Economic Success. Soetaert W. & Vandamme, E.J. (Eds.). Wiley-VCH. Weinheim, 385–412.

3. Viikari, Liisa; Suurnäkki, Anna; **Grönqvist, Stina**; Raaska, Laura; Ragauskas, Art (2009). Forest Products: Biotechnology in Pulp and Paper Processing. Encyclopedia of Microbiology. 3rd ed. Schaechter, M. (Ed.). Academic Press, 80–94.

4. Saarinen, Terhi; Orelma, Hannes; **Grönqvist, Stina**; Andberg, Martina; Holappa, Susanna; Laine, Janne (2009). Adsorption of different laccases on cellulose and lignin surface. BioResources 4(1), 94–110.

5. Buchert, Johanna; **Grönqvist, Stina**; Mikkonen, Hannu; Oksanen, Tarja; Peltonen, Soili; Suurnäkki, Anna; Viikari, Liisa. Process for producing a fibrous product. Pat. WO2005061790 A1, publication date 7 July 2005, application number FI04000795, application date 23 Dec. 2004, priority FI20031903 (2005).

6. **Grönqvist, Stina**; Hurme, Eero; Smolander, Maria; Suurnäkki, Anna; Viikari, Liisa. Method of producing a fibre products. Pat. WO2005060332 A2, publication date 7 July 2005, application number FI04000798, application date 23 Dec. 2004, priority FI 20031903 (2005).

7. Buchert, Johanna; **Grönqvist, Stina**; Mikkonen, Hannu; Oksanen, Tarja; Peltonen, Soili; Suurnäkki, Anna; Viikari, Liisa. Process for producing fibre composites. Pat. WO2005061791 A1, publication date 7 July 2005, application number FI04000794, application date 23 Dec. 2004, priority FI20031902 (2005).

8. Buchert, Johanna; **Grönqvist, Stina**; Mikkonen, Hannu; Viikari, Liisa; Suurnäkki, Anna. Process for producing a fibre composition. Pat. WO2005061568 A1, publication date 7 July 2005, application number FI04000793, application date 23 Dec. 2004, priority FI 20031901 (2005).

Contents

Appendices III–V of this publication are not included in the PDF version.

List of important symbols and abbreviations

AAS	atomic absorption spectroscopy
ABTS	2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
AFM	atomic force microscopy
DCS	dissolved and colloidal substances
EC	Enzyme Commission
EPR	electron paramagnetic resonance
ESCA	electron spectroscopy for chemical analysis
ESR	electron spin resonance spectroscopy
FE-SEM	field emission scanning electron microscopy
FTIR	fourier transform infrared spectroscopy
GC	gas chromatography
HPLC	high-performance liquid chromatography
ID	inner diameter
L	lumen
LiP	lignin peroxidase
LMS	laccase-mediator system
ML	middle lamella
MnP	manganese dependent peroxidase
MTBE	methyl *tert*-butyl ether
MWL	milled wood lignin
P	primary wall of wood cell wall
PGW	pressurized ground wood

RT	room temperature
S	secondary wall of wood cell wall
S_1	outer layer of secondary cell wall
S_2	middle layer of secondary cell wall
S_3	inner layer secondary cell wall
SEC	size exclusion chromatography
TMP	thermomechanical pulp
TOFA	tall oil fatty acids
Tyramine	3-hydroxytyramine hydrochloride
VP	versatile peroxidase
W	warty layer
XET	xyloglucan endo-transglycosylase
XPS	X-ray photoelectron spectroscopy

1. Introduction

During recent years, the traditional pulp and papermaking business in Europe has been striving to find new viable applications for wood fibres. Renewal of the business is needed in order to compensate for decreased sales due to competition from new pulp and paper producers outside Europe and changing consumer behaviour. The strategic target has been to improve the value and properties of traditional fibres and fibre products and to find new applications for wood fibres that would generate the needed growth and higher return on investments. At the same time, interest in the use of renewable materials in new applications is growing. In this respect, wood fibres represent an interesting raw material for various applications. However, the natural properties of wood fibres limit their use in many applications. Modification of fibre properties by chemical and biochemical means opens up new perspectives for utilisation of the fibres.

Fibre functionalization, in this work meaning adding new functional compounds to fibres, is a method to produce fibres with altered properties. The modification can be based on attachment of a compound with desired properties to the fibre, either directly or via a molecule that can act as a link between the fibre and the attached compound (Figure 1). In principle, all major components of wood, *i.e.* cellulose, hemi-celluloses and lignin, are potential targets for binding the new components to the wood fibre material. An interesting option is targeted chemo-enzymatic modification of fibre surface lignin by exploiting enzymatic radical formation with oxidative enzymes. The highly reactive radicals generated in the lignin can then be utilised in the bonding of new compounds to fibres.

When the current research was conducted, extensive research on laccase-aided modification of wood fibre materials was being conducted in several research groups. Today, due to intensive research in the field, the potential of laccase-aided oxidation for modification of wood-based materials has been widely reported and discussed. Despite extensive research, the underlying mechanisms of oxidative enzymes on the fibre-bound substrates studied in this work are still only partially understood.

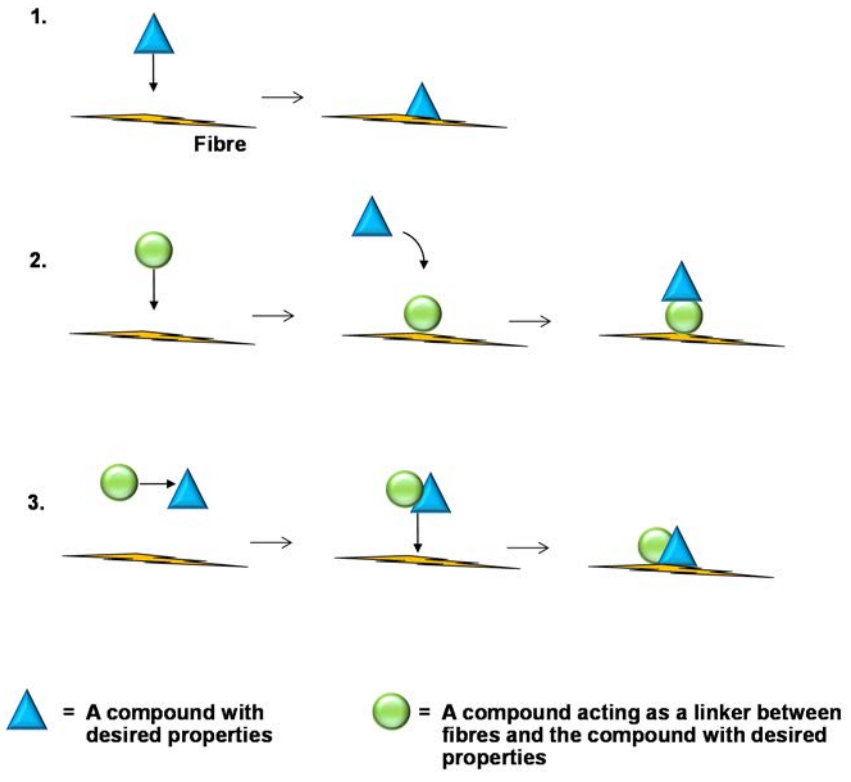


Figure 1. Functionalization of fibres. The modification can be based on direct attachment of a compound with the desired properties to the fibre (route 1) or via a molecule that can act as a linker between the fibre and the compound with the desired properties (routes 2 and 3).

2. Background

2.1 Wood and pulp structure and chemistry

2.1.1 Wood structure

Wood is a complex material, the major components being cellulose, hemicelluloses and pectins, lignin and extractives. It can be simplified that wood is a complex natural composite built up of fibres that are glued together by lignin. Fibres can also be regarded as composites, as they consist of fibrils that are held together by lignin and hemicelluloses (Figure 2).

Cellulose, being the main component in wood, forms the skeleton of all wood cells. A bundle of cellulose molecules, held together by hydrogen bonds, is proposed to form the smallest building element of the cellulose skeleton, *i.e.* the elementary fibril (Sjöström 1993, Alén 2000). The elementary fibrils are organised into stands called microfibrils that are approximately 5–30 nm wide. The microfibrils are combined to greater fibrils (fibril aggregates) and lamellae and act as building blocks of the different layers of the cell wall. It has been suggested that the microfibrils together with glucomannan form fibril aggregates with a diameter of 15 to 23 nm (Salmén, Olsson 1998, Åkerholm, Salmén 2001, Fahlén, Salmén 2003). The spaces between the microfibrils have been suggested to be filled by hemicelluloses (glucomannan and xylan) and lignin (Sjöström 1993, Alén 2000). This matrix has also been reported to contain some disordered cellulose (Sjöström 1993).

The wood cell wall is composed of two layers, *i.e.* primary wall (P), and secondary wall (S) (Figure 2). The thick secondary wall can be divided into three sub-layers, *i.e.* outer layer (S_1), middle layer (S_2) and inner layer (S_3) (Rydholm 1965, Ilvessalo-Pfäffli 1977, Sjöström 1993, Alén 2000). In some cases, the S_3 layer is covered with a warty layer (W). The hollow fibres have a central cavity called lumen (L). The cells (fibres) are bound together by the middle lamella (ML).

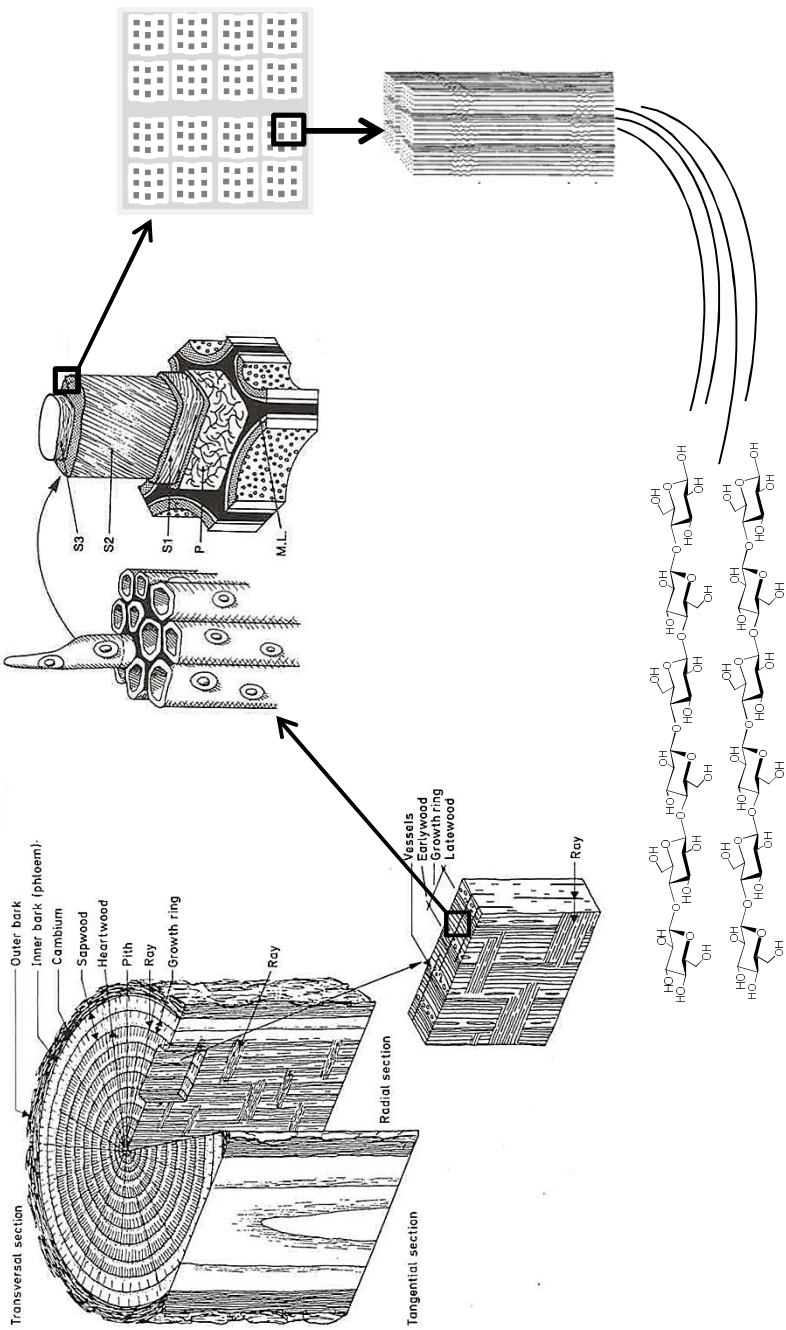
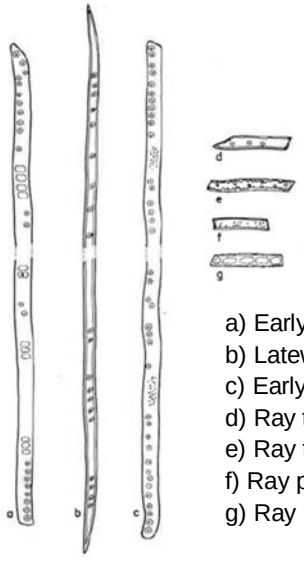


Figure 2. Structure of wood (Fengel, Wegener 1989, Kirk, Cullen 1998, Alén 2000, Fahlén, Salmén 2005).

The chemical composition and the orientation of structural elements in the cell layers differ from each other (Ilvessalo-Pfäffli 1977, Sjöström 1993, Alén 2000). In addition, the origin of the cells also strongly affects the cell characteristics (Ilvessalo-Pfäffli 1995). Separation of the different cell wall layers has been found to be very difficult (Sjöström 1993, Alén 2000). Thus, despite extensive studies, the distribution of the chemical constituents in the different layers is not yet fully understood. Based on current knowledge, the middle lamella consists mainly of lignin but also of some kind of irregular network composed of pectins and hemicelluloses. The primary wall consists of cellulose, hemicelluloses, pectins and protein that are completely embedded in lignin. Secondary walls consist mainly of cellulose but also of hemicelluloses and some lignin.

Based on their shape, wood cells can be divided into two very broad categories, *i.e.* thin and **long prosenchyma cells** and "**brick-like" parenchyma cells** (Sjöström 1993, Alén 2000). The wood cells have different principal functions related to conducting, support and storage. The conducting and supporting cells are water- or air-filled dead cells. Storage cells are parenchyma cells that distribute and store nutrients in the living parts of the wood via openings in the cell wall called pits. Pits occur usually as pairs of complementary pits in adjacent cells. The neighbouring pits form a bordered, half-bordered or a simple pit pair (Sjöström 1993, Alén 2000). Wood cells also contain some random single pits that form openings to the areas between the cells (Ilvessalo-Pfäffli 1977). The number, shape and orientation of pits are unique for each species, and thus pits are used as a diagnostic feature in identification of wood and fibres.

Softwood consists of 90–95% prosenchyma cells called tracheids (Figure 3) (Ilvessalo-Pfäffli 1995, Sjöström 1993). Most of these tracheids are longitudinal and can thus be classified as fibres. The length of these softwood fibres varies between 2 and 6 mm depending on the origin (wood species and location in the stem) (Alén 2000). In addition to the longitudinal tracheids, some species also contain short ray tracheids (Figure 3). The rest, 5–10% of the softwood cells, are ray parenchyma cells with an average length of 0.01–0.016 mm (Sjöström 1993). Ray parenchyma cells are predominantly oriented horizontally. The short ray tracheids can usually be found on the top and bottom of these rays. Softwoods also contain tube-like intercellular horizontal and vertical canals filled with resin.



a) Early wood pine tracheid
b) Latewood pine tracheid
c) Early wood spruce tracheid
d) Ray tracheid of spruce
e) Ray tracheid of pine
f) Ray parenchyma cell of spruce
g) Ray parenchyma of pine

Figure 3. Major cell types in softwoods modified from (Ilvessalo-Pfäffli 1977).

In contrast to softwoods, a greater variety of cells can be found in hardwoods, *i.e.* libriform cells, vessels, ray parenchyma cells and fibre tracheids that are hybrids of the above-mentioned cells (Sjöström 1993). The libriform cells and fibre tracheids constitute about 65–70% of the stem volume. The length of libriform cells and tracheids, the fibres of hardwood, vary depending on the species (Sjöström 1993, Alén 2000). The length of libriform cells of birch is typically 0.8–1.6 mm. The vessel elements form long tubes in the wood, providing an efficient transportation system for nutrients and water in the living tree. Hardwoods typically have a higher amount of parenchyma cells than softwoods (Alén 2000). The rays in hardwood, consisting exclusively of parenchyma cells, are wider than rays in softwood (Sjöström 1993, Alén 2000).

2.1.2 Main chemical components of wood

Cellulose

Cellulose comprises about 40–45% of the dry substance of wood (Sjöström 1993, Alén 2000). It is the supporting material of the cell wall. Cellulose is a linear homopolymer composed of β-D-glucopyranoside units linked together by β-(1→4)-glucosidic bonds. The degree of polymerisation of native wood cellulose is about 10 000 (Sjöström 1993). The functional groups of the cellulose chains, *i.e.* the hydroxyl groups, have a strong tendency to form intra- and intermolecular hydrogen bonds. Due to these bonds, cellulose molecules aggregate to form

microfibrils. In the aggregates, the highly ordered crystalline regions alternate with less ordered amorphous regions. The cellulose found in wood is closely associated with hemicelluloses and lignin (Sjöström 1993, Alén 2000).

Hemicelluloses and pectins

Hemicelluloses are a heterogeneous group of polysaccharides. Like cellulose, hemicelluloses also act as a supporting material in the cell walls. The amount of hemicellulose in wood is typically 20–30% (Sjöström 1993, Alén 2000). The structure and composition of hemicelluloses varies depending on the tree species and the location in wood. The building blocks of hemicelluloses are D-glucose, D-mannose, D-galactose, D-xylose, L-arabinose, D-arabinose, L-rhamnose, 6-deoxy-L-mannose, L-fucose and small amounts of uronic acids (Fengel, Wegener 1989, Alén 2000).

Hemicelluloses found in in softwood are mainly galactoglucomannans (15–20% of dry wood), whereas hardwood hemicelluloses are rich in xylans (15–30% of dry wood) (Sjöström 1993, Alén 2000). In addition to galactoglucomannans, softwoods also contain arabinoglucuronoxylan, arabinogalactan and other polysaccharides in minor quantities. Hardwood also contains unsubstituted glucomannan and minor amounts of other polysaccharides.

The other polysaccharides found in wood are pectic substances, starch and proteins (Sjöström 1993, Alén 2000). Pectic substances are comprised of galacturonans, galactans and arabinans (Fengel, Wegener 1989). Pectins are typically found in primary cell wall and in the middle lamella (Sjöström 1993).

Lignin

The third major component found in wood is lignin. About 26–32% of softwood and 20–25% of hardwood is lignin (Sjöström 1993). In wood, lignin is a structural part of the cell wall and forms together with hemicelluloses the matrix that embeds cellulose (Sjöström 1993, Alén 2000). As in the case of the other major components of wood, lignin is not uniformly distributed within the cell wall. Lignin is concentrated in ML and P, but as the S-layer has the largest volume, the major part of lignin is found in the S layer (Alén 2000).

Lignin is an aromatic polymer built up of three different types of phenyl propanoid units (ρ-hydroxycinnamon alcohols) via enzyme-catalysed radical coupling during biosynthesis. The building units (precursors of lignin), *i.e.* coniferyl alcohol, sinapyl alcohol and p-coumaryl alcohol, differ from each other in the amount of methoxy substituents (Figure 4). The coupling of the units in various ways becomes possible as the phenoxy radicals generated by enzymatic oxidation are delocalised in the structure as a result of resonance stabilization (Figure 5) (Fengel, Wegener 1989, Sjöström 1993, Alén 2000). The phenyl propanoids are linked together mainly by ether linkages (C-O-C), but also by carbon-carbon (C-C) bonds without any clear repeating system. The most prominent linkage in both soft- and hardwood is the β-O-4 linkage, accounting for 40–60% of all linkages (Fengel, Wegener 1989, Sjöström 1993, Alén 2000).

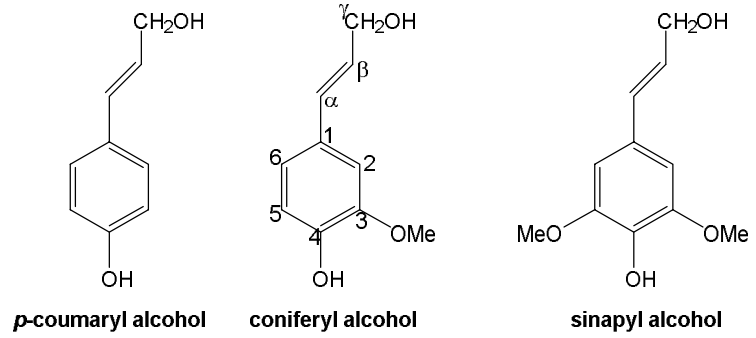


Figure 4. Molecular structures of p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Fengel, Wegener 1989).

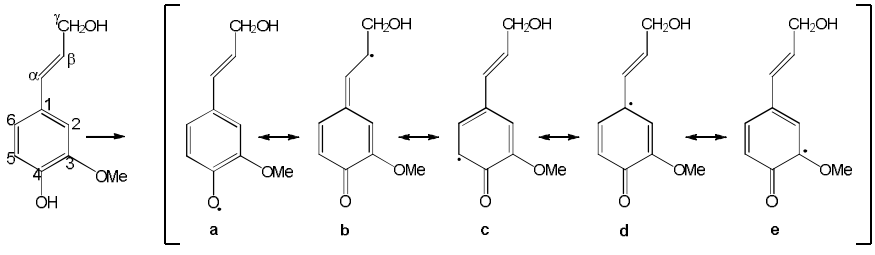


Figure 5. Delocalisation of radicals in lignin structure due to resonance stabilization (Sjöström 1993).

Due to the various possible linkages between the lignin units and the random occurrence of the different linkage types, the polymeric structure of lignin is very complex. The exact composition of lignin varies between wood species (Alén 2000). Softwood lignin contains mainly guaiacyl units, derived from coniferyl alcohol, whereas hardwood lignins are typically termed quaiacyl-syringyl lignins because they are built up of both coniferyl and sinapyl alcohols. Grass lignins contain all phenyl propanoid units and also p-coumaryl alcohols (Sjöström 1993, Alén 2000).

Since the first description of lignin by Payen in 1838, several hypothetical structural formulas for wood lignin have been suggested over the years (Freudenberg 1965, Adler 1977, Brunow *et al.* 1998, Ralph, Brunow & Boerjan 2007, Crestini *et al.* 2010) (Figure 6). In 1998 it was suggested that in addition to the widely accepted structural elements, softwood lignin contains some dibenzodioxocin structures (Brunow *et al.* 1998). Since then sprirodienes have also been identified in the lignin structure (Ralph, Brunow & Boerjan 2007). Common to most suggested structures is that lignin is presented as a phenolic, branched polymer. Based on recent research carried out on milled wood lignin (MWL), it has been proposed that lignin exists in fact as linear oligomers that interact with each other (Crestini *et al.* 2011, Lange, Decina & Crestini 2013).

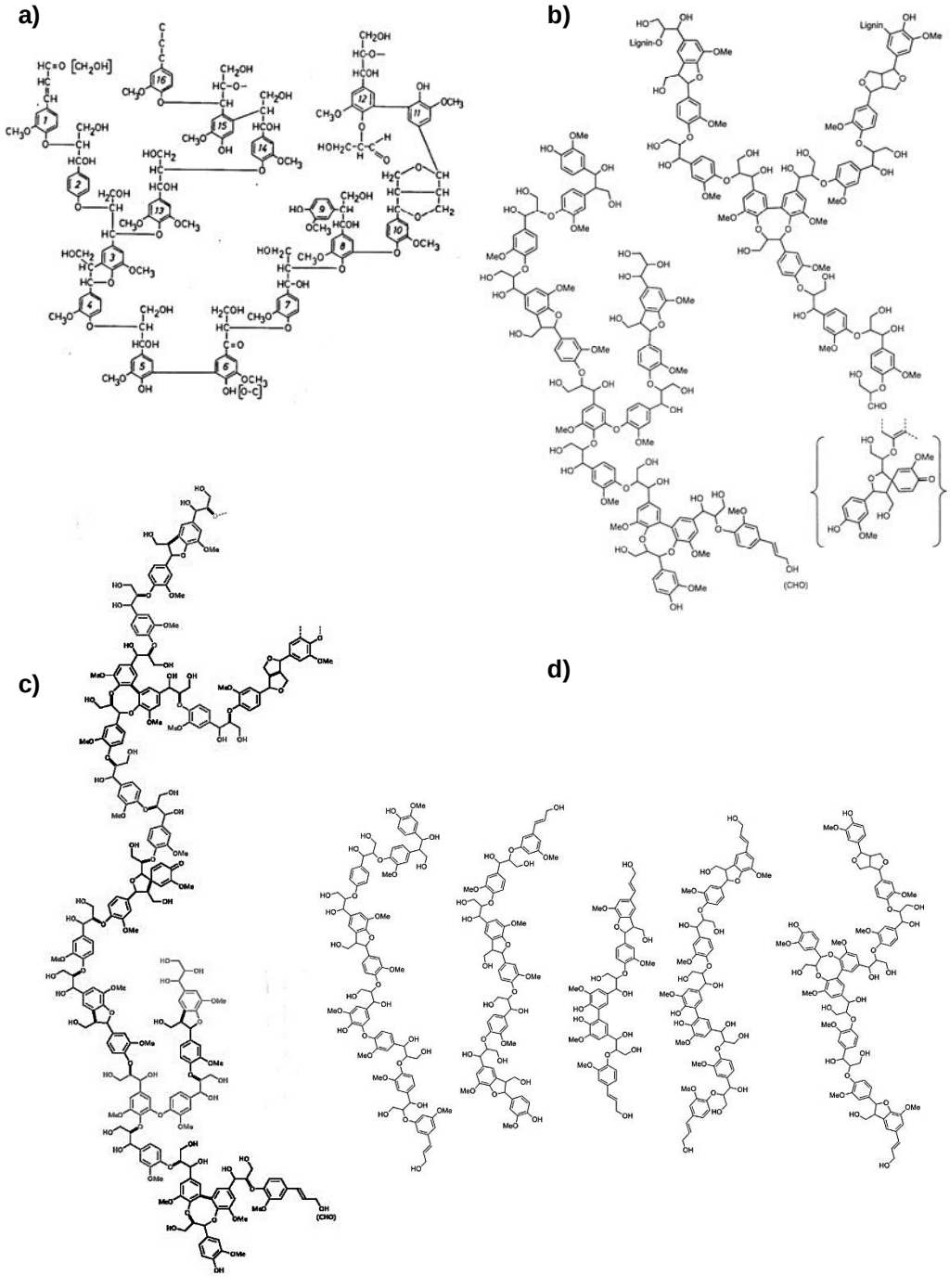


Figure 6. Suggested structures for lignin: a) Adler 1977, b) Brunow *et al.* 1998, c) Ralph, Brunow & Boerjan 2007 d) Lange, Decina & Crestini 2013.

Nevertheless, the exact structure and real molecular mass of native lignin still remain as open questions, as there is no analytical technique powerful enough to analyse lignin *in situ*, and lignin isolation prior to chemical characterisation always alters the polymeric structure (Rydholm 1965, Alén 2000, Crestini *et al.* 2011). However, isolation of lignin is often necessary for research purposes. Due to the close association of lignin with the other cell wall polymers, poor solubility in any commonly used solvents and its tendency to degrade or react upon isolation, the isolation of native lignin is very challenging (Fengel, Wegener 1989, Sjöström 1993, Sundholm 1999, Alén 2000, Hafrén *et al.* 2000, Fahlén, Salmén 2005, Guerra *et al.* 2006, Crestini *et al.* 2011, Lange, Decina & Crestini 2013).

Milled wood lignin, *i.e.* lignin isolated from milled wood by extraction with aqueous dioxane (Björkman 1956), is generally considered to be the best representative of native lignin, although extraction yields are low (<50%) and the obtained material is always to some extent contaminated with polysaccharides (Sjöström 1993). However, recent studies claim that milled wood lignin is only a good representative of the milled sample, not of lignin found in native wood prior to the milling (Lange, Decina & Crestini 2013). Another approach is to isolate lignin from kraft pulp by enzymatic hydrolysis of carbohydrates (Yamasaki *et al.* 1981). The use of various other isolated lignins from various separation processes has also been reported, *e.g*. acidolysis lignin, cellulolytic enzyme lignin, enzymatic mild acidolysis lignin, kraft lignin, sulphite lignin, organosolv lignin, pyrolysis lignin and steam explosion lignin (Guerra *et al.* 2006, Lange, Decina & Crestini 2013).

Extractives

Wood extractives, the extraordinarily large number (several thousands) of wood components that are soluble in neutral organic solvent or water, comprise from 1 to 4.5% of wood dry solids (Alén 2000). The amount and composition of extractives varies between different wood species but also between various parts of the same tree. In addition, growing conditions affect the amount of extractives (Ekman, Holmbom 2000). Extractives are regarded as non-structural wood constituents (Alén 2000).

Due to the large amount of extractives they can be classified in various ways. Extractives can be divided into terpenoids and stereoids (including terpenes), fats and waxes and their components, phenolic compounds and other *i.e.* inorganic components (Fengel, Wegener 1989, Sjöström 1993). Another way to classify the extractives is to group terpenes, terpenoids, esters of fatty acids (fats and waxes), fatty acids and alcohols as well as alkanes and to classify them according to their structure as aliphatic and alicyclic compounds. The remaining groups are then the phenolic compounds and other compounds (Alén 2000).

The individual components can also be sorted based on the type of solvent needed to extract the components, *i.e.* lipophilic extractives can be extracted by nonpolar and hydrophilic extractives by polar solvents (Sjöström 1993). The hydrophilic compounds include phenolic constituents (*e.g.* stilbenes, lignans, tannins and flavonoids), sugars and salts (Sjöström 1993, Holmberg 1999). The lipophilic extractives consist of fats and waxes and their components as well as of

terpenoids and sterols. The lipophilic extractives such as fatty and resin acids, sterols, steryl esters and triglycerides, are commonly referred to as wood resin or wood pitch (Alén 2000, Back 2000a). Some common fatty and resin acids found in wood are presented in Figure 7.

In wood, fats and waxes can be found in parenchyma cells, whereas resin acids are located in resin canals. During mechanical pulping, the various types of resin components are mixed and can be found on the surface of fibres and fines, inside parenchyma cells and in colloidal form (Allen 1980, Ekman, Eckerman & Holmbom 1990, Holmbom *et al.* 1991, Sjöström 1993). The wood resin is known to have a negative impact on paper machine operation and on paper quality (Dreisbach, Michalopoulos 1989, Back 2000b, Sundberg *et al.* 2000, Holmbom, Sundberg 2003).

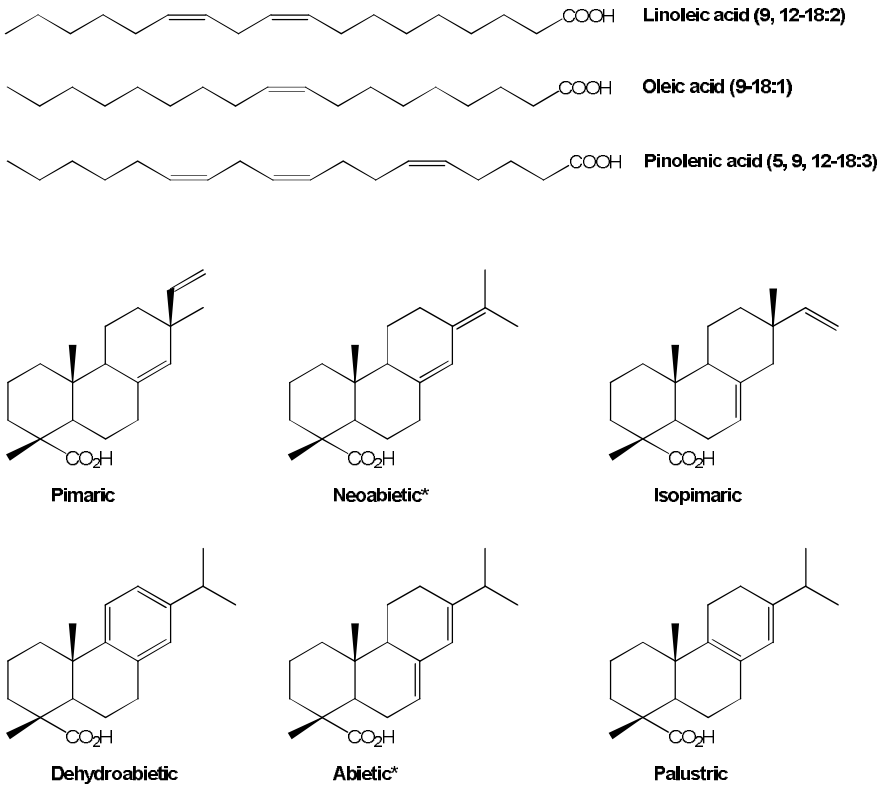


Figure 7. Structures for some common fatty and resin acids found in wood, *indicates conjugated resin acids (III).

2.1.3 Mechanical pulping

The main aim of pulping processes is to separate wood fibres from each other for further processing (*e.g*. papermaking, manufacture of composites etc.) (Rydholm 1965). The pulping stage can be carried out either chemically or mechanically. In chemical pulping, the separation of fibres takes place as the lignin and the major part of the hemicellulose are dissolved or degraded and removed by the cooking chemicals. In mechanical pulping, wood fibres are separated as the material between fibres is softened due to the mechanical forces combined with heat and pressure, in some cases with the help of accessory chemicals (chemi-mechanical pulp) (Sundholm 1999). As both cellulose and hemicelluloses are softened under mechanical pulping conditions already at 20°C, the softening of lignin is critical for the efficiency of the pulping process (Salmén *et al.* 1999).

In practice there are two methods to produce mechanical pulps, *i.e*. grinding or refining. In grinding, the wood logs are pressed against a rotating pulp stone. In refining, wood chips are disintegrated in a disk refiner. Mechanical pulps, *e.g*. thermomechanical pulp (TMP), the dominating refiner-based pulp (Tienvieri *et al.* 1999), or pressurized ground wood (PGW) pulps, have a high yield, typically 97–98% for Norway spruce (Sundholm 1999). The wood materials dissolved or dispersed into the process waters during pulping are mainly some hemicelluloses, lipophilic extractives, lignans and lignin-related substances (Holmbom *et al.* 1993, Örså *et al.* 1993, Manner *et al.* 1999).

The colour of the mechanical pulp is similar to that of the wood raw material (Lindholm 1999). Mechanical pulps are usually bleached with dithionite or with hydrogen peroxide in order to increase the brightness of the pulp. The bleaching aims at elimination of coloured groups, *i.e*. chromophores in lignin (Lindholm 1999). The most common chromophores found in wood lignin are: coniferylaldehyde, α-carbonyl groups and various quinone structures. The easily oxidisable chromopores are degraded, while only a negligible amount of lignin is released from the pulp (Holmbom *et al.* 1991, Holmbom, Sundberg 2003). Extractives can also have a negative effect on the colour of the pulp (Lindholm 1999). Thus, the aim of bleaching can also be to decrease the content of extractives (Lindholm 1999). The amount of coloured extractives in Norway spruce has, however, been reported to be small (Lindholm 1999). Additionally, the wood phenolic structures, which as such do not affect the colour, can at elevated temperatures change into coloured structures. Bleaching of mechanical pulps results only in a partial elimination of the coloured structures. Additional positive effects of bleaching are the enhanced fibre bonding and strength (Lindholm 1999). The positive effects of bleaching on colour tend to diminish with time, as reduced chromophores can be reoxidised to their coloured form.

2.1.4 The character and properties of mechanical pulps

The dimensions and properties of wood fibres are strongly dependent on how the wood is defiberized. Although the dimensions of a pulp fibre are affected by the dimensions of the original wood fibre, the characteristics of the separated fibres are determined by the pulping process (Salmén *et al.* 1999, Heikkurinen, Leskelä 1999). Due to the grinding and refining, the length and cross-sectional dimensions are changed (Heikkurinen, Leskelä 1999). Treatment conditions used in pulping strongly affect the fracture zones in the wood. It has been stated that for TMP the rupture of the fibre wall during refining most often takes place between the primary and secondary walls (Figure 8). As the fibres are both separated and further refined (fibre development) during mechanical pulping, lamellar cracks and peeling of the outer layers of fibres (depending on the fracture zone) can take place as well as external fibrillation of the remaining secondary wall, internal fibrillation of the fibre and formation of fines (Salmén *et al.* 1999, Sundholm 1999, Heikkurinen, Leskelä 1999). As a result of the mechanical pulping the pulp contains a mixture of intact fibres, fragmented fibres and fines.

The fraction of pulp passing through a round hole with a diameter of 76 µm or through a nominally 200 mesh screen is commonly defined as fines (Kleen, Kangas & Laine 2003). In mechanical pulps, the proportion of fines varies typically between 10% and 40%. Fines consist of various types of particles, *e.g.* broken fibres, cell wall fragments, middle lamella fragments, ribbons, fibrils, fibril bundles, bordered pits, ray cells and fragments of all these, having different chemical composition and physical properties (Heikkurinen, Leskelä 1999, Rundlöf 2002, Kleen, Kangas & Laine 2003, Sundberg, Pranovich & Holmbom 2003).

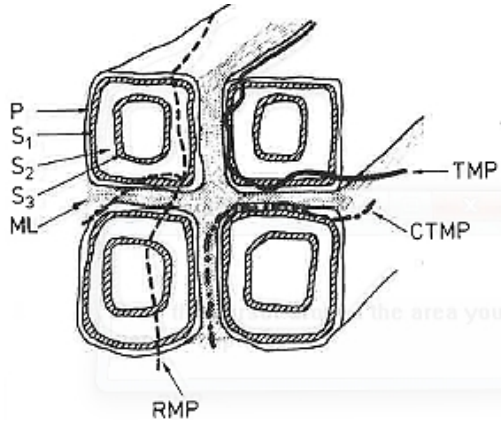


Figure 8. Schematic diagram of fracture zones in softwood as affected by different mechanical processes (Salmén *et al.* 1999). TMP = thermomechanical pulp, RMP = refiner mechanical pulp and CTMP chemithermomechanical pulp. P = primary wall, S_{1-3} = secondary walls and ML = middle lamella.

As the components found in wood can be found in about the same ratios in mechanical pulp, the chemistry of a mechanical pulp is determined by the chemical components of the raw material (carbohydrates, lignin, extractives and metals) (Sundholm 1999). However, due to the naturally heterogeneous structure of wood and due to the processing steps in mechanical pulping and bleaching, the chemical compositions of the bulk and the surface of the different fractions are not the same (Sundholm 1999, Koljonen *et al.* 2003, Kleen, Kangas & Laine 2003, Kangas, Kleen 2004).

After fibre development almost all of the outer layers, *i.e.* ML, P and outer S_1 layers are peeled off, leaving the S_2 layer exposed (Heinemann *et al.* 2011, Kangas, Kleen 2004, Kangas *et al.* 2004). This can explain the chemical composition of the pulps surfaces. It has been shown that in mechanical pulps the lignin and extractive contents on the surface of fibres are higher than in the bulk material (Koljonen *et al.* 2003, Kleen, Kangas & Laine 2003, Kangas, Kleen 2004). For example, the gravimetric lignin content for a TMP was reported to be 30%, whereas the surface lignin content as analysed by electron spectroscopy for chemical analysis (ESCA, also known as X-ray photoelectron spectroscopy, XPS) was clearly higher *i.e*. 40% (Koljonen *et al.* 2003). The difference in extractives content for the same pulp in the bulk (soluble in acetone) and on the surface (analysed by ESCA) was reported to be even higher, *i.e*. ~1% in the bulk versus up to 30% on the surface (Koljonen *et al.* 2003).

In another ESCA study, the surface coverage of lignin and extractives of the surface layers of TMP and peeled bulk TMP fibres was studied (Kleen, Kangas & Laine 2003). Based on this, the bulk versus surface coverage in TMP fibres was reported to be ~47 versus 57% for lignin and ~5 versus 14% for extractives. Both studies thus indicated that the fibre surface is rich in both lignin and extractives.

Although the lignin content of TMP fines has been reported to be higher than in TMP fibres (Sundberg, Holmbom 2004), the amount of surface lignin on fines and on fibres has been found to be approximately the same (Kleen, Kangas & Laine 2003). Interestingly, it appears that there are more quaiacylic units on the surfaces of TMP fibres than on the surfaces of fines (Kleen, Kangas & Laine 2003, Kangas, Suurnäkki & Kleen 2007). Thus, it has been concluded that TMP fibres and fines have different lignin structures (Kleen, Kangas & Laine 2003, Kangas, Suurnäkki & Kleen 2007). Furthermore, it has been reported that surface lignin contains less methoxyl groups and guaiacyl units than the bulk lignin (Kleen, Kangas & Laine 2003). Therefore, it appears that the different layers of the cell wall have different lignin compositions. Atomic force microscopy (AFM) studies have shown that lignin can be found as granular structures on the fibre surface (Koljonen *et al.* 2003). Besides the granulated structures, a non-granulated layer of lignin on the surface of fibres has been suggested (Kangas, Kleen 2004).

The extractive content has been reported to be somewhat higher on TMP fines than on the fibre surface or bulk fibres (Kleen, Kangas & Laine 2003). For example, canal resins are known to have a tendency to follow fines (Ekman, Holmbom 2000). The type of fines has been found to strongly affect the surface composition (Kangas, Kleen 2004).

As described in Section 2.1.3, bleaching of mechanical pulps aims at elimination of coloured groups. The effects of dithionite and peroxide on surface lignin and extractives content of mechanical pulps have been found to be very small or insignificant (Koljonen et al. 2003). Of the lipophilic extractives, peroxide bleaching has been reported to affect the resin acids with conjugated double bounds (Holmbom *et al.* 1991). In peroxide bleaching of spruce milled wood lignin, the coniferyl aldehydes have been reported to be effectively degraded, although the effects of peroxide bleaching were generally small (Holmbom *et al.* 1991).

2.2 Enzymes for pulp and paper applications

2.2.1 Basics of enzymes

Enzymes are proteins that act as catalysts, *i.e.* they lower the energy of activation required for a reaction to occur, but remain unaltered themselves. Enzymes have a key role in practically all biological processes. In contrast to many inorganic catalysts, enzymes are both substrate (the molecule which the enzyme acts on) and reaction specific (Stryer 2000a, Cavaco-Paulo, Gübitz 2003, Buchholz, Kasche & Bornscheuer 2005). The high substrate specificity of enzymes is due to the structure of the active site, into which only certain molecules can fit. Although all enzymes discriminate between molecules, the extent of discrimination varies between different enzymes. As most enzymes are very specific in respect to which groups and which bonds they act on, the products formed in enzymatically catalysed reactions are also highly specific.

The enzymatic reactions are also regulated by the surrounding conditions. Generally, enzymes catalyse reactions at ambient pH and temperature. In enzyme processes the process pH and temperature can thus be used to start, accelerate, inhibit and stop the enzymatic reactions (Godfrey, West 1996). Enzymatic reactions can also be inhibited irreversibly or reversibly by specific molecules that prevent the substrate from binding to the enzyme (Stryer 2000a).

In principle, enzymes are structured by folded amino acid chains with additional components, such as metals (Stryer 2000a, Stryer 2000b). The structure is determined by the amino acid sequence and varies widely between different enzymes. The active site is the most important part of the enzyme and contains the region that binds the substrate and the residues that participate in catalysis. Large enzymes usually have several structural domains which can can function and exist independently. For example fungal laccases are monomeric molecules with a three domain structure (Ducros *et al.* 1998), whereas fungal cellulases and some hemicellulases usually have a two-domain structure with one catalytic domain and a binding domain connected via a linker (Tenkanen, Buchert & Viikari 1995, Teeri 1997).

The Enzyme Commission (EC) has classified enzymes into six main classes based on the reactions they catalyse (Table 1).

Table 1. Major classes of enzymes (adapted from Buchholz, Kasche & Bornscheuer 2005).

Class number (EC)	Enzyme class	Reaction catalysed by the enzyme
1	Oxidoreductases	Oxidation – reduction
2	Transferases	Transfer of a group from one compound to another
3	Hydrolases	Hydrolytic cleavage of covalent bonds
4	Lyases	Non-hydrolytic bond cleavage
5	Isomerases	Internal rearrangement (geometric or structural) within a substrate (molecule)
6	Ligases	Joining of two molecules to form a larger molecule (ATP as co-substrate)

2.2.2 Enzyme toolbox for pulp and paper applications

Due to their natural origin, non-toxicity and the mild conditions needed for enzymes to function, enzymes have become a prominent alternative to chemicals in many areas of pulping and papermaking. As new and more cost-effective methods to produce enzymes in yeast and bacteria have been developed, the attractiveness of enzymes has further increased.

All the major chemical components in wood can be modified by enzymes (Viikari *et al.* 2009, Viikari *et al.* 2010) (Table 2). The majority of enzymes used in wood and pulp processing are hydrolases, *e.g.* cellulases and xylanases.

Table 2. Enzymes for processing of wood components (adapted from (Viikari *et al.* 2010, Viikari *et al.* 2009 and *Parikka *et al.* 2012).

Wood Component	Enzymes acting on the component			
	Group	**Class**	**Action**	**Potential exploitation/benefit**
Cellulose	Endoglucanases	3	Depolymerisation, structure modification	Pulping, paper machine runnability, drainage, enhancement of paper properties (strength), deinking
	Cellobiohydrolases	3		
	β-glucosidases	3		
Hemicellulose	Endoxylanases	3	Depolymerisation	Pulping, bleaching (xylanases), process control (degradation of glucomannas), paper machine runnability and drainage, deinking
	Endomannases	3		
	Assory enzymes: glucuronidase, arabinosidase, galactosidase, esterases	3	Side group cleavage	
	Transferases (Xyloglucan endotransglycosylase)	2	Transfer reaction	Fibre modification
	Galactose oxidase*	1	Oxidation	Modification of galactoglucomannan for enhancement of paper properties
Lignin	Laccases	1	Oxidation, depolymerisation with mediator	Pulping, process control, fibre modification, bleaching (laccase+mediator)
	Peroxidases	1		
Extractives	Lipases	3	Degradation by hydrolysis	Process control
	Laccases	1	Oxidation	
Pectin	Pectinases	2	Cleavage, demethylation	Pulping (savings in cationic chemicals)

2.2.3 Oxidative enzymes for pulp and paper applications

Oxidative enzymes, *i.e.* oxidoreductases, have a significant role both in lignin biosynthesis and in degradation of lignocellulosic biomass (Hatakka 1994). As an outcome of the research related to lignin biodegradation, the most essential enzymes in lignin degradation have been identified to be oxidases, peroxidases and hydrogen peroxide -generating enzymes (Hatakka 1994, Martínez *et al.* 2005). Of these enzymes, the utilisation of laccases and peroxidases, especially manganese-dependent peroxidases, has attracted considerable interest in various applications. Despite extensive research, the underlying mechanisms of oxidative enzymes on fibre-bound substrates are still only partially understood.

Laccases

Laccases (EC 1.10.3.2) are multi-copper proteins that catalyse the oxidation of various aromatic compounds, especially phenols as well as some non-aromatic compounds, by concomitant reduction of oxygen to water (Kawai *et al.* 1988, Bourbonnais, Paice 1990, Thurston 1994, Gianfreda, Xu & Bollag 1999, Xu 1999). Laccases are known to be able to catalyse the crosslinking of monomers, degradation of polymers and ring cleavage of aromatic compounds. The suitability of a substrate for oxidation by laccase mainly depends on the oxidation potential of the substrate, rather than on steric demands (Lange, Decina & Crestini 2013). The variety of substrates oxidised by laccase can be broadened by the use of small molecular mass compounds, mediators (Bourbonnais, Paice 1990, Bourbonnais, Paice 1992) (Figure 9). Laccases can be found in fungi, higher plants, insects and bacteria (Gianfreda, Xu & Bollag 1999, Claus 2003, Claus 2004, Dwivedi *et al.* 2011). The molecular size of laccase is typically about 50–100 kDa (Claus 2004).

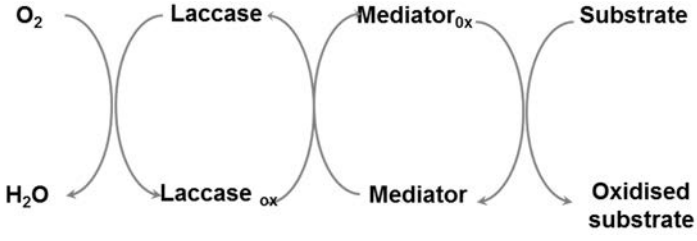


Figure 9. A schematic presentation of mediated oxidation of a substrate by laccase.

The laccase molecule is a dimeric or tetrameric glycoprotein with a minimum of four copper atoms (Thurston 1994, Gianfreda, Xu & Bollag 1999). The properties and structure of the copper centres of the laccase determine whether the laccase is a high (*e.g*. laccases from basidomycetes) or a low-redox potential laccase (*e.g.* bacterial and plant laccases) (Dwivedi *et al.* 2011).

The coppers are classified into three types, *i.e.* Type 1: blue copper, Type 2: non-blue copper and Type 3: a copper-copper pair (Gianfreda, Xu & Bollag 1999, Claus 2004). The oxidation of substrate, *i.e.* withdrawal of one electron from the substrate takes place at the Type 1 copper. The electrons are transported to the trinuclear cluster, formed by the Type 2 and 3 coppers. After four cycles of single electron oxidations, forming four free radicals in the substrate, the four electrons are donated to molecular oxygen, thus causing the reduction of molecular oxygen and formation of water (Claus 2004, Dwivedi *et al.* 2011). Although, the exact laccase reaction mechanisms are not yet fully understood, reduction of oxygen most probably takes place in two steps, since bound oxygen intermediates are involved. It has, however, been claimed that no release of toxic peroxide intermediates takes place (Thurston 1994, Claus 2004).

The overall reaction catalysed by laccases is: $4\ RH + O_2 \rightarrow 4\ R\bullet + 2\ H_2O$

In the laccase-catalysed oxidation, the substrate thus loses a single electron and forms a free radical. The unstable radicals formed in the oxidation may undergo further non-enzymatic oxidation or reduction reactions, couple to other phenolic structures or polymerize to produce intensely coloured products.

Peroxidases

Peroxidases are heme proteins, *i.e*. they have an iron-binding heme group in their active centre. They catalyse the oxidation of a large variety of substrates with hydrogen peroxide as electron acceptor through two one-electron oxidations (Banci 1997). Several peroxidases are known to participate in lignin degradation, the major groups being manganese-dependent peroxidase (EC 1.11.1.13), lignin peroxidase (EC 1.11.1.4), and versatile peroxidase (EC 1.11.1.16) (Jong, Field & de Bont 1992, Heinfling *et al.* 1998).

Manganese peroxidase (MnP) is the most common lignin-modifying peroxidase, produced by almost all wood-colonizing basidiomycetes causing white-rot and also by various soil-colonizing litter-decomposing fungi (Hofrichter 2002, Martínez 2002). As MnP has been detected in most of the studied lignin-degrading fungi, it has been suggested to have a crucial role in decomposing lignin. MnP oxidizes Mn^{2+} to Mn^{3+}. Chelation of Mn^{3+} by organic acids (*e.g.* oxalic, malic, lactic or malonic acid) is necessary in order to stabilize the ion and to promote its release from the enzyme. The chelated Mn3+ is a powerful oxidant that can oxidise phenolic moieties in lignin. As a result of the oxidation, phenoxy radicals are formed (Hofrichter 2002). MnP has also been reported to be able to oxidise unsaturated fatty acids, generating lipid radicals that are able to diffuse into wood to oxidise non-phenolic structures of wood (Kapich, Jensen & Hammel 1999).

Lignin peroxidases (LiP) are capable of oxidising various phenolic and non-phenolic lignin substructures. Characteristic for LiPs is that they are able to oxidise high redox-potential aromatic compounds (Kirk, Farrell 1987, Martínez 2002). The cation radical formation in non-phenolic lignin structures causes several unspecific

reactions, resulting finally in ring cleavage. LiPs have been found to be secreted by many white-rot fungi, although the secretion of LiP is less common than that of MnP. (Niku-Paavola *et al.* 1988, Lundell *et al.* 1993, Hatakka 1994).

Versatile peroxidases (VP) combine the substrate specificity characteristics of LiPs and MnPs (Camarero et al. 1999), and are thus able to oxidize a variety of high and low redox potential substrates including Mn2+, phenolic and non-phenolic lignin dimers, α-keto-γ-thiomethylbutyric acid (KTBA), veratryl alcohol, dimethoxybenzenes, different types of dyes, substituted phenols and hydroquinones. Only a few fungi, *e.g*. *Pleurotus* and *Bjerkandera,* have been reported to produce VP (Martínez 2002).

2.2.4 Monitoring the reactions of oxidative enzymes

The action of oxidative enzymes can be monitored by following the oxidation of the substrate or the reduction of the co-substrate, *i.e.* oxygen for laccase and hydrogen peroxide for peroxidases. Various types of detectors for analysing the oxygen and peroxide consumption are commercially available. Additionally, the co-substrate consumption can also be analysed by various chemical assays.

The oxidation reactions catalysed by oxidative enzymes can also be followed by monitoring the formation of radicals. Radicals can be followed by indirect chemical methods or directly using electron paramagnetic resonance (EPR) spectroscopy, also called electron spin resonance (ESR) spectroscopy. EPR spectroscopy is a method to detect unpaired electrons, *i.e.* radicals (Sundholm 1984).

An electron has an electric charge, and spinning around its own axis it creates a magnetic field (Sundholm 1984). An unpaired electron can have a spin of - ½ or +½. (Halliwell, Gutteridge 2000) Due to the “spin” the electron also has a magnetic moment. The magnetic moment of an electron is a vector sum of the magnetic moment of the electron in a circular orbit around a nucleus and the angular movement of the electron around its own axis. The magnetic moment makes the electron behave like a compass or a bar magnet when placed in magnetic field (Halliwell, Gutteridge 2000). When an external magnetic field is applied, the paramagnetic electrons can either orient in a direction parallel (lower energy level) or antiparallel (higher energy level) to the direction of the magnetic field (Figure 10). This thus creates two distinct energy levels for the unpaired electrons (Eaton *et al.* 2010). EPR spectroscopy detects the energy difference between the spins of an electron placed in an applied magnetic field.

Initially, there will be more electrons in the lower energy level than in the upper level (Halliwell, Gutteridge 2000, Eaton *et al.* 2010). Transitions between energy levels are in EPR spectroscopy generated by magnetic radiation with a frequency of 9.5–35 GHz (Hon 1992), usually at microwave frequencies. The frequency of the radiation is held constant while the magnetic field is varied in order to obtain an absorption spectrum. The paramagnetic system absorbs microwave energy at a fixed value and an ESR spectrum is registered.

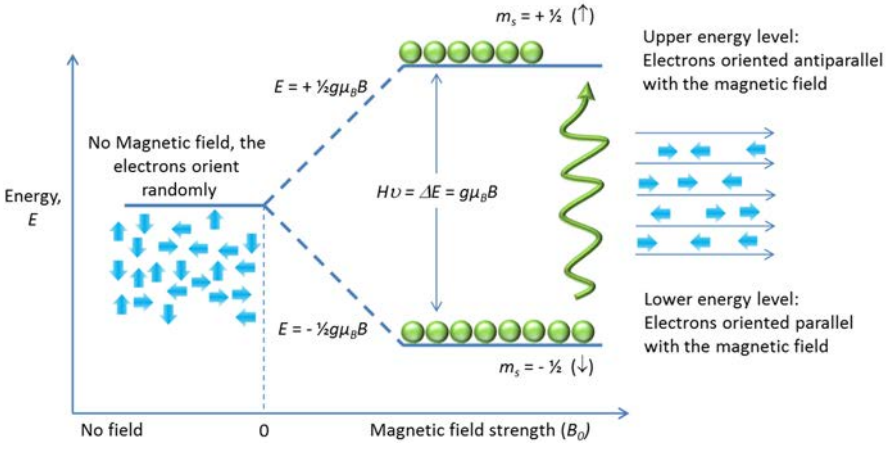


Figure 10. Variation of electron spin energy with the magnetic flux density.

2.2.5 Accessibility of mechanical pulp for enzymatic modification

In order to act, enzymes must be able to reach their substrate. Thus, the susceptibility of a substrate to enzymatic attack has generally been tied to the accessible surface area (Cowling, Brown 1969, Stone *et al.* 1969). A clear correlation between the pore volume and the enzymatic digestibility of lignocellulosic substrates by hydrolytic enzymes has been reported (Stone *et al.* 1969, Wong *et al.* 1988, Mooney *et al.* 1998).

Two types of capillary voids can be found in wood; gross capillaries *i.e*. cell lumina, pit apertures and pit membrane pores, and cell-wall capillaries, *e.g.* spaces between microfibrills (Cowling, Brown 1969). The diameter of gross capillaries is between 200 nm and 10 µm, whereas the size of cell wall capillaries varies according to the presence or absence of water. When saturated with water, the largest cell wall capillaries can expand to about 20 nm in diameter. The majority of the cell wall capillaries are, however, substantially smaller. The molecular masses of laccases are known to be between 50 and 100 kDa (Thurston 1994, Xu 1999, Claus 2004) and for example *Melanocarpus* laccase has been found to have dimensions of 6 x 7 x 9 nm (Hakulinen *et al.* 2002). The gross capillaries of wood are thus bigger than the dimensions of many enzymes (Cowling, Brown 1969). However, it has been stated that the diffusion of enzymes through pores in wood only takes place if the dimensions of the pores are at least as large as the largest dimension of the enzyme (Cowling, Brown 1969).

As described in Section 2.1.4, mechanical pulping affects the structure of fibres. Despite many efforts with various techniques, the structure of fibre cell walls of mechanical pulps has not yet been fully clarified. Mechanical pulps are known to have slightly higher swelling properties than native wood fibres (Salmén, Tigerström & Fellers 1985), indicating a somewhat more opened structure. However, the

swelling of mechanical pulps is clearly lower than that of chemical pulps (Salmén, Tigerström & Fellers 1985, Laivins, Scallan 1996). The difference in swelling can be explained by the presence of lignin in mechanical pulps. It is also known that fines from TMP swell about twice as much as fibres of mechanical pulps (Laivins, Scallan 1996).

Based on thermoporosity measurements of pore size distribution, it has been concluded that the swelling of mechanical pulps is due to formation of micropores (small pores closely associated with cell wall polymers) rather than macropores (larger pores not associated with the cell wall polymers) (Maloney 2000). Thus, the results indicated that macropores would not be formed due to splitting or delamination of the fibres. It was, however, pointed out that it is possible that pores bigger than the probe used in the analysis are formed (Maloney 2000). AFM measurements of the fibre wall structures of TMP fibres have revealed that the outer surfaces of TMP fibres are less porous than the corresponding S_2 layer (Heinemann *et al.* 2011). The difference was explained by the difference in chemical composition.

Simons staining has also been used as a tool to estimate the pore size distribution of various pulps (Chandra *et al.* 2008, Fernando, Daniel 2010). Based on reported results it is clear that mechanical pulps have clearly less and smaller pores than *e.g.* kraft pulps (Chandra *et al.* 2008). Field emission scanning electron microscopy (FE-SEM) studies have shown that the pores on the fibre surface of TMP are cracks rather than holes (Kangas *et al.* 2004).

Many studies on the accessibility of enzymes in wood concentrate on the accessibility of cellulose-degrading enzymes (Cowling, Brown 1969, Stone *et al.* 1969, Srebotnik, Messner & Foisner 1988, Wong *et al.* 1988, Mooney *et al.* 1998). As laccases have about the same or somewhat greater molecular size as cellulases and are not expected to be able to create pores as cellulases can (Grönqvist *et al.* 2014), it is expected that the reported poor accessibility of hydrolases to cellulose in wood applies also to the accessibility of lignin for laccases. The native structure of mechanical pulp fibres apparently limits the accessibility of enzymes on the fibre and fines surfaces (including surfaces of accessible pores) and to dissolved and colloidal material solubilized in process water. Therefore, it can be stated that the surface chemistry and morphology of TMP fibres define the action of enzymes and the possible modification routes.

2.3 Pulp and paper applications utilizing oxidative enzymes

Oxidation of fibre-bound lignin and as well as low-molecular mass lignin model compounds with laccase has been studied by several groups (Kaplan 1979, Felby *et al.* 1997, Felby, Pedersen & Nielsen 1997, Barsberg, Thygesen 1999, Niku-Paavola *et al.* 2002, Rittstieg *et al.* 2002, Lund, Eriksson & Felby 2003, Mattinen *et al.* 2008, Lahtinen *et al.* 2009, Mattinen *et al.* 2011). In addition to lignin, laccases have been reported to have activity on lipophilic extractives and hydrophilic lignans (Buchert *et al.* 1999, Zhang 2000, Buchert *et al.* 2002).

The oxidation of fibre-bound lignin by laccase is thought to be due to direct oxidation of surface lignin, or alternatively mediated by dissolved and colloidal material (Felby *et al.* 1997, Hassingboe, Lawther & Felby 1998). It has also been suggested that the presence of water-soluble extractives would be necessary for radical formation in lignin (Barsberg, Thygesen 1999). Despite extensive studies, the mechanisms of action of oxidative enzymes on fibre-bound lignin are still only partially understood.

The activation of TMP by laccase has previously been followed by spectroscopic methods; the activation was reported to decrease pulp brightness but otherwise only minor structural changes were observed (Lähdetie *et al.* 2009). Peroxidases have been studied mainly for lignin degradation.

2.3.1 Enhanced processing

The use of lignin-degrading fungi and enzymes capable of depolymerizing lignin by β-O-4 ether cleavage in pre-treatments of pulps prior to pulping has been shown to facilitate subsequent mechanical pulping processes (Bar-Lev, Kirk & Chang 1982, Hatakka *et al.* 2002, Mansfield 2002, Maijala *et al.* 2008). It has been reported that impregnation of radiata pine wood chips with a laccase preparation could reduce refining energy consumption by 5–8% in a TMP process (Mansfield 2002). However, more prominent energy savings have been obtained by cellulase treatment of the reject fraction prior to secondary refining (Pere *et al.* 2002). Both laccase and cellulase treatments have been reported to enhance the strength properties of paper (Mansfield 2002, Mohlin, Pettersson 2002).

Enzyme-aided bleaching sequences attracted considerable interest in the 1990s. Laccase can oxidise phenolic hydroxyl groups found in lignin, but due to the limited substrate range, laccase alone is not suitable for bleaching as it does not depolymerise lignin (Call, Mücke 1996).

In 1990, Bourbonnais et al (Bourbonnais, Paice 1990, Bourbonnais, Paice 1992) reported that lignin can be efficiently removed using a mediator oxidised by laccase. The discovery of the laccase-mediator system (LMS) resulted in widespread interest in studying the use of laccase in delignification of kraft pulps. The method has been demonstrated in pilot scale (Call, Mücke 1997). In addition to the reported potential in bleaching, LMS has also been found to have a positive impact on paper strength properties (Widsten, Kandelbauer 2008). Furthermore, LMS has also been suggested for deinking of recycled wood-based fibres (Nyman, Hakala 2011) and for removal of extractives (Gutiérrez *et al.* 2006). Despite intensive research, the cost and recyclability issues related to the mediator still need to be solved.

In addition to laccase, the use of MnP for bleaching has also been studied (Paice *et al.* 1993, Paice *et al.* 1995, Moreira *et al.* 2001). Unlike laccase, MnP uses a natural mediator, Mn(II). The drawback of the MnP-based method is that only phenolic structures can be attacked (Bao *et al.* 1994). It has, however, been suggested that other reactions initiated by MnP could be involved in the

degradation of non-phenolic structures. The use of MnP is limited by the specific reaction conditions required by the enzyme as well as the poor availability and hence also the price of the enzyme.

Laccases have also been found to affect lipophilic extractives without a mediator (Buchert *et al.* 1999, Beatson *et al.* 1999). Furthermore, laccases have been used to polymerise lignans found in process waters (Buchert *et al.* 2002). Laccase could thus be used as a tool for pitch control (Buchert *et al.* 1999, Buchert, Mustranta & Holmbom 2002, Zhang *et al.* 2002). A prominent amount of lipophilic extractives and lignans could be removed by combining the polymerising effect of laccase with microfiltration (Widsten *et al.* 2004).

2.3.2 Fibre modifications

Fibre modifications enhancing the natural fibre properties or creating completely new fibre properties could be used to broaden the application areas for wood fibres. An interesting option to modify fibre properties is through oxidative activation of fibre lignin. As described above, in the enzyme-catalysed oxidation of wood fibres the primary reaction of many oxidative enzymes, *i.e.* laccases, lignin peroxidases and manganese peroxidases is the formation of phenolic or cationic radicals in the lignin matrix (Kirk, Farrell 1987, Gianfreda, Xu & Bollag 1999, Thurston 1994, Kirk, Cullen 1998, Gajhede 2001, Widsten, Laine & Tuominen 2002). At the same time, solubilised lignans, dissolved lignins and some extractives are also radicalized (Buchert *et al.* 1999, Buchert *et al.* 2002). Due to the high reactivity of these radicals further polymerisation, depolymerisation and co-polymerisation can occur. Thus, radicals in the enzyme-activated fibres can further react with other radical-containing molecules.

Radical-based activation of surface lignin of fibres has been used for bonding of low-molecular mass compounds, such as polyphenolic dyes, syringic acid, vanillic acid and 4-hydroxybenzoic acid to lignin by laccase (Lund, Bjerrum & Felby 1998, Chandra, Ragauskas 2001, Chandra, Ragauskas 2002, Chandra, Felby & Ragauskas 2004, Chandra, Lehtonen & Ragauskas 2004).

This chemo-enzymatic functionalization of fibre surfaces opens up new eco-friendly routes to improve existing paper properties or to create completely new properties in fibres (Buchert *et al.* 2005a, Buchert *et al.* 2005b, Buchert *et al.* 2005c, Grönqvist *et al.* 2005). A great variety of new functional properties can be introduced to fibres by this method; *e.g.* hydrophobicity, charge, conductivity or anti-microbial properties. The method has been used to modify pulps (Buchert *et al.* 2005a, Buchert *et al.* 2005b, Buchert *et al.* 2005c, Buchert *et al.* 2005d, Grönqvist *et al.* 2005), and also for dip coating of paper (Elegir *et al.* 2008). Despite extensive studies, the mechanisms of action of oxidative enzymes on fibre-bound lignin are still only partially understood. As some of the areas of industrial interest have been patented, many results in the field of enzymatic functionalization are most probably not open for scientific discussion and evaluation.

Various chemical approaches have also been suggested, but the drawback of the chemical means is that they usually also affect the technical properties of the woody fibres. By contrast, the enzyme-aided methods affect only the fibre surface, leaving the fibre skeleton intact.

2.4 Oxidative enzymes in other applications

2.4.1 Board manufacture and veneers

The manufacture of fibreboards and other composites consumes huge amounts of petrochemical-based adhesives (Nyanhongo *et al.* 2011). Promotion of auto-adhesion of fibres by utilising enzyme-generated radicals has therefore attracted considerable interest. Radicals formed in laccase-aided activation of fibres have successfully been utilised for fibre board manufacture (Felby, Pedersen & Nielsen 1997, Kharazipour, Huettermann & Luedemann 1997, Hüttermann, Mai & Kharazipour 2001, Felby, Hassingboe & Lund 2002, Widsten *et al.* 2003, Felby *et al.* 2004). Compared to traditional manufacturing methods, based on the use of synthetic adhesives, the utilisation of oxidative enzymes enables a more environmentally friendly processing. The method has been tested in pilot scale (Felby, Hassingboe & Lund 2002). Studies carried out with wood particles have shown that water extractable lignin components can interact as redox mediators with the fibre surface lignin and the oxidizing enzyme (Felby *et al.* 1997).

Bonding of new compounds to wood particles by laccase-aided functionalization has been reported to increase the internal bonding of particle boards (Fackler *et al.* 2008). The laccase-aided bonding of fluorophenols (Kudanga *et al.* 2010) and tannins (Widsten *et al.* 2010) to wood veneer has also been reported. These results indicate that functionalization can also be used to upgrade the properties of various wood surfaces.

2.4.2 Textile industry

The pulp and paper industry and the textile industry have several similarities. Both use natural lignocellulosic fibres and the raw materials are processed in various ways. The chemical compositions of many of the natural fibres used for textiles are similar to the wood fibres of the pulp and paper industry, *e.g.* cotton contains cellulose and hemicelluloses, whereas the main components of flax are cellulose, hemicelluloses and lignin.

The textile industry, in contrast to the pulp and paper industry, has several well established enzyme-based processes and is one of the biggest consumers of industrial enzymes. Utilisation of laccases for dye removal has been found to be an interesting option, as laccases can be used both to degrade dyes and to mediate their coupling reactions (Campos *et al.* 2001, Benzina *et al.* 2013). Currently, laccases are used in industrial scale to bleach denim.

Laccase-aided bonding of new compounds to textile fibres has also been studied during recent years. Laccase-aided activation has been successfully utilised for bonding of chitosan and catechin into flax fibre (Silva *et al.* 2011). Additionally, modification of wool fabrics in order to produce a textile material with antimicrobial, antioxidant and water repellent properties has been reported (Hossain *et al.* 2009, Hossain *et al.* 2010a, Hossain *et al.* 2010b). In the case of flax, bonding is thought to take place via lignin, but as wool does not contain lignin the bonding mechanism must be different.

3. Aims of the present study

Fibre functionalization, *i.e.* by bonding of new compounds to the fibres, offers an interesting option to improve the value, properties and competiveness of wood-based fibre products.

To exploit the radicals formed in laccase-aided oxidation of lignin-containing fibres in fibre functionalization, deep understanding of the factors affecting the formation of phenoxy radicals in the fibres is needed. The aim of this study was to determine the activity of laccases on mechanical softwood pulp and its fractions. The activation of both isolated and fibre-bound lignin was determined by following the co-substrate (*i.e.* oxygen) consumption and by measuring the radical formation. The impact of pulp type (bleached or unbleached) on the reactivity was also assessed. As dissolved and colloidal substances (DCS) have been proposed to have a role in the laccase-catalysed oxidation of fibres, the action of DCS, especially fatty and resin acids, in the laccase-catalysed oxidation was clarified. Finally, utilisation of the formed radicals in fibre functionalization was assessed.

The aims, addressed in the five separate original publications, were:

1. To clarify the action of laccase on TMP and its fractions, *i.e.* fibres, fines and DCS (II, V)
2. To determine laccase action on isolated TMP components, *i.e.* fatty and resin acids (III) and milled wood lignin (IV)
3. To determine the factors affecting the stability of radicals formed in the laccase-catalysed oxidation of TMP fibres (II and V)
4. To evaluate the possibility to bond positively charged groups to activated pulp fibres (V)

4. Materials and methods

4.1 Pulps, pulp fractions and enzymes

Two unbleached and two peroxide-bleached thermomechanical pulps (TMP) from Norway spruce (*Picea abies*) were sampled from Finnish paper mills and used as such or after fractionation (Figure 11). Waters containing dissolved and colloidal substances (DCS-water) were prepared as described by Örså and Holmbom (Örså, Holmbom 1994) at pH 4.5 and pH 7. The pulps (TMP and bleached TMP) used to prepare the DCS-water were further washed and called “washed fibres”. The fines (<200 mesh) and fibre (>200 mesh) fractions were separated by a Super Dynamic Drainage Jar equipment, which is composed of a tank with a 200-mesh wire (wire hole diameter 76 µm) and a mixer. The chemical compositions of the DCS-water and the pulp fractions used are shown in Table 3. The carbohydrate compositions of the pulps and DCS waters were analysed by HPLC after acid or secondary enzymatic hydrolysis, respectively (Buchert *et al.* 1993, Tenkanen *et al.* 1999, Tenkanen, Siika-Aho 2000). Metal, lignin and extractive contents were analysed as described in Table 6.

Additionally, a lignin isolated from Norway spruce by the milled wood lignin (MWL) method (Björkman 1956) and commercial model fractions, *i.e.*, tall oil fatty acids (TOFA) and gum rosin representing fatty and resin acids found in pine and spruce wood, were utilised in the work. The chemical compositions of the fatty and resin acid preparations are presented in

Table 4 as analysed by gas chromatography (GC) after extraction with methyl *tert*-butyl ether (MTBE) (Örså, Holmbom 1994).

The laccases used in this work are presented in Table 5. The *Trametes hirsuta* laccases were experimental laccases, whereas *T. villosa* and *Myceliophtora thermophila* laccases were commercial preparations. Laccase and manganese peroxidase (MnP) activities of the enzyme preparations were determined using ABTS (2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) as substrate (Niku-Paavola *et al.* 1988). Peroxidase activity was analysed using guaiacol as substrate (Bergmayer 1974, Paszczynski, Huynh & Crawford 1985) and lignin peroxidase using veratryl alcohol as substrate (Tien, Kent Kirk 1983). The peroxidase activities were assayed using H_2O_2. H_2O_2 is the co-substrate that is needed in the peroxidase-catalysed oxidation and therefore, even though the laccase preparations

were found to contain some peroxidase activity, the peroxidase action was considered to be negligible in the laccase treatments as no H_2O_2 was added and no H_2O_2 is formed in the laccase-catalysed reaction.

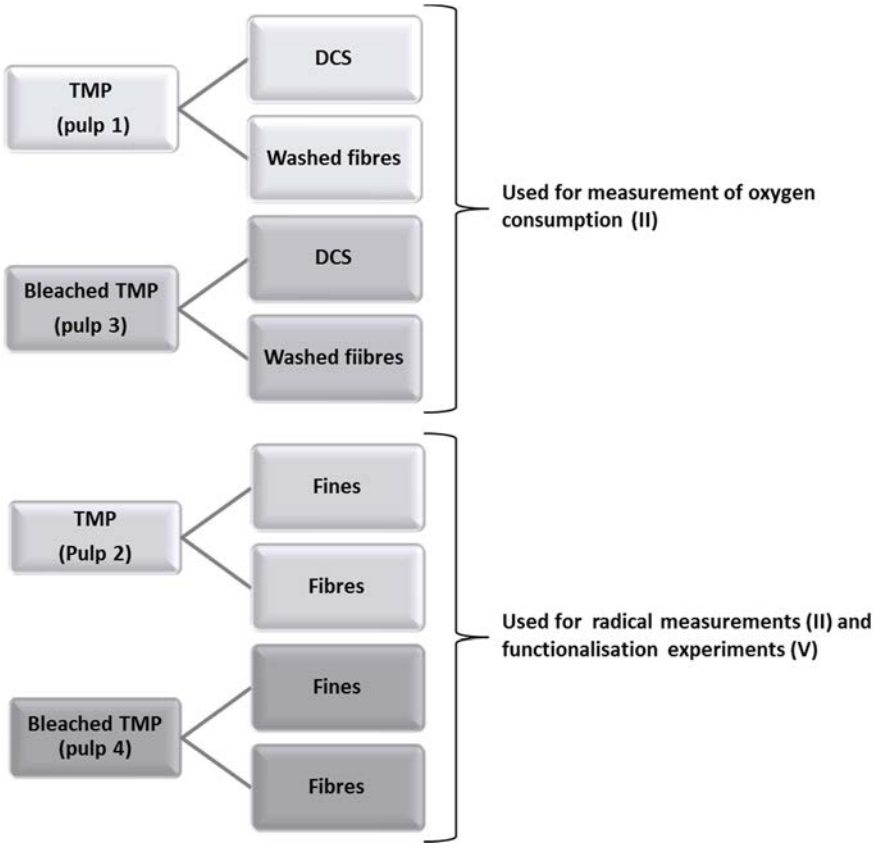


Figure 11. Pulps and pulp fractions used in the work.

Table 3. Chemical composition of the pulps and pulp fractions used in this work.

	Carbohydrates		Lignin		Extractives	Metals
	mg/100 mg	mg/L	mg/100 mg	mg/L	mg/g	mg/kg
TMP (Pulp 1)	71		27		7	2300
-DCS-water pH 4.5		≤ 96		63	59	nd
-DCS-water pH 7		≤ 99		91	69	nd
TMP (Pulp 2)	72		26		7	2020
-fines	63		35		9	1860
-fibres	73		26		2	1290
Bleached TMP (Pulp 3)	71		28		4	5310
-DCS-water pH 4.5		≤ 40		48	25	nd
-DCS-water pH 7		≤ 48		44	29	nd
Bleached TMP (Pulp 4)	73		27		5	6550
-fines	63		33		6	4150
-fibres	75		25		3	290

nd = not determined

Table 4. Chemical composition of commercial model fractions used to represent the fatty and resin acids present in pine and spruce wood (V).

Name	Components	Identified components (% of d.w.)	Major components	% identified
Tall oil fatty acids	Fatty acids	84.4	Linoleic acid	53.1
			Oleic acid	29.3
			Pinolenic acid	11.0
			Others	6.6
Gum rosin	Resin acids	58.8	Conjugated resin acids	67.9
			Pimaric acid	9.3
			Dehydroabietic acid	7.2
			Isopimaric acid	5.4
			Others	10.2

Table 5. Laccases used in the experiments. One nanokatal (nkat) is defined as the amount of enzyme activity that converts 1 nanomol of the substrate per second.

Laccase	Supplier	Redox potential (V)	Protein (mg/mL)	Activity (nkat/mL)			
				Laccase	Manganese peroxidase	Lignin peroxidase	Xylanase
Trametes hirsuta[1]	VTT	0.78 (pH 4.9)[a]	5.7	7600*	4.3	0	4.1
Trametes hirsuta[2,5]	VTT		12.5	4400*	16.4	86.6	nd
Trametes hirsuta[3]	VTT		12.4	5200*	5.9	0.7	1.7
Trametes hirsuta[4]	VTT		nd	9571*	122	19	nd
Myceliophtora thermophila[2]	Novozymes	0.47 (pH 6)[b]	13.5	1020*, 1150**	0*	0*	nd
Trametes villosa[3]	Novo Nordisk	0.79[b]	nd	2100	nd	nd	nd

[1] used in oxygen measurements in paper II, [2] used in radical measurements in paper II, [3] used in paper III, [4] used in paper IV, [5] used in paper V,

[a] (Rebrikov *et al.* 2006), [b] (Kumar *et al*. 2003)

* at pH 4.5, **at pH 7

nd = not determined

4.2 Laccase treatments of pulp material monitored by oxygen consumption (II)

The activities of *T. hirsuta* (at pH 4.5) and *M. thermophila* laccases on DCS-waters, pulps, fibre and fines fractions were analysed by measuring the consumption of dissolved oxygen in samples during laccase treatment at 40°C under agitation at 500 rpm. The substrates were diluted to 0.5% consistency with 0.1 M sodium citrate buffer (pH 4.5) or sodium phosphate buffer (pH 7).

The DCS-waters prepared at pH 4.5 and 7 were used as such. The laccase dosage in pulp, fibre and fines treatments was 1 000 nkat/g of the dry sample, whereas in treatments of DCS-waters, the dosage was 10 000 nkat/L. The dissolved oxygen measurements were made in a closed vessel with a SensorLink PCM800 meter using a Clark oxygen electrode.

4.3 Laccase treatments of pulps prior to detection of radicals in dried samples (II)

The radicals generated in unbleached and bleached TMP and fibre and fines fractions during laccase treatment were studied by electron paramagnetic resonance spectroscopy (EPR spectroscopy) from dried samples. Prior to measurement of radical species by EPR spectroscopy, pulps or pulp fractions were treated with *T. hirsuta* laccase using a dosage of 1 000 nkat/g of the dry sample at 1% consistency, pH 4.5, at 40°C (for pulps) or at room temperature (RT) (for fines and fibre fractions) for 1h with extra oxygen supply. Immediately after the treatments, the fibre material was filtered, washed with distilled water (20 x dry weight of the sample) and hand sheets were prepared according to SCAN M 5:75 on wire cloth. The hand sheets were dried at room temperature. As the test tube for EPR spectroscopy had to be filled uniformly, small discs punched from hand sheets made of the treated pulps were used to fill the test tube. The “long-living” radicals were detected from dried samples by EPR spectroscopy within two days of the laccase treatment.

In order to study further the role of DCS material in the laccase-catalysed oxidation of fibre fraction, the fibres were diluted to 1% consistency with water or DCS – water made using 2% pulp and thereafter treated with laccase (1 000 nkat/g) for 1 h at RT, pH 4.5 (unpublished results).

4.4 Laccase treatments of pulps prior to detection of radicals in frozen samples (V)

The radicals generated in unbleached and bleached TMP in laccase treatment were studied by EPR spectroscopy immediately after the treatment. Prior to measurements of radical species by EPR spectroscopy, unbleached and bleached TMPs were treated with *T. hirsuta* laccase at 1% consistency, pH 4.5 and at 20°C

for 30 minutes. In order to study the effect of laccase dosage on the formation of radicals, dosages from 10 to 10 000 nkat/g of the dry sample were studied. After the treatments, the pulps were washed and filtered and packed into capillary quartz tubes (2.4 mm ID). After packing, the tubes were transferred to liquid nitrogen (-196°C).

The stability of radicals was studied from samples treated with a laccase dosage of 2000 nkat/g. The amount of radicals was measured with EPR spectroscopy for bleached and unbleached TMP for 0–1440 minutes and for 0–1500 minutes, respectively.

4.5 Laccase treatments of fatty and resin acids (III)

The oxidation of fatty and resin acids by laccase treatment was studied. The fatty and resin acid substrates were dissolved in acetone to a concentration of 20 g/l. For the enzymatic treatment, acetone solution was mixed into 25 mM ammonium acetate buffer to obtain a colloidal dispersion with a concentration of 400 mg/l. A low amount of acetone remained in the system, but was regarded as not affecting the experimental procedure. Laccase (5 000 nkat/g) treatments were carried out at 40°C and at pH 4.6 for 4 h. Oxygen was bubbled through the reaction vessel during the reaction. After the enzymatic treatment, the dispersions were heated in boiling water for 10 min in order to inactivate the laccase.

4.6 Laccase treatments of MWL (IV)

The effects of laccase oxidation on MWL were studied. For the laccase treatments, MWL was dissolved in a small volume of 0.1 M NaOH. The dissolved lignin was mixed with 25 mM citric acid buffer to obtain a dispersion with a concentration of 1 mg/ml. Laccase (1 000 nkat/g) treatments were carried out at 20°C, pH 4.5. The oxygen consumption was detected for the first 30 minutes as described above, whereafter the reaction was continued up to 24 h in contact with air. The effects of the enzymatic treatment were analysed by monitoring the changes in the molecular mass of the substrate by size exclusion chromatography (SEC) (Hortling, Turunen & Kokkonen 1999). Changes in the amount of phenolic hydroxyls were analysed spectroscopically (Tamminen, Hortling 1999). Formation of radicals in an MWL sample (1 mg/ml) treated with 2500 nkat/g of laccase at RT was detected by EPR spectroscopy in frozen samples.

4.7 Laccase treatment of tyramine (V)

The ability of laccase to oxidise 3-hydroxytyramine (hereafter tyramine, Figure 12) was analysed by measuring the consumption of oxygen in a solution of 2.65 mM tyramine dissolved in 0.1 M citric acid buffer treated with 100 nkat/g of laccase at pH 4.5 and RT. The measurements were made in a closed vessel as described above.

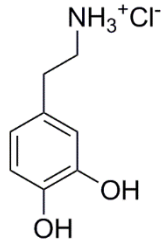


Figure 12. Structure of 3-hydroxytyramine.

4.8 Laccase-aided functionalization of pulps (V)

The pulps were first activated by laccase treatment (1000 nkat/g) for 30 minutes (RT, pH 4.5, constant mixing), whereafter 3-hydroxytyramine hydrochloride dissolved in water was added (0.33 mmol tyramine/g pulp). The total treatment time including mixing was 1 h for samples analysed by ESCA and 3 h for samples analysed by FTIR. The final pulp concentration after all additions was 7.5%. After the treatment the pulp was filtered twice and washed with distilled water (20 x dry weight of the sample).

4.9 Reference treatments

The reference treatments for all experiments were performed under identical conditions as described above, but without addition of laccase. For the functionalization experiments reference samples without addition of laccase and/or tyramine were prepared.

4.10 Analytical methods

The analytical methods used in this work are summarised in Table 6.

Table 6. Analytical methods used in the experiments.

Method	Detection of	Details	References	Publication
High-performance liquid chromatography (HPLC)	Carbohydrate composition of pulps	Analysis after acid hydrolysis	(Tenkanen *et al.* 1999, Tenkanen, Siika-Aho 2000)	II
HPLC	Carbohydrates in DCS	Analysis after secondary enzymatic hydrolysis of oligomers to monomers	(Buchert *et al.* 1993)	II
Atom absorption spectroscopy (AAS)	Metals	Analysed after ashing		II
Klason lignin	Lignin in pulps	Analysed after acid hydrolysis (KCL method 115b:82)		
Gas chromatography (GC)	Extractives	After methyl *tert*-butyl ether (MTBE) extraction	(Örså, Holmbom 1994)	II, III
Measurement of dissolved oxygen	Laccase activity based on co-substrate consumption	The oxygen measurements were made in a closed vessel with a Sensor Link PCM800 meter using a Clark oxygen electrode.		II, IV, V
Electron paramagnetic resonance spectroscopy (EPR spectroscopy)	Laccase action based on radical formation	Detected in dried samples Detected in frozen samples		II IV, V
Size exclusion chromatography (SEC)	Molecular mass	Three TSK-gel columns (G3000, G2500 and G1500HXL), tetrahydrofuran as an eluent. Detection was carried out with RI.		III
		Four polystyrene sulphonate Na-salts (MW 4 800, 17 000, 41 000 and 35 000) were used as standards.	(Hortling, Turunen & Kokkonen 1999)	IV

UV spectroscopic analyses	Lignin content in aqueous phase Conjugated phenolic structures and total content of phenolic structures		(Örså, Holmbom 1994, Tamminen, Hortling 1999)	II IV
Electron Spectroscopy for Chemical Analysis (ESCA)	Surface composition	Analysis of handsheets with a Kratos Analytical AXIS 165 electron spectrometer using a monochromated A1 Kα X-ray source		V
Fourier transform infrared spectroscopy (FTIR)	Detection of covalent chemical linkages.	Bruker Equinox 55 Irscope FTIR Microscope (Germany) FTIR spectra of the samples were measured using transmission technique and a diamond cell. The spectral resolution was 4 cm^{-1} and the number of scans was 200. FTIR spectra of the samples were measured using transmission technique and a diamond cell.		V

5. Results and discussion

New properties can be introduced to mechanical pulp fibres by functionalization, i.e. by bonding of new functional components to the fibre surfaces. The presence of lignin on the fibre surfaces offers possibilities for formation of radicals by oxidative enzymes. Radical formation is the first step in the oxidative enzyme-aided bonding of new compounds to the fibre surface. To control the extent of modification, the formation of radicals should be controlled.

5.1 Action of laccase on mechanical pulps (II, III, V)

The action of laccases from *T. hirsuta* (at pH 4.5) and *M. thermophila* (at pH 7) on bleached and unbleached TMP, fibres and fines fractions and DCS-water (Figure 13) was studied by following the consumption of laccase co-substrate, *i.e.* oxygen, in the reaction system.

The *T. hirsuta* (at pH 4.5) and *M. thermophila* (at pH 7) laccases were selected for the pulp and pulp fraction treatments as they have different pH optima and could thus be used in different pH conditions (Table 5). This was of interest as pH is known to have a strong effect on the dissolution of DCS material *i.e.* lipophilic extractives, lignans, carbohydrates and some lignin from wood (Sundberg *et al.* 2009, Strand *et al.* 2011). The selected enzymes also differed from each other in their oxidation potential and molecular size. The *T. hirsuta* laccase (62 kDa) is a basidomycetes laccase with high oxidation potential (0.78 V), whereas the *M. thermophila* laccase (85 kDa) is an actinomycetes laccase with low oxidation potential (0.47 V) (Lähdetie *et al.* 2009, Berka *et al.* 1997).

Due to the molecular size of oxidative enzymes (diameter ~10 nm), DCS have been suggested to have a role in the enzyme-aided radical formation in fibre-bound lignin (Felby *et al.* 1997, Hassingboe, Lawther & Felby 1998, Barsberg, Thygesen 1999). Therefore, the effect of DCS on the laccase-catalysed oxidation of TMP was evaluated by comparing the oxidation of unwashed pulps with oxidation of washed pulps containing less DCS (Figure 13, a versus b).

The “long-living”, stable radicals created in pulps and fines and fibre fractions by *T. hirsuta* laccase at pH 4.5 were analysed from dried samples by EPR spectroscopy within two days of the laccase treatment. In order to determine the

more labile radicals, radicals were also measured from pulp samples directly after laccase treatment and freezing. Additionally, the oxidation of fatty and resin acids model fractions by two *Trametes* laccases having about the same redox potential was assessed.

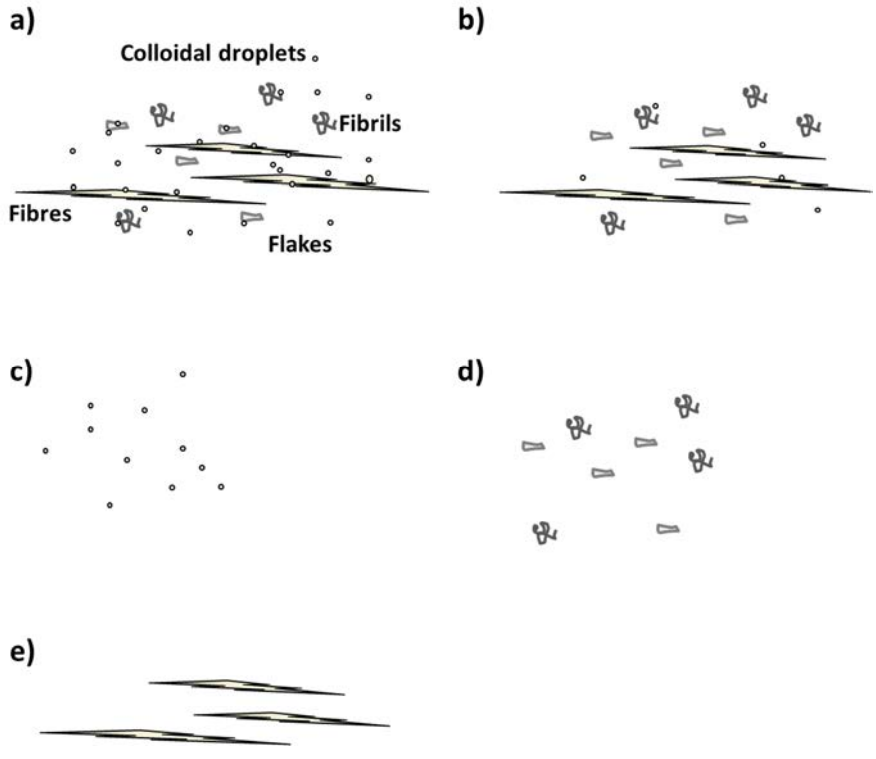


Figure 13. Pulps and pulp fractions from unbleached and bleached TMP used to evaluate the effects of laccases from *T. hirsuta* (at pH 4.5) and *M. thermophila* (at pH 7 a) TMP, b) washed TMP, c) DCS-water, d) fines fraction and e) fibres fraction of TMP.

5.1.1 Action of laccase on TMP and the role of DCS material on the oxidation of TMP (II)

Both *T. hirsuta* (at pH 4.5) and *M. thermophila* (at pH 7) laccases were found to catalyse oxidation of the unbleached TMP as such and the DCS-fraction made thereof as seen by the consumption of oxygen (Figure 14). Interestingly, even though the *M. termophlia* laccase has a lower oxidation potential, more oxygen was consumed at pH 7 than with *T. hirsuta* laccase at pH 4.5, indicating increased oxidation of the pulp and the DCS fraction. The main reason for the observed higher reactivity was most probably the availability of more reactive material at pH 7,

as more DCS and lignin are dissolved from the pulp at pH 7 than at pH 4.5 (Örså *et al.* 1993). In addition, the different substrate specificities of the used laccases may have affected the degree of detected oxidation.

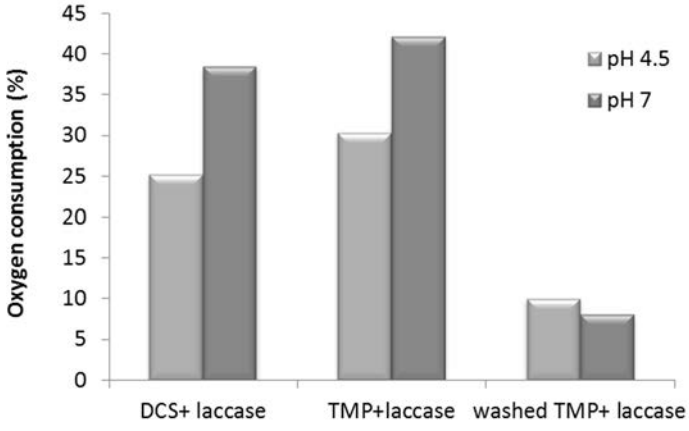


Figure 14. Consumption of oxygen in laccase-catalysed oxidation of DCS-water and unwashed and washed TMP at pH 4.5 (*T. hirsuta*) and pH 7 (*M. termophlia)*, 40°C, 30 min.

The ability of laccase to catalyse the oxidation of fibre-bound lignin is well known and the action of laccases on DCS material has also been reported previously (Felby *et al.* 1997, Hassingboe, Lawther & Felby 1998, Buchert *et al.* 1999). The aim of this study was to further clarify the role of DCS in the oxidation. Thus, the role of DCS material in the oxidation of fibre-bound material was studied by washing the pulp at pH 7 to remove excess DCS material from the pulp. Dilution of the pulp in warm water at pH 7 has been reported to remove readily liberated material from the surface of the fibres (Örså, Holmbom 1994). It is, however, also known that even after effective washing more low-molecular mass substances are dissolved from the mechanical pulp when the pulp is mixed with fresh water (Ekman, Eckerman & Holmbom 1990). Thus, the washed TMP studied in this work can be considered to be “low DCS” pulp. The activities of both *T. hirsuta* and *M. thermophila* laccases on the washed pulp were clearly lower than on the original TMP, indicating that the washing step had removed most of the material readily oxidised by these laccases (Figure 14). However, it could not be concluded how much of the detected oxygen consumption was due to oxidation of material further dissolved and dispersed and how much was due to oxidation of fibre-bound material.

The enzyme-catalysed oxidation of the pulps was also followed by detection of radicals by EPR spectroscopy. The presence of water is known to weaken the signal obtained in the EPR spectroscopy analysis. Thus, dried samples were utilised in the measurements. As the radicals were measured within two days, the detected radicals were concluded to be “long-living” radicals.

Less radicals were observed by EPR spectroscopy in the pulp samples from which the dissolved and colloidal substances were removed prior to laccase treatment by washing (Figure 15). In the unwashed pulp, the amount of radicals was found to increase by about 25% as a result of the laccase treatment, whereas the increase obtained by laccase treatment was only 16% for the washed pulp. A similar effect of removing the DCS by washing was thus observed both in oxygen consumption and EPR spectroscopy measurements.

It was concluded that the radicals detected by EPR spectroscopy were probably located in fibres and fines of both unwashed and washed pulps, as the loose DCS was expected to be washed away when the laccase treatment was stopped by washing and filtration and additionally with cold water during the hand sheet preparation. As more radicals were found in the unwashed TMP compared to the washed TMP, the previous suggestion that DCS material might have a mediating role in the laccase-catalysed oxidation of the fibre-bound material was supported (Felby *et al.* 1997, Hassingboe, Lawther & Felby 1998). However, it is also possible that the difference in radical content of pulps can be explained by radicals formed in DCS material reattached to the fibre mat during filtration as the treatment was stopped.

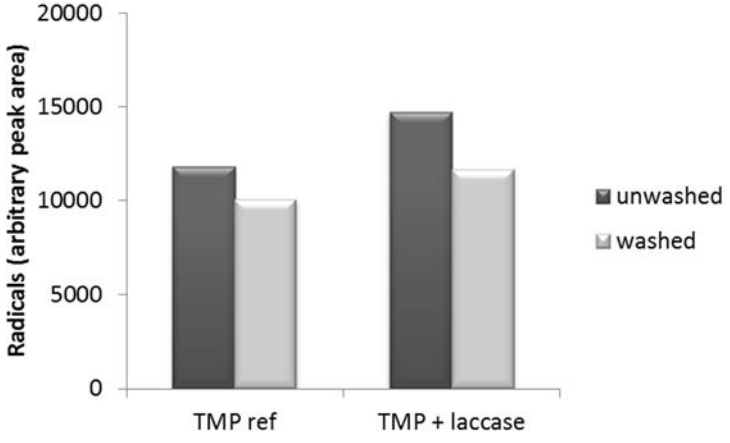


Figure 15. Radicals detected by EPR spectroscopy in the unwashed and washed TMP after laccase treatment (*T. hirsuta* laccase (1 000 nkat/g), 1h, 40°C, pH 4.5, 1% consistency).

The effects of the laccase on bleached TMP and DCS water made thereof were also studied. No clear laccase activity on the bleached TMP or on the DCS prepared thereof was detected by measurement of oxygen consumption. On the basis of information from the literature, the effect of peroxide bleaching on the surface lignin content of mechanical pulps has been found to be very small or insignificant, whereas a small reduction in the amount of extractives by peroxide bleaching has been reported (Koljonen *et al.* 2003). However, in peroxide

bleaching of spruce milled wood lignin, the coniferyl aldehydes have been reported to be effectively degraded (Holmbom *et al.* 1991). The analysis of chemical composition of DSC waters revealed that the amount of lignin and extractives in the DCS-water prepared from bleached TMP was lower than in that prepared from unbleached TMP (Table 3). The altered chemistry of lignin after peroxide bleaching and the lower amount of DCS had the result that no changes were detectable by measurement of oxygen consumption.

5.1.2 Effect of laccases on fatty and resin acids (III)

As shown above, the presence of DCS clearly affects the degree of oxidation of TMP by laccase. Previously published research has shown activity of laccases especially on lignans, but has also demonstrated that oxidation of triglycerides, steryl esters and resin acids is possible (Buchert *et al.* 1999, Buchert *et al.* 2002a). Free and esterified fatty acids are the major group of extractives found in DCS. The effect of laccases on fatty acids had not been reported before this research, making the oxidation of fatty acids an interesting area. Resin acids, found in resin canals, dissolve and disperse quickly from mechanical pulps to water (Örså *et al.* 1993). Besides being one of the major causes of pitch problems (Matsui *et al.* 1998), fatty and resin acids have also been proposed to have a mediating role in the laccase-catalysed oxidation of wood lignin (Felby *et al.* 1997, Barsberg, Thygesen 1999).

Two *Trametes* laccases (*i.e. T. hirsuta* and *T. villosa*) were used for oxidation of fatty and resin acid model fractions. Both *Trametes* laccases, although having different oxidation potentials, appeared to be able to act on fatty acids containing several double bonds. *T. hirsuta* laccase was found to decrease the amount of pinolenic and linoleic acids by about 20%, whereas the effect of *T. villosa* laccase was slightly less pronounced (Figure 16). SEC analysis also showed the formation of reaction products of higher molecular mass in the laccase reaction, indicating formation of oligomers (III). However, the oligomers formed were not identified. The possible effect of enzyme (protein) addition on the solubility of fatty acids was not studied.

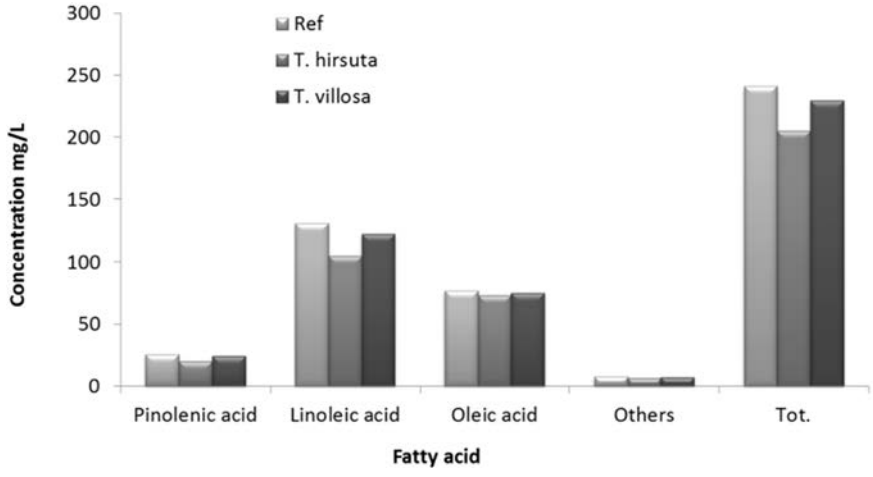


Figure 16. Effects of *Trametes* laccases (5 000 nkat/g) on fatty acids found in TOFA (4 h, 40°C, pH 4.6 with extra oxygen supply).

When the resin acid fraction was treated with the laccases, a clear decrease in the amount of resin acids with conjugated double bonds was observed with both laccases (Figure 17). Thus, the effect of laccases appeared to correlate with the type of chemical linkages present in the fatty and resin acids. As the obtained results and the results reported in the literature clearly show that laccases act on lignans, triglycerides, steryl esters and free fatty and resin acids, it is possible, as suggested, that DCS could have a role in the oxidation of fibre-bound lignin.

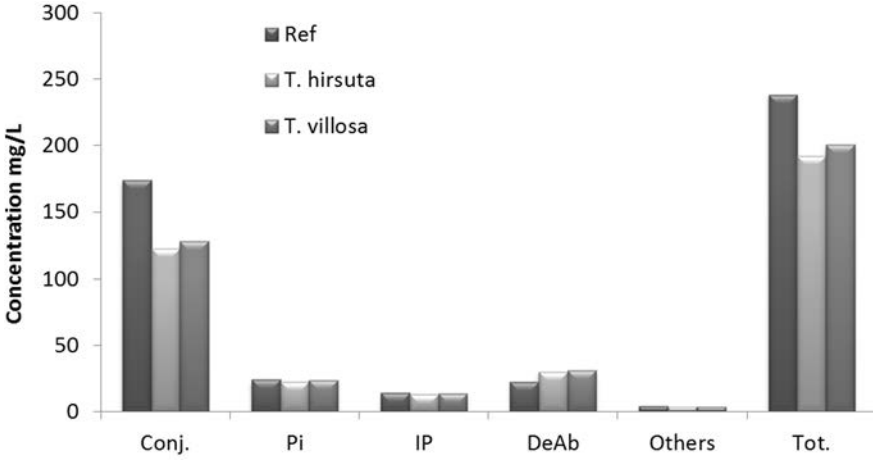


Figure 17. Effects of *Trametes* laccases (5000 nkat/g) on resin acid model fraction (4 h, 40 °C. pH 4.6 with extra oxygen supply). Conj. indicates conjugated resin acids: abietic, neoabietic and Pi pimaric acid.

5.1.3 Activity of laccase on fibres and fines fraction (II)

The laccase-catalysed oxidation of fractionated TMP fibres and fines was studied by oxygen consumption measurements during oxidation and EPR analysis after oxidation. During fractionation the fibres and fines were extensively washed, resulting in removal of extractives (Table 3). Interestingly, after fractionation of the unbleached and bleached TMP to fines and fibres, practically no oxygen consumption was detected by oxygen consumption measurements, although the materials are known to be rich in lignin and have a high surface lignin content (Kleen, Kangas & Laine 2003, Koljonen *et al.* 2003, Kangas, Kleen 2004).

Although oxidation of fines and fibres could not be detected by oxygen consumption measurements, the EPR measurements of samples prepared from dried hand sheets revealed that both fines and fibres of the unbleached pulp were oxidised (Figure 18). The radical content of the fines fraction, rich in lignin and extractives, was increased by 30%, whereas the increase in the fibres fraction was 20% as compared to the reference treated samples. The higher amount of radicals in fines can be explained by the reported higher lignin content of fines (Sundberg, Holmbom 2004). Although the amount of surface lignin on fines and on fibres, as analysed by ESCA, has been found to be about the same (Kleen, Kangas & Laine 2003), the higher amount of radicals can be explained by stabilisation of radicals via migration mechanism (Barsberg, Thygesen 1999) in the lignin structure, enabling further oxidation. Additionally, the surface area analysed by ESCA was expected to be smaller than the surface area accessible for laccase.

In order to further study the role of DCS in the laccase-catalysed oxidation of lignin-rich pulp material, extra DCS material was mixed with the fibres fraction prior to treatment with *T. hirsuta* laccase (unpublished results). The EPR measurements showed that the amount of radicals was higher in the reference sample diluted with extra DCS compared to the sample diluted with water. The laccase treatment resulted in a 17% increase in radicals for both the samples, with and without the extra DCS. Thus, the degree of laccase-aided oxidation could not be affected by the addition of extra DCS.

The increase in the amount of radicals obtained by laccase-catalysed oxidation was negligible for peroxide-bleached fibres, whereas a 25% increase was observed for the fines fraction (Figure 18). Peroxide bleaching is known to cause slight modification of lignin (Holmbom *et al.* 1991). According to the literature, it appears that the fibres and fines have different lignin structures (Kangas, Suurnäkki & Kleen 2007, Kleen, Kangas & Laine 2003). This difference can explain why only oxidation of fines was observed.

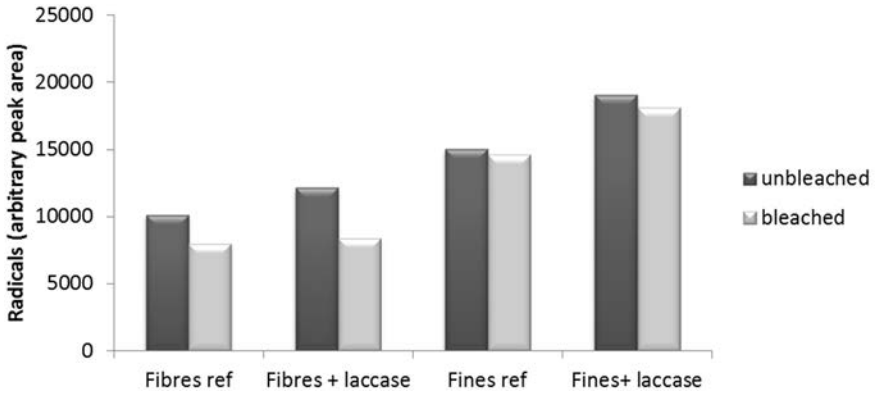


Figure 18. Radicals detected by EPR spectroscopy in laccase-treated fines and fibre fractions of unbleached and bleached TMP (*T. hirsuta* laccase (1000 nkat/g), 1 h, 40°C, pH 4.5, 1% consistency).

On the basis of analyses carried out by ESCA, measuring the surface to a depth of 5–10 nm, the effect of laccase treatment on the surface properties of TMP fibres and flakes has been reported to be minor (Kangas, Suurnäkki & Kleen 2007). However, laccase treatment was claimed to lower the surface coverage of extractives on fibrils and to increase the surface coverage of lignin of fibrils.

Felby and co-workers (1997a) reported a linear relationship between free radical formation and oxygen consumption in beech wood fibres treated with laccase. Our results for laccase-treated TMP and washed TMP support the reported correlation. However, as no oxygen consumption was detected during laccase-catalysed oxidation of the unbleached and bleached TMP fibres and fines fractions, even though radicals were found to be generated, it appears that the oxidation of pulps detected by the dissolved oxygen detection equipment used in our work actually only reveals oxidation of material dissolved from the pulp. The amount of accessible surface lignin in pulp, fibre and fines fractions is probably too small for the oxidation to be detected by oxygen consumption.

5.1.4 Effect of sample drying, storage time and enzyme dosage on the detected amount of radicals (V)

Radicals are significantly dependent on storage conditions and time and the presence of water. Felby and co-workers (Felby, Hassingboe & Lund 2002) reported a clear difference between the amount of radicals measured in wet and dry laccase-treated fibre samples. The levels of radicals in samples dried with a flash drier were lower than those in wet samples. No information was provided about the storage time used in the experiments.

Oxidation of unbleached and bleached TMP with *T. hirsuta* laccase was studied further by measuring radicals in the samples by EPR spectroscopy directly after

laccase treatment and freezing. The laccase treatment temperature was here only 20°C. The stability of the generated radicals was studied as a function of storage time. Additionally, the formation of radicals as a function of laccase dosage was studied.

Interestingly, when the frozen samples were analysed by EPR spectroscopy, the amount of radicals generated in the bleached pulp by laccase-catalysed oxidation was higher than that generated in the unbleached TMP (Figure 19). This result was not in line with the findings discussed in Sections 5.1.1 and 5.1.3 according to which, on the basis of oxygen consumption measurements and radical measurements carried out with dried samples, the unbleached TMP was oxidised more efficiently than bleached TMP. However, as concluded above, the observed consumption of the co-substrate, *i.e.* oxygen may actually have been only due to oxidation of DCS. Further, in the EPR spectroscopy measurements carried out on dried samples, only the stable radicals could be measured, whereas in these new measurements of wet and frozen samples the more labile radicals were also included. Thus, the difference in the results for the dried and wet samples suggests rapid decay of some of the radicals formed. As the amounts of radicals detected in dried samples do not correlate with those measured in wet samples, radicals detected in dried and stored samples can only be seen as a proof of radical formation, but should not be used to design further treatments utilising the formed radicals. One possible explanation for the higher amount of radicals in frozen bleached pulp sample found in the literature (Suurnäkki *et al.* 2010) is that the amount of radicals generated in various pulps is dependent on the amount of phenoxy groups rather than on the total amount of lignin in the pulp.

A clear decrease in the total amount of radicals in both pulps was detected as a function of time (Figure 19). The peak areas in the bleached and unbleached TMPs were decreased by ~ 43 and 55%, respectively, within 75 minutes and were only about 10% of the initial values after 24 hours. As expected, the results show that the delay between sample preparation and actual measurement strongly affects the amount of detected radicals.

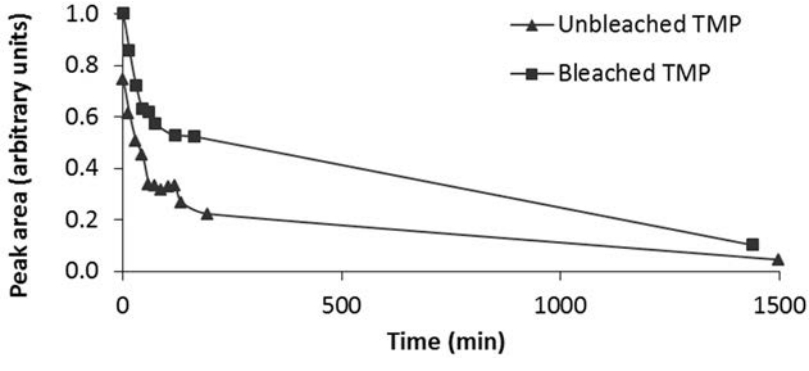


Figure 19. Stability of laccase-generated radicals in unbleached and bleached TMPs as a function of the storage time at RT after the laccase treatment (*T. hirsuta* laccase (2000 nkat/g), 30 min, RT, pH 4.5, 1% consistency).

As expected, the laccase dosage was found to have a clear influence on the amount of radicals formed in both unbleached and bleached TMPs (Figure 20). The amount of radicals generated in the pulps by laccase was found to increase when the dosage was increased at a given consistency. However, the increase in radical content was not directly proportional to the increase of dosage. The effect of laccase dosage on the amount of formed radicals has also been reported by Suurnäkki and co-workers (Suurnäkki *et al.* 2010).

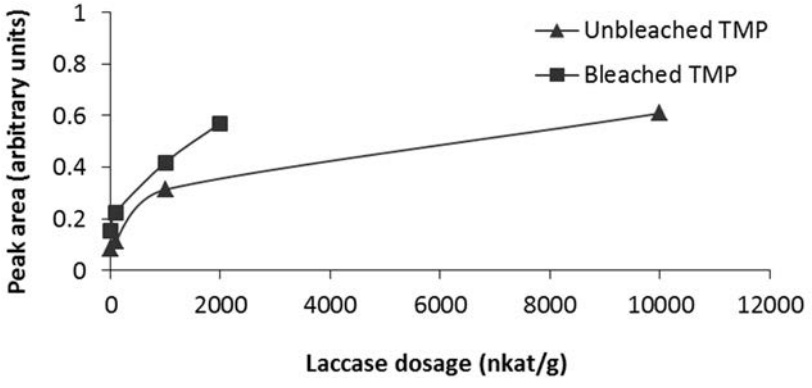


Figure 20. Radicals detected by EPR spectroscopy in unbleached and bleached TMP samples treated with *T. hirsuta* laccase for 30 min at RT, pH 4.5 at 1% consistency (samples were transferred into liquid nitrogen immediately after the treatments).

Based on the obtained results and reports found in the literature (Felby, Hassingboe & Lund 2002, Barsberg, Thygesen 1999), it appears that two types of radicals can be detected after laccase treatments of wood fibres; *i.e.* "short-living" radicals that can only be detected immediately after the laccase treatment in wet fibre samples and stable," long-living" radicals which can be detected in dried samples even days after the treatment. The stable radicals detected in dry samples represent only a small proportion of the originally generated radicals.

Suurnäkki and co-workers (Suurnäkki *et al.* 2010) studied the absolute amount of phenoxy radicals formed in oxidation of both TMP and long fibres from TMP. The time needed to reach the maximum level of detectable radicals in the whole pulp (TMP) was up to 30 minutes, whereas the maximum level was reached in the long fibres fraction in just 5 minutes. The authors suggested that in the presence of DCS, the laccase-catalysed oxidation mechanism of TMP would be different than without DCS. It was suggested that the low-molecular mass compounds could act as mediators, as well as cross-linking both with each other and with the fibre moiety (Suurnäkki *et al.* 2010). As the amount of radicals was so much lower in the whole pulp than in fibres, coupling of low-molecular mass compounds (and fines) with each other and with lignin appears to be the most likely explanation.

5.2 Effect of laccase on isolated lignin (MWL) (IV)

As laccase can oxidise both extractives and lignin, studies with isolated lignin excluded the role of extractives. In the *T. hirsuta* laccase-catalysed oxidation of MWL, consumption of oxygen was observed, indicating oxidation of the MWL (Figure 21). Laccase-catalysed oxidation of MWL was also demonstrated by EPR spectroscopy, as formation of radicals was detected (Figure 22). When the effect of treatment time on the laccase-catalysed oxidation was studied, no additional effect on the amount of radicals was observed by prolonging the treatment time from 20 minutes to 2 hours. However, a prolonged treatment of 24 h was found to result in clear polymerisation of MWL (Figure 23). The molecular mass of the fraction with the largest molecules was found to increase by 40% (from 9200 to 12 900). The 24 h laccase treatment was not found to decrease the amount of total phenols in lignin, whereas the amount of conjugated phenols in lignin was found to increase (Table 7). Similar results have been obtained with residual lignin isolated from kraft pulp (Niku-Paavola *et al.* 2002). However, oxidation of a lignan compound (hydroxymatairesinol) by laccase has been reported to result in a 40–50% decrease in the phenolic hydroxyl content (Buchert *et al.* 2002a). Thus, it appears that the effect of laccase on low-molecular mass substrates, such as lignans, is different to that on the more complex lignin.

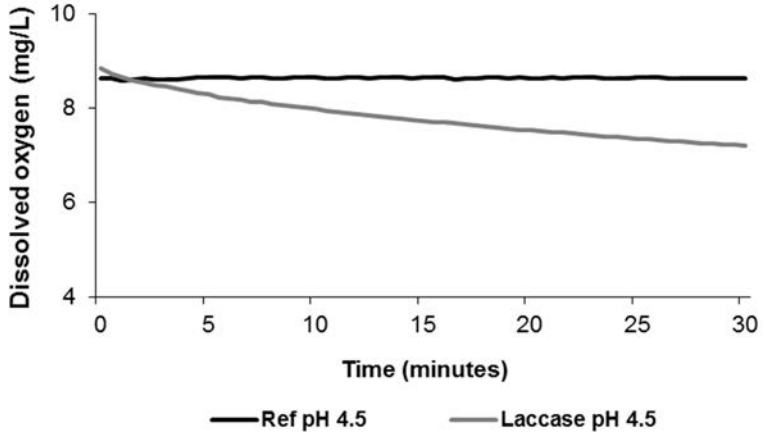


Figure 21. The reactivity of MWL in the laccase-catalysed oxidation as analysed by co-substrate (*i.e.* oxygen) consumption. Treatment conditions: 0.1% MWL solution, pH 4.5, 20°C, *T. hirsuta* laccase (1000 nkat/g).

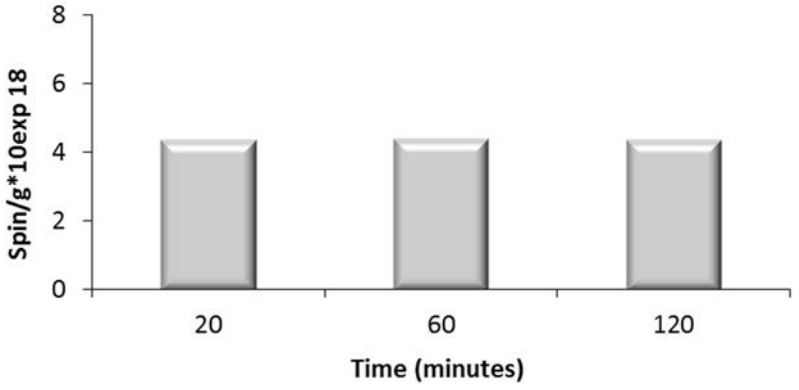


Figure 22. The absolute amount of phenoxy radicals measured by EPR spectroscopy in MWL treated with laccase. Treatment conditions: 0.1% MWL solution, pH 4.5, 20°C, *T. hirsuta* laccase (1000 nkat/g).

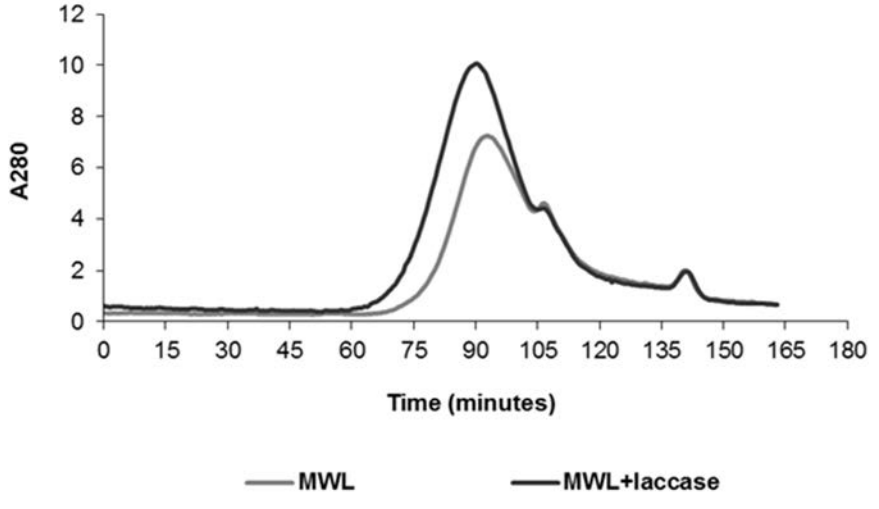


Figure 23. Gel permeation chromatograms curves of MWL and MWL treated with laccase. A 0.1% MWL solution, pH 4.5, 20°C, was treated with *T. hirsuta* laccase (1000 nkat/g) for 24 h.

Table 7. Effect of laccase treatment on the structure of lignin. A 0.1% MWL solution, pH 4.5, 20°C, was treated with *T. hirsuta* laccase (1000 nkat/g) for 24 h.

Treatment	Conj. Phenols (mmol/g)	Tot. phenols (mmol/g)	Conj./Tot phenols (%)
Ref, pH 4.5	0.141	1.09	13
Laccase, pH 4.5	0.162	1.11	15

Apparently, in larger lignin structures, the formed radicals can migrate into the structure (Barsberg, Thygesen 1999) and thus no decrease in the amount of phenolic hydroxyls is detected. It is also possible that in accordance with a previous finding that when a phenoxy radical delocalized into the phenol ring reacts with another radical, a hydroxyl group is regenerated at its original site (Hüttermann, Mai & Kharazipour 2001). The results also correlate well with those on the stability of radicals discussed in Section 5.1.4, showing that the amount of radicals decreases as a function of time.

In order to utilise the radicals formed during laccase-catalysed oxidation in functionalization of mechanical pulps, the bonding of new compounds to fibre surfaces by radical reactions should be performed within an appropriate short time after activation, before the radicals are migrated in the structure.

5.3 Functionalization (V)

The possibility to utilise the radicals formed in the laccase-catalysed oxidation of unbleached and bleached TMP was studied by bonding of tyramine to the activated pulps. As laccase-catalysed oxidation of both TMP (Figure 14) and tyramine (Figure 24) with *T. hirsuta* laccase was found to be rapid, the bonding of tyramine by radical coupling to TMP was expected to be possible. The degree of bonding was analysed by ESCA as increased nitrogen content on the surface of the fibres.

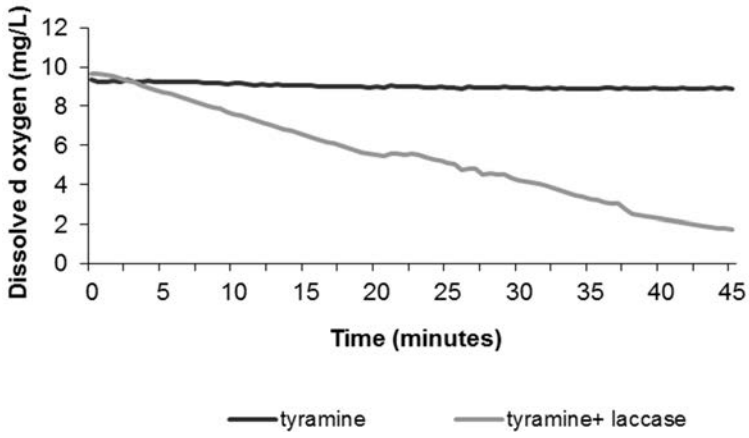


Figure 24. The reactivity of 3-hydroxytyramine hydrochloride in the laccase-catalysed oxidation as analysed by co-substrate (*i.e.* oxygen) consumption. Treatment conditions: 2.65 mmol/L of tyramine, pH 4.5, RT was treated with *T. hirsuta* laccase (1000 nkat/g).

ESCA analysis of the TMP pulps indicated that tyramine was bonded to both unbleached and bleached TMP (Table 8). The degree of bonding to the bleached pulp was significantly higher than to the unbleached pulp. The ESCA results revealed that the amounts of C-O and C=O were increased by the laccase treatment, indicating that laccase had oxidised the surface lignin in the pulp. The nitrogen content analysed by ESCA was used to estimate the surface coverage of tyramine. Nitrogen content of 3-hydroxytyramine is 9.1% as calculated from the molecular mass of nitrogen divided by the molecular mass of 3-hydroxytyramine (14.01 g/mol / 153.18 g/mol). Thus as the surface coverage of nitrogen in the modified unbleached and bleached pulps were +0.5% and +1.4%, respectively, the surface coverage of tyramine in the unbleached pulp would be about 6% and in the bleached pulp 15% after the laccase aided modification.

The bonding of tyramine to TMP was further studied by FTIR (Figure 25). A differential spectrum was produced of the spectra for the reference and tyramine-bonded samples. A band at 1060 indicated the formation of ether linkages.

Table 8. Surface composition (as analysed by ESCA) of handsheets to which 3-hydroxytyramine hydrochloride was bonded by laccase.

	Elemental composition (%)*			Carbon deconvolution (%) **			
Treatment	**O1s**	**C1s**	**N1s**	**C-C**	**C-O**	**C=O**	**C- =O**
Unbleached TMP							
REF	28.3	71.6	**0.10**	44.7	44.7	7.9	2.8
+ tyramine	30.5	69.2	**0.29**	45.4	44.3	7.6	2.7
+ laccase	30.5	69.2	**0.10**	39.6	49.1	9.0	2.6
+ tyramine and laccase	27.8	71.6	**0.61**	46.6	42.8	7.8	2.8
Bleached TMP							
REF	31.0	68.9	**0.11**	36.7	52.5	9.0	1.8
+ tyramine	30.3	69.5	**0.15**	38.9	50.6	8.9	1.7
+ laccase	32.7	66.7	**0.54**	30.2	57.5	10.7	1.6
+ tyramine and laccase	30.3	68.2	**1.51**	35.3	52.3	10.6	1.6

* Relative amounts of oxygen (O 1s), carbon (C 1s) and nitrogen (N 1s) of samples.
** Relative amounts of differently bound carbons (%) measured from high resolution C 1 s spectra.

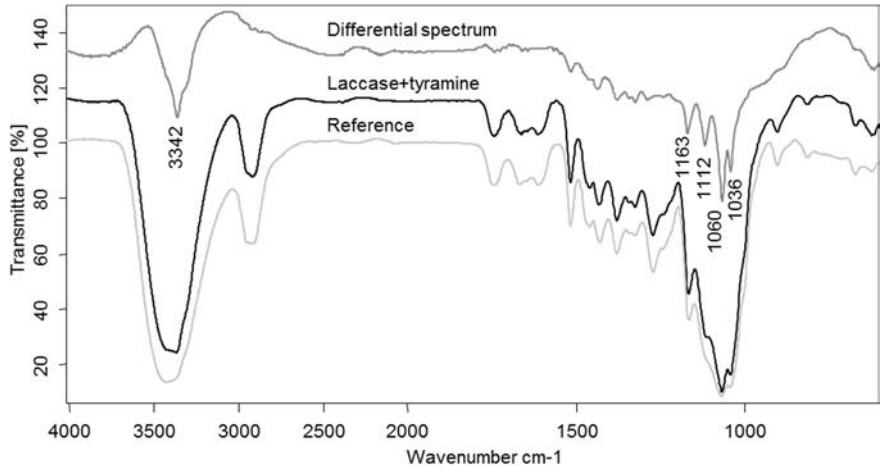


Figure 25. Differential IR spectrum obtained by subtraction of the spectra reference sample from the tyramine+ laccase-treated sample. Aromatic ethers: C-O-C stretching vibration: =C-O-C stretching, 1270–1230 cm^{-1} (strong absorption band) 1050–1010 cm^{-1} (medium strong absorption band). Phenols: O-H stretching vibration (intermolecular H-bonds, polymeric), near 3320 cm^{-1} (broad strong intensity). C-OH stretching vibration: C-OH stretching, 1260–1180 cm^{-1}

Based on earlier findings on MWL and lignans (Buchert *et al.* 2002) and on the FTIR results, a hypothetic mechanism for the bonding of tyramine to lignin is suggested (Figure 26). Laccase catalyses first one electron oxidation of phenolic hydroxyl groups to the corresponding radicals both in lignin and tyramine. The radical formed in a phenolic hydroxyl group of lignin then drifts either to the aromatic ring of the phenol unit (ortho or meta position) or to the aliphatic part. This radical then reacts via a coupling reaction with the phenoxy radical in the tyramine. In principle, the same reaction takes place in nature during the biosynthesis of lignin (Brunow *et al.* 1998). The mechanism provides the possibility to bond new compounds that can be activated by laccase to fibre-bound lignin.

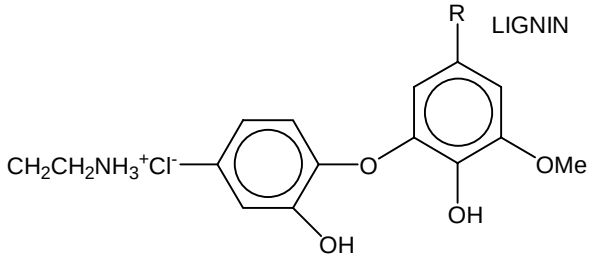


Figure 26. Hypothetical mechanism for bonding of tyramine to lignin by laccase.

The laccase-aided modification of wood fibres by bonding of new molecules has gained considerable interest, and various application areas have been identified and methods patented (Buchert *et al.* 2005a, Buchert *et al.* 2005b, Buchert *et al.* 2005c, Grönqvist *et al.* 2005). Even utilisation of the method for non-wood pulp fibres, *e.g.* flax and sisal fibres has been reported (Aracri *et al.* 2010). Work to identify factors affecting the degree of bonding has been started. Extra oxygen supply has not been found to have any effect on the degree of bonding, whereas reaction consistency and the amount and addition method of the new compound affected the degree of modification (Chandra, Felby & Ragauskas 2004). It has also been claimed that the nature and type of the lignin polymer determines its reactivity and thus how well it can be modified (Nyanhongo *et al.* 2010). Furthermore the type of laccase used has been claimed to affect the degree of bonding (Saarinen *et al.* 2009).

The method has been reported to have potential for bonding of phenol compounds with antimicrobial activity, aiming at *e.g.* novel antimicrobial packages (Elegir *et al.* 2008, Widsten *et al.* 2010, Fillat *et al.* 2012). Bonding of hydrophobic compounds has been reported to result in fibres with hydrophobic properties (Suurnäkki, Mikkonen & Immonen 2011, Reynaud *et al.* 2013, Garcia-Ubasart *et al.* 2013). The yellowing tendency of mechanical pulps has been successfully retarded by bonding of linoleic acid (Buchert *et al.* 2005d, Liitiä *et al.* 2007). The possibility to enhance paper strength with different phenolic compounds has also

been evaluated (Chandra, Lehtonen & Ragauskas 2004, Na, Shulan & Menghua 2008, Liu, Qin & Li 2013). Bonding of amino acids via Michael addition to laccase-oxidised softwood kraft pulps has been found to have a positive effect on the strength properties of paper made from the modified pulp (Witayakran, Ragauskas 2009).

6. Conclusions and future perspectives

The main aim of this thesis was to elucidate the effects of laccases on softwood TMPs and their fractions. Furthermore, utilisation of the radicals formed by laccase-catalysed oxidation in fibre functionalization was to be assessed. The main conclusions answering the aims specified in Section 3 of the study were as follows:

1. The studied laccases were reactive with the unbleached TMPs and their fractions. The degree of oxidation of TMP was found to be influenced by the presence of dissolved and colloidal substances (DCS). However, the results did not conclusively confirm the previously suggested role of DCS in the laccase-catalysed oxidation of fibre-bound lignin.

2. It was concluded that measurement of oxygen consumption could only be used to analyse the oxidation of DCS, as the apparatus used in this work was not sensitive enough to detect oxidation on fibre material. EPR spectroscopy measurements were needed to obtain information about the oxidation of fibres.

3. Based on the results laccase appeared to be able to catalyse the oxidation of free fatty and resin acids. The type of chemical linkages present in fatty and resin acids was found to define the effect of laccase. It seems that laccases can be used to oxidise fatty acids with several double bonds and resin acids with conjugated double bonds. However, as the possible effect of enzyme protein on solubility was not studied, the effect should be confirmed with further studies.

4. Laccase treatment of MWL was not found to decrease the amount of total phenols in lignin, whereas the amount of conjugated phenols in lignin was found to increase. It was concluded that the effect of laccase on low-molecular mass substrates, such as lignans, is different from that on the more complex lignin. Apparently, in larger lignin structures, the radicals formed can migrate into the structure and thus no decrease in the amount of phenolic hydroxyls can be detected.

5. Two types of radicals can be detected after laccase treatments in wood fibres. “Short-living” radicals can only be detected immediately after the laccase treatment in wet fibre samples, whereas stable, “long-living” radicals can be detected in dried samples even days after the treatment. The stable radicals detected in dry samples represent only a small proportion of the originally generated radicals. As it appears that the amounts of radicals detected in dried samples do not correlate with those measured in wet samples, radicals detected in dried and stored samples can only be seen as a proof of radical formation, and should not be used to design practical treatments utilising the formed radicals.

6. In order to utilise the radicals formed during the laccase-catalysed oxidation in functionalization of mechanical pulps, bonding of new compounds to fibre surfaces by radical reactions should be performed right after the activation, before the radicals decay or migrate into the structure.

7. Bleaching of TMP affects the amount and stability of radicals formed in the laccase-catalysed oxidation. More radicals were generated in the laccase-catalysed oxidation on bleached TMP than on unbleached TMP. Peroxide bleaching was also found to cause changes in lignin chemistry so that “long-living” radicals could only be detected in the fines fraction. This might indicate that the possible levels of modification of unbleached and bleached fines and fibres are different.

8. Bonding of 3-hydroxytyramine hydrochloride to TMP was demonstrated, which suggests that compounds containing functional groups can be bonded to wood fibres via laccase-catalysed oxidation of surface lignin.

Although the laccase-aided fibre functionalization method is limited to lignin-rich pulps, its potential is remarkable. It has been shown that the method can be used to create completely new properties in lignin-containing fibres.

Traditionally, high value products of the pulp and paper industry have been produced from fully bleached kraft pulps. It is unlikely that the upgraded lignin-rich pulps would compete with the traditional high-value pulps in existing applications. Due to the current price of enzymes, it is also unlikely that the method would be used to enhance the properties of the fibres in the low-value products currently utilising lignin-rich fibres. Rather, it is more likely that the potential of the modified pulps is in completely new high-value products.

Although the laccase-aided fibre functionalization method could in principle be introduced quite easily to existing processes, the method has not been utilised in industrial scale. The modification method could be used *e.g.* to create new types of plastic-free food packaging materials. However, the possible compounds fulfilling the requirements set for materials in contact with food are rather expensive. Another potential application area is fibre-polymer composites, the modification of which could be used to increase their compatibility. The current price of laccases also affects the price of the modified fibres, making them too expensive compared to the currently used materials. Another reason for the lack

of a breakthrough is that the potential of the method has not been demonstrated in such a way that the utilisation of wood fibres in new applications would become an attractive alternative for the industry. The lack of an external driver such as legislative or customer behaviour also limits interest in the use of new alternative materials in different applications.

For effective exploitation of the laccase-aided functionalization method, both understanding of the activation and bonding mechanisms and optimisation of the targeted fibre modification are essential. It appears that a variety of factors such as pulp and enzyme type, enzyme dosage and treatment conditions affect the activation of pulp fibres. Further research on the effect of structure of the compound to be added on the degree of modification needs to be carried out. It is also most likely that the treatment conditions must be adjusted for each type of pulp and modification compound.

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Errata to articles

Paper II, Activity of laccase on unbleached and bleached TMP

Section 1
Reads: Acidomycetes, should read: ascomycetous

Section 4.3
Reads: According to the EPR measurements, no radicals were formed on pulp

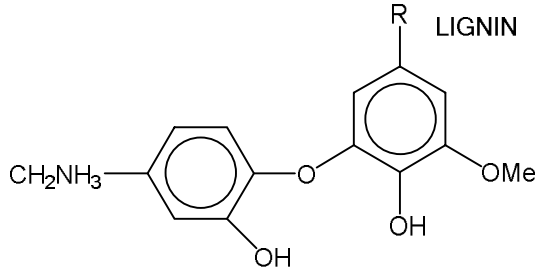
Should read: According to the EPR spectroscopy measurements, no radicals were formed on the bleached pulp

Paper III, Reactivity of Trametes laccases with fatty and resin acids

Legends of Figures 3 and 4 in paper III have been transposed.

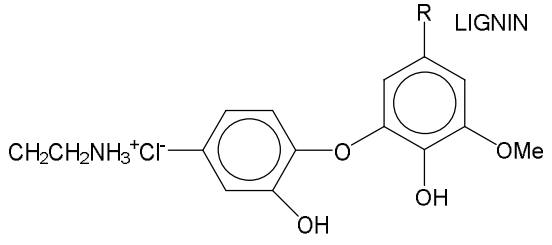
Paper V, Laccase-catalysed functionalisation of TMP with tyramine

Figure 6 looks like this:



TYRAMINE

Figure 6 should look like this:



TYRAMINE

PUBLICATION I

Lignocellulose processing with oxidative enzymes

Applications of Enzymes to Lignocellulosics

In: Mansfield, S. D. & Saddler, J. N. (Eds.)
ACS Symp. Ser. 855.
American Chemical Society.
Washington, DC (2003), pp. 46–65.

Chapter 3

Lignocellulose Processing with Oxidative Enzymes

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Since the successful introduction of commercial hydrolytic enzymes to lignocellulose processing, the next generation of oxidative enzymes are now entering the markets. Significant progress in molecular biology have enabled us to better understand the electron transfer mechanisms in the lignocellulosic substrates and improve the production of these enzymes at a commercial scale. The most intensively studied application is enzyme catalysed delignification, for which several concepts have been introduced. Recently, other applications, such as oxidative fibre modification or activation of lignin to replace traditional adhesives have been actively studied. However, in spite of extensive research, the underlying mechanisms are still only partially understood. This paper reviews recent advances in the application of oxidative enzymes for lignocellulose processing.

Oxidative enzymes have potential in several applications in various industrial areas, such as the cosmetic, food, textile, chemical and pulp and paper sectors. In lignocellulose processing oxidative enzymes can be used for modification of lignin and extractives. The enzymology of lignin modification has been the focus of scientists for more than 30 years. Due to the mostly

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promising results that have been obtained in bleaching and pulping, laccases and manganese-dependent peroxidases have been the most extensively studied groups of enzymes in this area. The first laccases have been on the market for some years, and laccase-based mediated bleaching systems have been developed and tested at a pilot-scale. The ability of laccases to oxidize lignin is currently being evaluated in the activation of fibre surfaces for bonding, grafting or glueing applications.

Enzymatic modification of fibre bound substrates represents a continuous challenge for scientists. Besides the diverse chemical compositions of the major components, the fibre cell wall matrix embeds these fractions to produce the rigidity and resistance typical of plants. The structures of carbohydrates; cellulose and hemicellulose, are chemically well understood, whereas lignin forms an undefined structure. In spite of extensive research, the mechanisms of enzymatic modification, especially degradation of lignin are not yet fully understood. This article reviews the latest achievements in oxidative modification of fibre components, while primarily focusing on lignin.

Oxidative Enzymes

Research related to lignin biodegradation has resulted in the identification of the essential enzymes in lignin degradation including oxidases, peroxidases, dehydrogenases and hydrogen peroxide generating enzymes. The only organisms capable of efficiently mineralising lignin are basidiomycetous white-rot fungi and related litter-decomposing fungi (1). Physiological conditions for lignin degradation, as well as secretion patterns of the lignolytic enzymes vary substantially among different fungal species (2). The most extensively studied lignolytic enzymes for various biotechnical applications include laccases and manganese-dependent peroxidases of white-rot fungi. Promising results with these enzymes have been obtained in pulp and textile dye bleaching as well as fibre modification (3).

Peroxidases

Fungal peroxidases participating in lignin biodegradation include lignin peroxidase (LiP, EC 1.11.1.14) (4), manganese-dependent peroxidase (MnP, EC 1.11.1.13) (5) and peroxidases having properties of both LiP and MnP and being either manganese-independent peroxidase (MIP) (6), LiP-like (7) or versatile peroxidase (8). There are also nonligninolytic fungal peroxidases, which do not have the characteristic substrate oxidation sites of either LiP or MnP (9). Purified ligninolytic enzymes have been shown to cause limited delignification

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provided that additives are supplemented; veratryl alcohol and H_2O_2 for LiP (10) and manganese, H_2O_2, organic acids and surfactants for MnP (11, 12).

Since the discovery of LiP and MnP in *Phanerochaete chrysosporium* (4, 5) these enzymes have been found to be secreted by many white-rot fungi, usually in multiple isoenzymes. LiP and MnP are heme-containing glycoproteins using hydrogen peroxide as an electron acceptor. LiP oxidizes nonphenolic subunits of lignin by a one-electron transfer mechanism resulting in formation of cation radicals, which are further decomposed chemically (13). It has been shown by various analysis with synthetic lignin and lignin model compounds that LiP is responsible for C_α-C_β bond cleavage, ring opening as well as many other reactions.

MnP oxidizes Mn(II) to Mn(III), which organic acids e.g. oxalic, malic, lactic, or malonic acid stabilize by chelating. Chelated Mn(III) oxidizes phenolic subunits in lignin and forms phenoxy radicals, which may further cleave bonds between aromatic rings and the C_α carbon atoms (1). Chelated Mn(III) is in general a powerful oxidant, which may also oxidize some nonphenolic aromatics such as dyes. Mn(III) also creates radicals from the co-oxidants present in the medium. Thioyl and peroxyl radicals, formed from thiols and unsaturated fatty acids respectively, are highly reactive and mediate oxidation towards nonphenolic lignin structures (14, 15). However, Mn(III) is not capable of oxidizing recalcitrant nonphenolic units of lignin. The capacity of MnP to oxidize lignin is limited because the phenolic structures constitute only 10-15 % of all units in lignin (16). However, the research on various white-rot fungi has shown that MnP is more common than LiP (2, 17) and that it has an essential role in depolymerization of lignin (18).

In the past few years several crystal structures of peroxidases from different sources have been reported. The three-dimensional structures of both LiP (19, 20) and MnP have been solved (21). Interestingly, the overall folding and the secondary structure of peroxidases are highly conserved despite their low sequence homology. The structure information, as well as the vast sequence data have increased our knowledge of the action of peroxidases on aromatic substrates. However, the way these enzymes act towards polymeric lignin is still not fully understood. Significant progress in production of recombinant peroxidases (22) has recently been obtained. Further enhancement of production might also be possible by means of the genomic data of *Phanerochaete chrysosporium,* which has recently been made available (23).

Laccases

Laccases (EC 1.10.3.2) are probably the most commonly occurring oxidoreductases in white-rot fungi (24). Most of the isolated and characterised

laccases are from fungal origin. Well known laccase producers include *Trametes, Pleurotus, Coprinus, Myceliophthora, Phlebia, Pycnoporus, Rhizoctonia,* and *Schizophyllum* (25). Laccase or laccase-like activity has also been demonstrated by plants, some insects and a few bacteria (26). It is well recognised that laccases are involved in both polymerisation and de-polymerisation processes of lignin. The plant origin laccases are reported to have an important role in wound response and lignin biosynthesis (27) whereas in fungi they are involved in lignin degradation, as well as in several other functions including pigmentation, fruiting body formation, sporulation, and patogenesis (26, 28). A biological role of laccases in the oxidation of Mn^{2+} has also recently been proposed (29).

Laccases belong to the blue multi-copper oxidase family. The catalytic site of laccases contains four copper atoms per laccase molecule. The copper atoms can be classified into three types: one type 1 Cu, one type 2 Cu, and two type 3 Cu. The mononuclear site (type 1 Cu) functions as the primary electron acceptor, extracting electrons from the substrate. The copper is coordinated by two histidine nitrogens and a cysteine sulfur with a highly covalent Cu-S bond giving rise to the pronounced blue color of laccases. Type 2 and two type 3 Cu form the trinuclear center, where reduction of molecular oxygen takes place. It is not fully understood how the electrons are transferred from the mononuclear site to the trinuclear site. It has been proposed that the electrons are extracted through a conserved Cys-His pathway from the mononuclear site to the trinuclear site (30).

Laccases catalyze the four-electron reduction of dioxygen to water with four concomitant one-electron oxidation of the reducing substrate. The mononuclear site functions as a primary electron acceptor whereas the trinuclear center, the binding site of the dioxygen, accepts electrons from the mononuclear site. The exact nature of the reaction mechanism is still controversial and debated. The most widely accepted mechanism is that proposed by Messerschmidt *et al.* (30). Although laccases have been extensively studied, thus far only two crystal structures are available, namely the type 2 copper depleted laccase from *Coprinus cinereus* (31) and a recently published laccase from *Trametes versicolor* in four copper form (32). In addition, the crystallisation of laccases from *Trametes versicolor* and *Pycnoporus cinnabarinus* have been reported (33).

Laccases display a surprisingly broad specificity towards the reducing substrate. They catalyse oxidation of a wide variety of aromatics, especially phenolic, and inorganic substrates. Simple diphenols like hydroquinone and catechol, polyphenols, diamines, and aromatic amines are good substrates for most laccases. Since the description of the mediator concept in the early nineties (34-36), the range of potential mediator substrates has continued to increase. Mediators are small molecular weight compounds, which can be oxidised by laccase. The oxidised mediator then oxidises the actual substrate. A typical

example of a mediator is hydroxybenzotriazole (HBT), which has been studied intensively for delignification together with laccase (37). Laccase alone can only oxidise phenolic subunits in lignin. However, when combined with a mediator, non-phenolic groups can also be oxidised. Promising results with mediated oxidation (Figure 1) have been obtained in pulp delignification. In principle, different types of monomers, as well as polymers, can be oxidised by a suitable enzyme mediator combination. Because of the broad substrate specificity range of laccases, they possess great biotechnological potential. The most intensively studied applications for these enzymes include pulp delignification, textile dye bleaching, effluent detoxification as well as biopolymer modification (3).

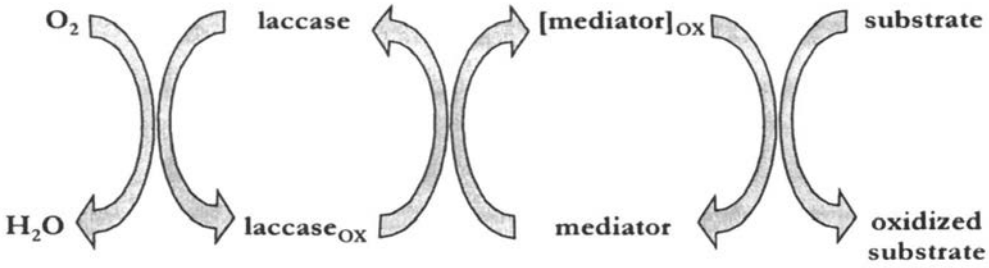

Figure 1. A schematic presentation of mediated oxidation by laccase.

Surface Chemistry Of Pulp Fibres

Wood fibres are mainly composed of cellulose, hemicellulose, *i.e.* xylan and glucomannan, lignin and extractives (38). As oxidative enzymes react with lignin both the chemistry and location of the lignin in the pulp fibres is of most importance for the enzyme activity. In mechanical pulping no major chemical changes in the fibre components occur, whereas during alkaline chemical pulping, *i.e.* kraft cooking, about 90 % of lignin is removed from the fibres. The larger average pore size of chemical fibres renders them more susceptible to the action of macromolecular enzymes. The enzymatic action even in chemical pulps is, however, limited to accessible surfaces, *i.e.* to fines and to the outermost surface and accessible pores of long fibres (39).

In addition to fibres process waters in paper manufacture also contain various wood derived compounds, *i.e.* dissolved and colloidal substances (DCS). These are also potential substrates for enzymatic oxidation. These wood components, such as extractives, carbohydrates and lignin are dissolved and

dispersed into the process waters (40, 41) during mechanical pulp production and bleaching. In chemical pulping and bleaching wood extractives are extensively modified and degraded (38).

The surface composition of different types of fibres has been analysed by ESCA (Electron Spectroscopy for Chemical Analysis). Unbleached softwood kraft pulps are reported to have a surface coverage of lignin of about 10-30% depending on the pulp kappa number (42). Surface coverage of lignin in unbleached hardwood kraft pulp is reported to be about 20 % (43). Partial enzymatic removal of xylan results in increased surface coverage of lignin in conventional unbleached pine kraft pulps (43). Concentrations of both lignin and xylan in primary and secondary fines of unbleached kraft pulps have been visualised by mechanical peeling techniques (44). In mechanical pulps about 33 % of the surfaces are covered by lignin and this surface coverage of lignin is not changed in the bleaching due to non-delignifying bleaching (45, 46). As oxidative enzymes are particularly active on the fibre surfaces, both mechanical and chemical pulps contain potential substrates for oxidation.

Bleaching Of Chemical Pulp

Today, bleaching of kraft pulps is mainly carried out with chlorine dioxide, hydrogen peroxide, oxygen and ozone in variable sequences. During the search for environmentally sound alternatives for chlorine based chemicals, enzymatic methods were also developed and commercialised. Enzyme-aided bleaching is used today in the pulp and paper industry to improve the bleachability of kraft pulps through the action of xylanases or other enzymes affecting the extractability of lignin. The effect of xylanase pretreatment on bleachability is, however, limited. The most promising direct enzymatic bleaching system is based on the use of oxidative enzyme, laccase, together with a mediator directly degrading lignin. In addition to laccase, the potential of MnP has been studied in chemical pulp bleaching. The effect of oxidative enzymes on the potential for lignin preserving bleaching of mechanical pulps has also been studied (47).

Laccase-Mediator Concept In Chemical Pulp Bleaching

In the laccase-mediator concept, the mediator oxidised by laccase enzyme acts directly on lignin and results in efficient delignification (Figure 1). In the initial study, the common substrate of laccases, ABTS was used as the mediator (35). The search for a more suitable mediator resulted in discovery of 1-hydroxybenzotrizole (HBT) (36). This delignification procedure is commonly referred to as the LMS (laccase-mediator-system) or Lignozyme process and it

has been demonstrated in pilot scale in totally chlorine free (TCF) bleaching sequence (37). A number of other mediators with great structural variety have been studied. The most effective mediators in delignification usually contain N-OH functional groups (48, 49), such as the most promising current mediators, violuric acid (VIO) and N-hydroxy-N-phenylacetamide (NHA). The latter mediator results in extremely fast delignification with no significant impact on cellulose structure (50). The performance of NHA was further improved by implementing a slow-release mediator system, based on a precursor of NHA (DiAc, N-acetoxy-N-phenylacetamide) (51). The delignification degree of laccase-HBT after an alkaline extraction has also been reported to be high, up to 40 % in low-kappa number softwood and hardwood kraft pulps (52). In high kappa number softwood kraft pulps, violuric acid has been reported to be as twice as efficient a mediator as both HBT and NHA in the LMS bleaching (53). In addition to nitrogen based mediators, inorganic mediators such as transition metal complexes or polyoxometalates containing *e.g.* molybdenium ion have recently been successfully tested for laccase-mediator bleaching (54, 55). Until now, only one fungal metabolite, 3-hydroxyanthranilate, has been introduced as a natural mediator (56). Other potential natural mediators are siderophores, which are strong iron chelating agents, have also been studied for lignin degradation (57). Several studies on the mechanisms of laccase-mediated delignification of pulps have been published (*e.g.* 58-66).

The efficiency of laccase-DiAc stage was recently demonstrated in pilot scale in an ECF bleaching sequence, $LaEpD_0EoD_1$ (67). The pilot scale bleaching trial with laccase-DiAc stage required 24 % less chlorine dioxide than the reference mill sequence ($D_0EopD_1EpD_2$) without strength loss, suggesting that laccase-DiAc stage could be an alternative for oxygen delignification stage in ECF bleaching. Optimisation of alkaline extraction stages and further development of enzyme suitable for alkaline conditions required in DiAc conversion to NHA and high shear forces were, however, found to be prerequisites for economical viability of the system. The LMS system has been shown to be able to replace either the oxygen delignification or ozone stage, (68-70). The development of the laccase-mediator concept is presented in Table I.

In addition to delignification, the effects of LMS on the physical properties of pulps have been determined. In high kappa number chemical pulps, both laccase-HBT treatment and HBT treatment alone enhanced the handsheet densification during PFI refining (71). The use of laccase with NHA and violuric acid resulted in similar bonding strength as compared to oxygen delignification, but without reduction in viscosity (50).

The combination of xylanase and laccase-mediator bleaching systems either sequentially or simultaneously has been reported to result in additive enhancement of bleachability (72-74). The application of LMS system employing HBT as mediator with xylanase treatment in one single stage was

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found to be ineffective, apparently due to the inactivation of xylanase by the HBT (72). This inactivating effect of HBT has also been observed towards laccases (49) as HBT radicals undergo chemical reactions with the aromatic amino acid side chains of many laccases. Studies on new mediators have revealed that NHA caused less damage to enzymes (72, 75). In practice, it would be beneficial to combine the indirect xylanase treatment with the delignifying laccase-mediator treatment as the target substrates of these treatments are different and thus the maximal effect of both treatments could be exploited.

Table I. Steps in the development of laccase-mediator systems

When	*Description*
1986	Enzyme-mediator concept (ref. by 37)
1990, 1991	Redox cascade, often in the presence of chelating agents (ref. by 37)
1992	Laccase and ABTS as mediator (35)
1992	Laccase and HBT as mediator (ref. by 37)
1993, 1994	Laccase and mediators containing N-OH, N-oxide, oxime or hydroxamic acid-compounds (ref. by 37)
1994	Pilot plant trial with HBT in TCF sequence, degree of delignification > 50% (ref. by 37)
1997	Laccase and NHA: Lower costs, reduced laccase inhibition, biodegradable and higher selectivity (48)
2000	Slow-release mediator (NHA- DiAc) Better cost efficiency, less mediator carryover (51)
2000	Metal-complex mediator (54) Completely reversible mediation, only catalytic amounts of mediator needed
2001	Pilot plant trial with slow- release mediator (NHA- DiAc) in ECF sequence, high brightness (88 % ISO) without strength loss (67)

MnP In Pulp Bleaching

The use of manganese peroxidase (MnP) has also been studied for bleaching of chemical pulps (11, 12, 76-78). In small scale tests, demethoxylation, delignification and an increase of about 10 ISO units in brightness after alkaline stage has been reported (12, 76, 77). Unlike the laccase-mediator system, the MnP based system uses a natural mediator, Mn(II). MnP uses hydrogen peroxide as the electron acceptor and oxidises chelated Mn(II) to Mn(III). Stable chelated Mn(III) can then diffuse to the fibre matrix, which then leads to the formation of

phenoxy radicals on the phenolic structures within the lignin. However, the nonphenolic lignin structures are not attacked. It has, however, been suggested that other reactions, such as peroxidation of lipids (including certain extractives) initiated by MnP could also be involved in degradation of both phenolic and non-phenolic units in lignin (15). The applicability of MnP in pulp delignification on industrial scale is limited mainly due to the strictly controlled reaction conditions demanded by MnP, as well as to the obviously high price and limited availability of the enzyme. Calculations on the costs of the components needed (peroxide, additives, enzyme) have not been published.

Oxidative Enzymes In Fibre Modification

The properties of fibre products are determined both by the physical and chemical properties of the fibres and the chemical additives used in processing. Upgrading of fibre properties is in many cases of great interest. The availability of oxidative enzymes such as laccase, capable of radicalising papermaking fibres, has raised the idea of an alternative, environmentally sound approach to wood fibre upgrading by targeted modification of fibres by enzymatic or chemo-enzymatic methods (Table II).

The primary reaction of laccase and other phenoloxidases is the formation of phenolic or cationic radicals. The oxidative enzymes initiate radical formation in solubilised lignans and colloidal lignin, as well as in fibres that will react further without additional enzymatic action (79-82). Due to the high reactivity of these radicals (either with each other or with a secondary substrate), reactions such as polymerisation, depolymerisation, co-polymerisation and grafting can occur. The size of oxidases limits the range of the enzyme on the fibre surface (83). Hence, such enzymes can be used to carry out surface specific modification of fibres. When more extensive modification is needed, small molecular weight mediators can be used together with laccase.

The ability of laccases to oxidise fibre bound lignin is somewhat unclear. The most probable primary substrates are the colloidal and solubilised lignin fragments present in pulp suspensions or attached to the fibres. In addition, extractives have been proposed to act as mediators in the oxidation reaction (79-81) Indeed, it has been suggested that the presence of water-soluble extractives would be essential for radical formation in fibre bound lignin (81). The activity of laccases on lipophilic extractives and hydrophobic lignans has been reported in several papers (84-87).

It has been proposed that the state of lignin in wood fibres determines the dominating pathway of oxidation (81). Depending on the chemical and physical structure of the lignin polymer, different types of modifications of lignin can take place. Laccase treatments have been found to generate two oxidation species in

lignin, *i.e.*, via oxygen chemically transformed lignin products and initial oxidation radicals that have gained stabilisation (81, 88). The radicals formed are phenolic and they can be observed directly (80).

Functionalization Of Fbres By Oxidative Enzymes

The ability of oxidative enzymes to create long-living radicals to fibre surfaces can also be exploited as such or after further functionalisation of fibres with specific chemical components. The chemical changes caused by the enzymatic modification may include macro-scale modifications caused by radical-initiated polymerisation or depolymerisation reactions (Table II). Thus, it can be envisioned that the presence of surface lignin in mechanical and lignin-rich chemical pulp fibres offers possibilities for producing tailor-made or completely novel paper and board products.

Laccase catalysed radical coupling of compounds to lignin has been mostly carried out with defined substrates (89-96). Mai and Hütterman (91) suggest that organic peroxides are needed to start the copolymerisation of acrylamide with lignin oxidised by laccase. Success in grafting low molecular weight compounds to surface lignin activated by laccase has also been reported. According to Chandra and Ragauskas (97), laccase facilitates the coupling of phenolic acids to fibre surfaces. Lund *et al.* (95) reported attempts to graft phenolic monomers onto softwood kraft pulp. They (95) questioned, however, whether laccase is actually bound to the lignin in fibres. As consequence of laccase activation, increases in sheets strength have been reported (98). Improvement in wet strength of kraft pulp fibres with laccase in the presence of lignin rich extractives has also been reported (99).

Adhesion Of Fibres By Oxidative Enzymes

In conventional production of lignocellulose based composites, such as fibre or particle boards, synthetic adhesives are used in combination with hot pressing. Alternative enzymatic methods may allow the production of particle and fibreboards with less or even totally without hazardous adhesives, such as urea-formaldehyde, phenol-formaldehyde or isocyanide. Laccase catalysed bonding can be achieved by activation of additional lignin by the oxidative enzyme (two component system) or by the enzymatic activation of the lignin present in fibres (one component system). Besides laccases, peroxidases have been studied for activation of lignin (100, 101). In their first experiments, Haars and Hüttermann (102) used the two component system where laccase treated lignosulphonate was used as an additive to bond wood fibres. In later experiments Haars and

Table II. Fibre modification with oxidative enzymes

Aim	*Description*
Modification of pulp properties	Generation of bonding strength on woody fibres by enzymatic phenol polymerisation with dehydrogenases (107)
	Modification of chemical and mechanical pulp fibres by laccase (108)
	Detection of laccase induced modification by luminescence spectroscopy (109)
	Studies on oxidative species generated in the lignin of wood fibres by a laccase catalysed treatment (81)
	Effect of cellulase, laccase and proteinase on papermaking properties of mechanical pulp fibres (110)
	Peroxidase treatment of pulp led to enhanced beating (111)
	Improved wet strength of paper material by the combined action of laccase and mediator (99, 112)
	Laccase treatment of mechanical pulp improves refining efficiency (113)
Activation of wood fibres	Enzymatic activation of middle lamella lignin of wood fibres as means for the production of binder-free fibre boards (114)
	Spectroscopic properties of oxidation species generated in lignin of wood fibres (81)
	Activation of lignin leads to copolymerisation with carbohydrates (115)
	Activity of laccase on TMP (116)
Bonding of wood fibres	The use of peroxidases and hydrogen peroxide in bonding of particle boards was suggested in 1972 (106)
	The use of laccase as radical donor in bonding of fibre boards was suggested in 1996 (106)
	Enzymatic activation of middle lamella lignin of wood fibres as means for the production of binder-free fibre boards (114)
	Enhanced autoadhession of fibres by laccase (80, 105)
	Lignocellulose-derived adhesive for bonding of wood boards (117)
	Influence of extractives on enzymatic catalysed bonding (79)
	Properties of fibreboards obtained by peroxidase catalysed reaction (101)
	Phenol oxidising enzymes in production of fiberboards (118)
	Enzymatic activation of lignin leads to copolymerisation with carbohydrates (115)
	Bonding of MDF boards and lineboards (119)
	Method to manufacture fibreboards (120)
Bonding of model compounds to lignin	Copolymerisation of lignin with low-molecular weight compounds (96)
	Copolymerisation of phenolic compounds to lignin (95)

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Table II continues. Fibre modification with oxidative enzymes

Aim	*Description*
	Studies of the reactions of activated lignin and nucleophiles (90)
	Peroxides in enzymatic copolymerisation of lignin with acrylates (91)
	Effect of ions in the enzymatically induced synthesis of lignin graft copolymers (121)
	Copolymers from lignin and acryl compounds (92, 93)
	Oxidative coupling of water-soluble phenols with lignin (94)
Bonding of model compounds to fibre bound lignin	Precipitation of laccase polymerised vanillic acid, cathecol, mimosa tannin and tannin acid dehydrogenatively to TMP (122)
	Grafting of N-contaning phenolic monomers onto softwood pulp (95)
	Bonding of 4-hydrohyphenol acetic acid to fibres (97)

coworkers (spent sulphite liquor was used with laccase for particle board and wood laminate production (103). Kraft lignin, as well as concentrated process water from thermomechanical pulp (TMP) refining have also been studied for additives in glueing experiments using *Trametes hirsuta* laccase to prepare particle boards and MDF boards (104). Tensile strength measurements from the test fibre boards showed clearly that laccase treatment was comparable to a process where a synthetic reference adhesive, urea formaldehyde resins, was used. Fibreboards with better modulus of rupture and elasticity have generally been reported (105).

In the one component system, enzymatic activation of surface lignin has been exploited to enhance the adhesion between fibres through activation of surface lignin in production of binderless fibreboards (88, 89, 105, 106). The use of both laccases and peroxidases in the activation has been reported (101, 105). The improved bonding is thought to be due to physical changes on the fibre surface caused by the phenoxy radicals (105). Although the mechanism is not completely understood, it presumably involves direct oxidation of fibre surface lignin and the parallel radicalization of solubilized or colloidal lignin (80). These radicals will react further without enzymatic action. During hot pressing, fibre to fibre bondings are formed between radicals and other reactive groups situated on separate fibres (105).

Oxidative Enzymes In The Hydrolysis Of Lignocellulose

The interest in replacing fossil fuels with biofuels derived from lignocellulosic raw materials is increasing due to the worldwide concern about green house gases. The enzymatic hydrolysis of lignocellulosic materials has been studied in detail since the 1950's and significant advances in basic and applied enzymology have been achieved. The molecular structures, catalytic mechanisms and substrate specificities of major cellulases have been elucidated in detail. However, the heterogenous nature of the lignocellulosic matrice makes it difficult to understand the interactions of enzymes and their substrates, containing also lignin and hemicellulose. The accessibility of the substrate plays a key role in hydrolysis and is improved by using different pretreatment techniques (123). The role of residual hemicellulose and lignin as limiting factors in enzymatic hydrolysis has recently been reviewed in detail (124). The exact role of lignin in limiting hydrolysis, however, has been difficult to define. According to Mooney *et al.* (125), one of the most remarkable restrictions is the effect of lignin on fibre swelling and its resulting influence on the accessibility of cellulose. Obviously, the removal of both lignin and hemicellulose would leave the cellulose more accessible to contact with cellulases. Lignin is, however, thought to influence cellulase accessibility to cellulose in more ways than just acting as a barrier to prevent the enzymes from effectively binding to cellulose. Thus, it has been shown that the increase in pore volume observed after lignin removal corresponds to the increased accessibility of the substrate (126, 127). Lignin is also thought to negatively influence the hydrolysis reaction by irreversibly adsorbing the cellulase enzymes, thus preventing their action (128, 129). It has been observed that the extent to which lignin adsorbs cellulases, depends on the nature of the lignin (130). Therefore, lignin may be a rate limiting factor in the hydrolysis of cellulose.

In the present steam pretreatment technology, lignin is not dissolved from the fibrous material and may comprise up to 40% of the raw material. In comparative studies, lignin has been extracted to verify its role in hydrolysis (125). Although it may not be feasible to extract lignin during the pretreatment phase, the role of partial lignin removal during the hydrolysis of cellulose is interesting. The compatibility of enzymatic lignin degradation with cellulose hydrolysis has been studied using the laccase-mediator system on steam-pretreated softwood. Thus, it was observed that the degree of hydrolysis was improved significantly by combining the two enzymatic treatments (131). The inhibitory effects of the LMS system on cellulase activity decreased slightly the effect. Therefore, the slow-release medior was also tested. The mechanism was expected to be based on removal of lignin fragments with sterical hindrance, modification of fibre surfaces improving cellulase desorption and eventually decreasing the inhibitory effect of aromatic compounds on cellulases.

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PUBLICATION II

Activity of laccase on unbleached and bleached thermomechanical pulp

In: Enzyme and Microbial Technology
2003: 32(3–4), pp. 439–445.

Available online at www.sciencedirect.com
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ENZYME and MICROBIAL TECHNOLOGY
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Activity of laccase on unbleached and bleached thermomechanical pulp

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Accepted 23 November 2002

Abstract

The introduction of value-added properties to pulp fibres is an attractive proposition. One interesting option is targeted modification of fibre surfaces by enzymatic activation. In this work, the activity of laccase on pulp and pulp fractions from thermomechanical pulp (TMP) and peroxide bleached TMP was studied on the basis of consumption of the co-substrate oxygen in the reaction and by studying the formation of radicals in the pulp material as analysed by electron paramagnetic resonance spectroscopy (EPR). Laccases obtained from *Trametes hirsuta* and *Myceliophthora thermophila* were used in the study. Laccases were found to be active on pulp material of unbleached TMP, whereas only fines from bleached TMP reacted in laccase treatment. Dissolved and colloidal substances (DCS) were assumed to have a mediating role in the laccase-catalysed oxidation of the fibre-bound material.

Keywords: Activation of fibres; Activity of laccase on pulp; Laccase

1. Introduction

The properties of fibre products, e.g. paper and board are determined by the physical and chemical characteristics of the pulp material and also by the chemical additives used in processing. The physical properties of a pulp are affected by the properties of the fibres and by the pulping conditions [1], whereas the chemistry is determined by the chemical components of the wood raw material, i.e. carbohydrates, lignin, extractives and metals [2]. Especially the presence of lignin gives mechanical pulps their characteristic properties.

The presence of lignin is generally considered as a drawback as it causes brightness reversion typical for mechanical pulps [3]. Recently, attempts to utilise lignin for different enzymatic fibre modification applications have been made. For example, promising results in mechanical fibre bonding have been achieved by using laccase for activation of surface lignin [4]. Radical-based activation of surface lignin has also been exploited for bonding of low molecular weight compounds to surface lignin by laccase [5,6]. In addition to enzymatic activation of surface lignin the surface lignin or cellulose can also be activated by using different types of chemicals. However, the chemical activation of fibres by oxidants such as ozone results in undesired delignification. Therefore, enzymatic activation of fibre surfaces is of great interest due to its specificity. As a result of activation, radicals are generated in the fibre surfaces. The radicals formed can possibly be exploited in functionalisation of fibres by various means.

Laccase is a multi-copper oxidase catalysing oxidation of various aromatic compounds, especially phenols by concomitant reduction of oxygen to water. The molecular size of laccase, i.e. 60–100 kDa corresponding to 70 Å × 50 Å × 45 Å, limits the extent of oxidation in pulp applications to the surface of pulp material [7–10]. In laccase-catalysed oxidation of wood fibres, phenoxy radicals are formed in the lignin matrix [11,12]. The oxidation is thought to be due to direct oxidation of surface lignin or alternatively mediated by dissolved and colloidal material [12,13]. It has even been suggested that the presence of water-soluble extractives is necessary for radical formation in lignin [14]. Activity of laccase on lipophilic extractives and hydrophilic lignans has also been reported [15–18].

In this work, the activity of laccase on TMP and bleached TMP and on pulp fractions of these pulps was studied by measuring the consumption of the co-substrate oxygen and by EPR analysis. Laccases obtained from *Trametes hirsuta* and *Myceliophthora thermophila* were used in the study. *T. hirsuta* laccase used at pH 4.5 is a Basidomycetes laccase with high oxidation potential, whereas *M. thermophila* laccase used at pH 7 is an Acidomycetes laccase with low

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0141-0229/02/$ – see front matter © 2002 Elsevier Science Inc. All rights reserved.
doi:10.1016/S0141-0229(02)00319-8

oxidation potential [19,20]. The used laccases also differed in molecular size: *T. hirsuta* laccase is smaller, about 60–70 kDa whereas the size of *M. thermophila* laccase is about 85 kDa [21].

2. Materials

2.1. Pulps

Two TMPs (pulps 1 and 2) and two peroxide bleached TMPs (pulps 3 and 4) produced from Norway spruce (*Picea abies*) were obtained from Finnish paper mills. The pulps were never-dried pulps, which were stored in a freezer before the treatments. The chemical compositions of the pulps were very similar. Pulps 1 and 3 were used in measurements of oxygen consumption and pulps 2 and 4 in radical measurements. Fibre (>200 mesh) and fines (<200 mesh) fractions of pulps 2 and 4 were also used.

2.2. DCS—water and washed pulp

Model waters containing dissolved and colloidal substances (DCS) were prepared from TMP (pulp 1) and bleached TMP (pulp 3) at pH 4.5 and 7 according to Örså and Holmbom [22]. The model waters were made from 1% pulps. The waters will be referred to in the text as DCS—waters. The fibres used in preparation of DCS—waters at pH 7 were washed with amount of water equal to 20× the dry weight of the pulp used. These fibres will be referred to in the text as washed fibres.

2.3. Enzymes

Two different laccase preparations were used in pulp material treatments. *T. hirsuta* laccase was produced and partially purified as described by Poppius-Levlin et al. [23]. According to Kojima et al. [24] this strain has an allelic gene pair encoding laccase. No other genes have been reported for *T. hirsuta*. *M. thermophila* laccase was kindly supplied by Novozymes and partially purified at VTT. The *M. thermophila* laccase was heterologously produced in *Aspergillus* and the protein showed a single band on SDS-PAGE with Coomassie staining.

Laccase activity was determined using ABTS (2,2-Azinobis(3-ethylbenzothiazoline)-6-sulfonic acid) as substrate [25]. The enzyme activities of the preparations are presented in Table 1 [25–29]. The cellulase activity of the preparations was not assayed.

3. Methods

3.1. Analysis of the chemical composition of pulp material

The chemical compositions of the pulps and pulp fractions used in this study were analysed. The carbohydrate compositions of the pulps were analysed by HPLC after acid hydrolysis [30,31], carbohydrates in DCS—waters by HPLC after secondary enzymatic hydrolysis of oligomers to monomers [32], metals after ashing with AAS, Klason lignin after acid hydrolysis (KCL method 115b:82), extractives after MTBE extraction by GC [22] and lignin in aqueous phase by UV-absorption at 280 nm after MTBE extraction [22].

3.2. Measurement of laccase activity on the basis of oxygen consumption

The activities of *T. hirsuta* (pH 4.5) and *M. thermophila* (pH 7.0) laccases on DCS—waters, pulps, fibre fractions and fines fractions were analysed by measuring the consumption of dissolved oxygen in samples during laccase treatment at 40 °C with agitation of 500 rpm. For the laccase treatment the pulps and fines and fibre fractions were diluted to 0.5% consistency with 0.1 M citric acid buffer (pH 4.5) or Na-phosphate buffer (pH 7). The DCS—waters were used as such. The laccase dosage in pulp, fibre and fines treatments was 1000 nkat/g, whereas in treatments of DCS—waters the dosage was 10 000 nkat/l. The measurement was made in a closed vessel with SensorLink PCM800 meter using a Clark oxygen electrode. The reference treatments were performed under similar conditions but without addition of enzyme.

Table 1
Enzyme activities in the laccase preparations

Laccase origin	Protein (mg/ml)	Activity (nkat/ml)			
		Laccase	Manganese peroxidase	Lignin peroxidase	Xylanase
Trametes[a]	5.7	7600	4.3	0	4.1
Trametes[b]	12.5	4400	16.4	86.6	nd
Myceliophthora	13.5	1020[c], 1150[d]	0[c]	0[c]	nd

nd: not determined.
[a] Used in oxygen measurements.
[b] Used in radical measurements.
[c] At pH 4.5.
[d] At pH 7.

Table 2
Parameters used in the EPR-measurements

Parameter	Value
Microwave power	2 mW
Frequency	9.420 ± 9 MHz
Centre field	3350 G
Modulation amplitude	5 G
Modulation frequency	100 kHz
Receiver gain	2.5×10^3
Sample size	325 mg/std volume

3.3. Measurement of laccase activity on the basis of radical formation

Prior to radical measurement, pulps or pulp fractions were treated with *T. hirsuta* laccase. The pulp was homogenised by cold disintegration before the treatments. Treatment conditions were: 1% consistency, treatment time 1 h, pH 4.5, treatment temperature 40 °C or RT with extra oxygen supply. The laccase dosage was 1000 nkat/g. Immediately after the treatments, the fibre material was filtered, washed with distilled water (20× o.d.) and handsheets were prepared according to SCAN M 5:75 on wire cloth. The handsheets were dried at room temperature. The reference treatments were carried out correspondingly but without laccase addition. The long living radicals were detected from dried samples by electron paramagnetic resonance spectroscopy (EPR) [33] within two days from laccase treatment. The handsheets were kept in the dark between the treatment and the EPR measurement. The EPR parameters used are presented in Table 2.

4. Results and discussion

4.1. Chemical composition of pulp material

The chemical compositions of the pulps and pulp fractions used in this study were typical for Finnish TMP material produced from Norway spruce (Table 3). The two TMPs (TMPs 1 and 2) had similar chemical compositions although the pulps were taken from different mills. The lignin contents of TMPs 1 and 2 were 27 and 26%, respectively. The chemical compositions of the two bleached pulps (bleached TMP 3 and bleached TMP 4) were also similar and the lignin contents were 28 and 27%. The TMP 2 and bleached TMP 4 were also fractionated and the chemical compositions of the fractions were analysed. The fines contained more lignin and extractives than fibres (Table 3).

4.2. Activity of laccase on TMP

The activities of *T. hirsuta* and *M. thermophila* laccases on TMP material were analysed by monitoring the oxygen consumption during laccase treatment. The radicals formed in fibre material in *T. hirsuta* laccase treatment were detected by EPR-measurement. On the basis of oxygen consumption measurements, *T. hirsuta* laccase was active on TMP (pulp 1) (Fig. 1). *T. hirsuta* laccase was also found to be very active on DCS—water (made from pulp 1) (Fig. 1). A similar result on the reactivity of DCS material has been reported previously [12,13,15]. The role of DCS material in the oxidation of fibre-bound material was studied further by washing the pulp (pulp 1) at pH 7 to remove the excess DCS material from the pulp. It is known that dilution of the pulp in warm water at pH 7 removes much of the readily liberated material from the surface of the fibres [34]. The activity of *T. hirsuta* laccase on the washed pulp was clearly lower than on the original TMP (Fig. 1), indicating that the washing step had removed most of the material readily oxidised by laccase. However, some oxidation was observed by oxygen measurement, but whether this was oxidation of the material further dissolved and dispersed from the washed fibres or that in the fibre-bound material could not be concluded. Even after effective washing of the pulp, more low-molecular weight substrates are dissolved from the pulp when the pulp is mixed with pure water. However, the washing at pH 7 had probably removed most of the lignans, as up to 90% at pH 8 and

Table 3
Chemical compositions of the pulps used in this work

	Carbohydrates (mg/100 mg)	Lignin (mg/100 mg)	Extractives (mg/g)	Metals (mg/kg)
TMP (pulp 1)	71	27	7	2300
DCS—water pH 4.5	≤96[a]	63[a]	59	nd
DCS—water pH 7	≤99[a]	91[a]	69	nd
TMP (pulp 2)	72	26	7	2020
Fines	63	35	9	1860
Fibres	73	26	2	1290
Bleached TMP (pulp 3)	71	28[a]	4	5310
DCS—water pH 4.5	≤40[a]	48[a]	25	nd
DCS—water pH 7	≤48[a]	44[a]	29	nd
Bleached TMP (pulp 4)	73	27	5	6550
Fines	63	33	6	4150
Fibres	75	25	3	290

nd: not determined.
[a] In mg/l.

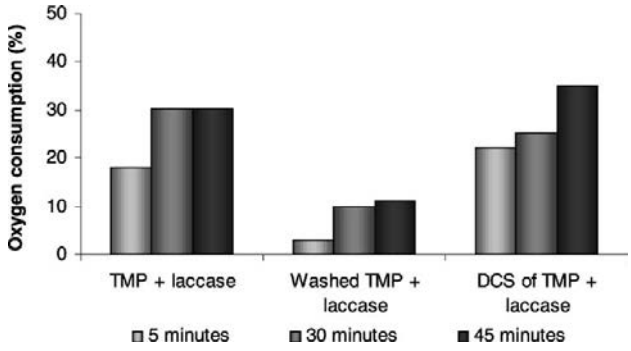

Fig. 1. The activity of *T. hirsuta* laccase (1000 nkat/g) on pulp material of unbleached TMP (pulp 1) at pH 4.5, 40 °C, measured by consumption of oxygen dissolved in the pulp suspension at 40 °C.

about 85% at pH 5.5 of low molecular lignans are released in warm water [35]. Therefore, the observed laccase activity on washed pulp is expected to be due to other material than lignans, e.g. lipophilic compounds present in colloidal material and fibre-bound lignin.

According to EPR measurements the laccase treatment of TMP (pulp 2) was found to increase the amount of radicals in handsheets by about 25% as compared with the reference handsheets, whereas radical formation was less significant in washed TMP (Fig. 2). The measured radicals were in both cases mainly those bound to fibre and fines material, as most of the loose DCS material was washed out during the handsheet preparation. These results support the suggestion that DCS material may have a mediating role in the laccase-catalysed oxidation of fibre-bound material [12,13]. However, the results do not reveal weather the presence of dissolved and colloidal material is necessary for the formation of fibre-bound radicals in the laccase treatment.

Because pH affects the solubilisation of material from wood, the activity of laccase was also studied at pH 7 using *M. thermophila* laccase. The oxygen consumption of TMP material treated with *M. thermophila* laccase was similar to or somewhat higher than that of pulp treated with *T. hirsuta* laccase at pH 4.5 (Figs. 1 and 3). The whole pulp slurry and the DCS—water were oxidised in the presence of *M. thermophila* laccase, whereas only moderate oxidation of the washed pulp was observed. However, *M. thermophila* laccase at pH 7 appeared to be more effective in catalysing the oxidation of pulp and DCS material than *T. hirsuta* at pH 4.5. The main reason for the observed higher activity was most probably the availability of more reactive material for laccase action at pH 7 than at pH 4.5. The lower redox potential of *M. thermophila* laccase than that of *T. hirsuta* laccase would suggest a lower oxidation potential of substrates. However, in addition to redox potential, substrate specificity also affects the oxidation potential of laccase.

In order to study further the activity of laccase on fibres or fines (pulp 2), their treatment with *T. hirsuta* laccase was monitored by dissolved oxygen measurement. Practically no activity was observed on the fibre and fines fractions

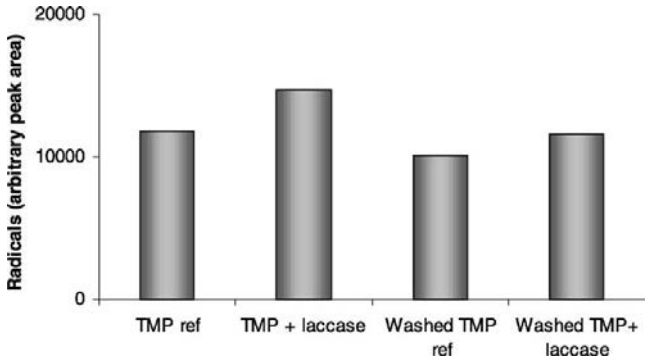

Fig. 2. Radicals found in laccase-treated TMP (pulp 2) (1000 nkat/g of *T. hirsuta* laccase, 40 °C, pH 4.5, 1 h).

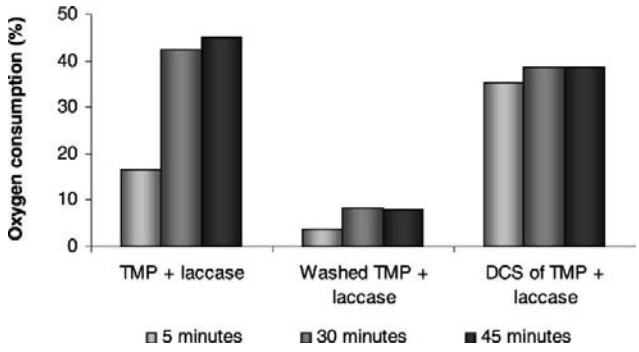

Fig. 3. The activity of *M. thermophila* laccase (1000 nkat/g) on pulp material of unbleached TMP (pulp 1) at pH 7, 40 °C, measured by consumption of oxygen dissolved in the pulp suspension at 40 °C.

(results not shown). EPR measurements, however, revealed that the laccase had acted on both the TMP fines fraction and the fibre fraction (Fig. 4). The radical content had increased by 20 and 30% in fibres and fines, respectively, by laccase treatment as compared to the corresponding references. It appears that the fines fraction, rich in lignin and extractives, is slightly more reactive than long fibres in laccase treatment.

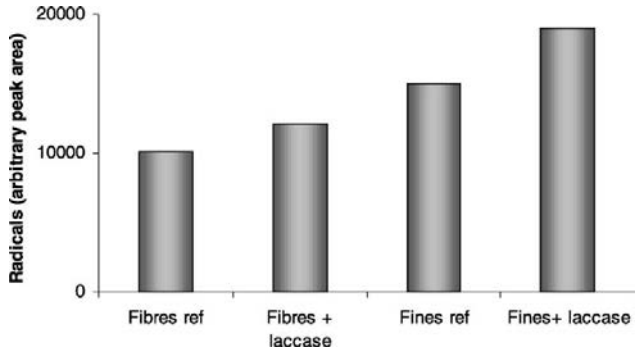

Fig. 4. Radicals found in laccase-treated fibres and fines fractions of TMP (pulp 2) (1000 nkat/g of *T. hirsuta* laccase, RT, pH 4.5, 1 h).

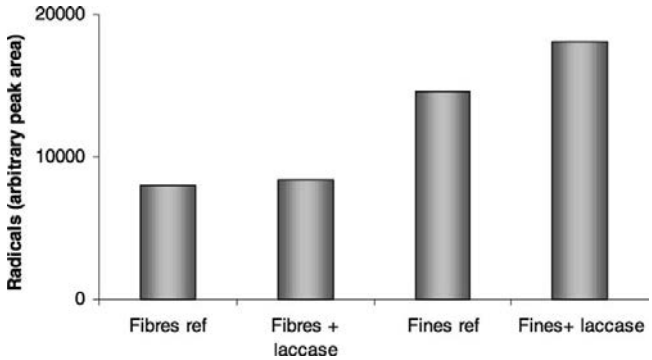

Fig. 5. Radicals found in laccase-treated fibres and fines fractions of bleached TMP (pulp 4) (1000 nkat/g of *T. hirsuta* laccase, RT, pH 4.5, 1 h).

4.3. Activity of laccase on bleached TMP

During peroxide bleaching, the chemical composition of lignin changes as some of the phenolic structures are opened by the oxidation [36]. The bleaching step also decreases the concentration of extractives [37]. To elucidate whether laccases are able to catalyse the oxidation of peroxide bleached material, the activities of *T. hirsuta* and *M. thermophila* laccases on pulp material were monitored by measurement of oxygen consumption at pH 4.5 and 7, respectively. No clear laccase activity was observed at pH 4.5 or at pH 7 on the bleached TMP 3, the washed pulp or the fractions (DCS, fibres and fines) (results not shown). Clearly, the changes in the chemical composition of the pulp material caused by peroxide bleaching decrease the amount of suitable substrate for laccase. According to the EPR measurements, no radicals were formed in the TMP fibres by *T. hirsuta* laccase, whereas radicals were formed in the fines fraction. The 20% increase in the radical content of the fines fraction (pulp 4) by laccase treatment indicated that even after bleaching, the fines contained material reactive in laccase-catalysed oxidation (Fig. 5).

5. Conclusions

According to the results both *T. hirsuta* and *M. thermopila* laccases are active on TMP and its different fractions. The laccase treatments were found to increase the amount of radicals in the TMP fibre material. DCS containing readily oxidisable components might have a mediating role in the formation of radicals. During peroxide bleaching the surface composition of pulp is modified and the amount of substrate suitable for laccase is decreased. As a result, the extent of laccase-catalysed oxidation is lower in bleached TMP. The capability of laccase to oxidise surface lignin in mechanical pulp fibres offers a means to functionalise fibres for customised paper and board products.

Acknowledgments

This work is part of the VTT technology theme “Clean world”. The project was partially financed by The National Technology Agency (TEKES). The technical assistance of Tiina Leppänen and Kati Uotila is gratefully acknowledged. The laccase expertise of Dr. Kristiina Kruus is acknowledged.

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PUBLICATION III

Reactivity of *Trametes* laccases with fatty and resin acids

In: Applied Microbiology & Biotechnology
2001: 55, pp. 317–320.

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PUBLICATION IV

Oxidation of milled wood lignin with laccase, tyrosinase and horseradish peroxidase

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2005: 67(4), pp. 489–494.

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PUBLICATION V

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In: Holzforschung 2006: 60(5), pp. 503–508.

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Series title and number
VTT Science 60

Title	**Action of laccase on mechanical softwood pulps**
Author(s)	Stina Grönqvist
Abstract	During recent years, the traditional pulp and papermaking business in Europe has been striving to find new viable applications for wood fibres. The target has been to improve the value and properties of traditional fibres and fibre products and to find new applications for wood fibres that would support much-needed growth in the industry. At the same time, interest in using renewable materials in new applications has increased. However, the natural properties of the fibres limit their use in many applications. Fibre functionalization, i.e. bonding of new compounds to the fibres, is a method to produce fibres with altered properties. An interesting option is targeted modification of fibre surface lignin via enzymatic radical formation with oxidative enzymes. The highly reactive radicals generated on the fibre surface can be utilised in the bonding of new compounds. In order to exploit the laccase-based functionalization method, deep understanding of factors affecting the formation of phenoxy radicals in fibres is needed. Furthermore, factors affecting the degree of bonding need to be clarified. The main aim of this thesis was to elucidate the effects of laccase treatments on softwood TMPs and their fractions. Furthermore, potential utilisation of the radicals formed by laccase-catalysed oxidation in fibre functionalization was assessed. The studied laccases were found to be reactive with the studied TMPs and their fractions. The degree of oxidation of TMP was found to be influenced by the presence of dissolved and colloidal substances (DCS). However, the results did not confirm the previously suggested role of DCS in the laccase-catalysed oxidation of fibre-bound lignin. Laccase appeared to be able to catalyse the oxidation of free fatty and resin acids. The type of chemical linkages present in fatty and resin acids was found to define the effect of laccase. It seems that laccases can be used to oxidise fatty acids with several double bonds and resin acids with conjugated double bonds. Laccase treatment of milled wood lignin (MWL) was not found to decrease the amount of total phenols in lignin, whereas the amount of conjugated phenols in lignin was found to increase. It was concluded that the effects of laccase on low- molecular mass substrates, such as lignans, are different to those on the more complex lignin. Apparently, in larger lignin structures, the formed radicals can delocalise into the structure. Two types of radicals can be detected after laccase treatments in wood fibres, i.e. “short-living” radicals that can only be detected immediately after the laccase treatment and stable, “long-living” radicals that can be detected in dried samples even days after the treatment. The stable radicals detected in dry samples represent only a small part of the originally generated radicals. The formed radicals should be utilised in bonding of the new compounds within an appropriate short time after activation, before the radicals are delocalised in the structure. Bleaching of TMP affects the amount and the stability of radicals formed in the laccase-catalysed oxidation. More radicals were generated in the laccase-catalysed oxidation on bleached TMP than on unbleached TMP. Peroxide bleaching was found to cause changes in surface chemistry so that “long-living” radicals could only be detected in the fines fraction. This might indicate that the possible levels of modification of unbleached and bleached fines and fibres are different. Bonding of 3-hydroxytyramine hydrochloride to TMP could be demonstrated, which suggests that compounds containing functional groups can be bonded to wood fibres via laccase-catalysed oxidation of surface lignin. Even though the laccase-aided fibre functionalization method is limited to lignin-rich pulps, its potential is remarkable. It has been shown that the method can be used to create completely new properties in lignin-containing fibres.
ISBN, ISSN	ISBN 978-951-38-8269-3 (Soft back ed.) ISBN 978-951-38-8270-9 (URL: http://www.vtt.fi/publications/index.jsp) ISSN-L 2242-119X ISSN 2242-119X (Print) ISSN 2242-1203 (Online)
Date	August 2014
Language	English, abstracts in Finnish and Swedish
Pages	94 p. + app. 53 p.
Keywords	fibre activation, fibre functionalization, surface modification, oxidative enzymes, laccase, lignin, TMP
Publisher	VTT Technical Research Centre of Finland P.O. Box 1000, FI-02044 VTT, Finland, Tel. +358 20 722 111

Julkaisun sarja ja numero
VTT Science 60

Nimeke	**Lakkaasin vaikutukset mekaanisiin havupuumassoihin**
Tekijä(t)	Stina Grönqvist
Tiivistelmä	Eurooppalainen massa- ja paperiteollisuus on viime vuosien aikana etsinyt uusia kannattavia sovelluksia puukuiduille. Tavoitteena on ollut parantaa nykyisten kuitujen ja kuitutuotteiden ominaisuuksia sekä löytää kuiduille uusia sovelluskohteita, jotka tarjoaisivat alalle toivottua kasvua. Samaan aikaan kiinnostus hyödyntää uusiutuvia raaka-aineita erilaisissa sovelluksissa on kasvanut. Puukuitujen luontaiset ominaisuudet rajoittavat kuitenkin niiden hyödyntämistä monissa sovelluksissa. Kuidun funktionalisonnilla, eli liittämällä kuidun pintaan uusia yhdisteitä, voidaan parantaa puukuitujen ominaisuuksia, nostaa niiden arvoa ja siten parantaa massa- ja paperiteollisuuden kilpailukykyä. Kun puukuidun pinnan ligniiniä muokataan hapettavilla entsyymeillä, muodostuu kuidun pintaan reaktiivisia radikaaleja. Syntyneiden radikaalien avulla kuituihin voidaan liittää yhdisteitä, jotka antavat kuidulle uusia ominaisuuksia. Menetelmän tarjoamien mahdollisuuksien hyödyntämiseksi tarvitaan tietoa kuidun radikalisointiin ja yhdisteiden liittämiseen vaikuttavista tekijöistä. Tämän väitöskirjan tavoitteena oli selvittää lakkaasin vaikutuksia kuusen TMP-massoihin ja niiden fraktioihin. Lisäksi työssä arvioitiin lakkaasin avulla hapetuksessa syntyneiden radikaalien hyödyntämistä kuidun funktionalisoinnissa. Tutkitut lakkaasit hapettivat tutkittuja TMP-massoja ja niiden fraktioita. Hapetuksen todettiin olevan riippuvainen liuenneiden ja kolloidaalisten aineiden määrästä. Tulokset eivät todentaneet aiemmin esitettyjä väitteitä liuenneiden ja kolloidaalisten aineiden roolista ligniinin lakkaasiavusteissa hapetuksessa. Saatujen tulosten perusteella voidaan olettaa, että lakkaasin avulla vapaiden rasvahappojen sekä hartsihappojen konjugoituneiden kaksoissidoksien hapettaminen on mahdollista. Puusta eristetyn ligniinin lakkaasiavusteisessa hapetuksessa fenolien kokonaismäärän ei todettu vähenevän, mutta konjugoituneen ligniinin määrän havaittiin kasvavan. Tässä työssä ja kirjallisuudessa esitettyjen tulosten perusteella voitiin todeta, että lakkasi vaikuttaa eri tavalla substraatteihin, joilla on korkea moolimassa (ligniini) ja alhainen moolimassa (lignaanit). Korkean moolimassan rakenteissa, kuten ligniinissä, hapetuksessa muodostuneet radikaalit voivat stabiloitua siirtymällä rakenteessa. Saatujen tulosten perusteella voitiin päätellä, että puukuituihin syntyy sekä lyhytkestoisia että pitkäkestoisia radikaaleja. Lyhytkestoiset radikaalit voidaan havaita kuidussa vain hetki hapetuksen jälkeen, kun taas pitkäkestoiset radikaalit voidaan havaita vielä useamman päivän säilytyksen jälkeen. Kuivatuista näytteistä mitatut pitkäkestoiset radikaalit edustavat vain pientä osaa alkuperäisestä radikaalien kokonaismäärästä. Lakkaasiavusteisessa hapetuksessa syntyneet radikaalit tulisikin hyödyntää uusien komponenttien liittämiseen suhteellisen nopeasti radikaalien muodostumisen jälkeen. TMP:n valkaisun todettiin vaikuttavan lakkaasiavusteisessa hapetuksessa syntyvien radikaalien määrään ja niiden stabiilisuuteen. Valkaistusta TMP:stä voitiin mitata suurempia määriä radikaaleja kuin valkaisemattomasta TMP:stä. Valkaisun vaikutuksesta, säilytyksen jälkeen, ainoastaan hienoaineesta voitiin mitata radikaaleja. Saatujen tulosten perusteella on syytä epäillä, että valkaistujen kuitujen ja hienoaineen hapettumisessa on suuria eroja. Näin ollen on myös mahdollista, että kuidut ja hienoaines ovat eri tavoin muokattavissa. Työssä voitiin osoittaa 3-hydroksityramiinihydrokloridin sitoutuminen TMP:hen lakkaasiavusteisesti. Tulos osoittaa, että uusia funktionaalisia ryhmiä voidaan sitoa ligniinipitoisiin puukuituihin aktivoimalla kuitujen pinnan ligniiniä lakkaasilla. Vaikka menetelmä soveltuu ainoastaan ligniinipitoisten puukuitujen muokkaukseen, avaa menetelmä täysin uudenlaisia mahdollisuuksia puukuitujen hyödyntämiselle.
ISBN, ISSN	ISBN 978-951-38-8269-3 (nid.) ISBN 978-951-38-8270-9 (URL: http://www.vtt.fi/publications/index.jsp) ISSN-L 2242-119X ISSN 2242-119X (painettu) ISSN 2242-1203 (verkkojulkaisu)
Julkaisuaika	Elokuu 2014
Kieli	Englanti, tiivistelmä suomeksi ja ruotsiksi
Sivumäärä	94 s. + liitt. 53 s.
Avainsanat	fibre activation, fibre functionalization, surface modification, oxidative enzymes, laccase, lignin, TMP
Julkaisija	VTT PL 1000, 02044 VTT, puh. 020 722 111

Series namn och nummer
VTT Science 60

Namn	**Lackasens inverkan på mekaniska massor framställda av barrträd**
Författare	Stina Grönqvist
Referat	Under de senaste åren har den europeiska massa- och pappersindustrin sökt nya lönsamma tillämpningar för träfibrer. Målet har varit att förbättra de traditionella fibrernas och fiberprodukternas egenskaper, samt att hitta nya tillämpningar för träfibrerna. Nya fiberegenskaper och -tillämpningar skulle kunna ge sektorn den tillväxt som behövs. Samtidigt har intresset för att använda förnyelsebara råvaror i en mängd olika tillämpningar ökat. Träfibrernas naturliga egenskaper begränsar dock deras användning i många tillämpningar. Modifiering av träfibrerna skulle kunna vidga fibrernas andvändbarhet, öka fibrernas värde och därmed förbättra massa- och pappersindustrins konkurrenskraft. Träfibrernas egenskaper kan modifieras genom att binda nya komponenter med önskade egenskaper till fiberns yta. Ett sätt att utföra modifieringen är att med hjälp av oxiderande enzymer, såsom lackas, bilda reaktiva radikaler i ligninen på fibrernas ytor och vidare utnyttja de bildade radikalerna till att binda komponenter med nya egenskaper till fiberytan. För att kunna utnyttja den fulla potentialen av den lackasbaserade modifierings-metoden, behövs mera information om både de faktorer som påverkar bildningen av radikaler samt om mekanismerna hur nya komponenter binds till fibrerna. Syftet med denna avhandling var att undersöka effekterna av lackas på TMP av gran och olika fraktioner av TMP. Därtill undersöktes modifiering av fibrerna genom bindning av nya komponenter via radikalerna som uppstått under lackasbehandlingen. De undersökta lackaserna kunde oxidera TMP-massor och deras fraktioner. Lösta och kolloidala substanser hade en klar inverkan på oxidationen. På basen av resultaten i detta arbete kan man anta att lackas kan oxidera fria fettsyror och hartssyror med konjugerade dubbelbindningar. Efter lackasbehandling av isolerat lignin förblev den totala mängden fenoler oförändrad, medan andelen konjugerade strukturer i lignin ökade. På basen av resultaten som presenterats i detta arbete och de resultat som hittats i litteraturen, kunde man konstatera att lackas har olika effekt på substrat som har en hög molmassa (t.ex. lignin) och tydligt lägre molmassa (t.ex. lignaner). I de högmolekylära strukturerna, såsom lignin, stabiliseras radikaler in i strukturen. På basen av resultaten kunde man dra slutsatsen att oxideringen av fibrer med lackas resulterar i att både kortvariga och långvariga radikaler bildas. Kortvariga radikaler kan upptäckas i fibern bara en kort tid efter oxideringen, medan de långvariga radikalerna kan observeras ännu efter flera dagars förvaring. Långvariga radikaler, som kunde mätas i proverna efter förvaring, utgjorde endast en liten del av det ursprungliga antalet radikaler. På grund av att en stor andel av de bildade radikaler snabbt stabiliseras in i ligninens struktur, bör bindning av nya komponenter ske relativt snabbt efter att radikalerna bildats. Blekning av TMP visade sig påverka både mängden och stabiliteten av radikaler som bildas i de lackas katalyserade reaktionerna. Mängden radikaler var högre i blekt massa. Peroxid blekningen påverkade ytkemin så att efter lagring kunde radikaler mätas endast i finmaterialet. Enligt resultaten finns det anledning att tro att möjligheterna att modifiera blekta fibrer och finmaterial är olika. I detta arbete kunde det bevisas att bindning av 3-hydroxythyramineklorid till fibrer är möjligt. Resultatet kan ses som ett bevis att nya funktionella grupper kan bindas till träfibrerna med hjälp av lackas. Även om denna metod är endast lämplig för ligninhaltiga träfibrer, öppnar metoden helt nya möjligheter för utnyttjande av träfibrer.
ISBN, ISSN	ISBN 978-951-38-8269-3 (Print) ISBN 978-951-38-8270-9 (URL: http://www.vtt.fi/publications/index.jsp) ISSN-L 2242-119X ISSN 2242-119X (Print) ISSN 2242-1203 (Online)
Datum	Augusti 2014
Språk	Engelska, referat på finska och svenska
Sidor	94 s. + bil. 53 s.
Nyckelord	fibre activation, fibre functionalization, surface modification, oxidative enzymes, laccase, lignin, TMP
Utgivare	VTT PL 1000, 02044 VTT, puh. 020 722 111

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ISBN 978-951-38-8269-3 (Soft back ed.)
ISBN 978-951-38-8270-9 (URL: http://www.vtt.fi/publications/index.jsp)
ISSN-L 2242-119X
ISSN 2242-119X (Print)
ISSN 2242-1203 (Online)

VTT

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