Trametes versicolor as biodegrader and biocatalyst when using lignocellulose for ethanol production

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I never loose. I either win or learn.
Nelson Mandela

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List of publications

This thesis is based on the following papers, referred to by Roman numerals in the text:

I - Identification of white-rot fungi and soft-rot fungi increasing ethanol production from spent sulfite liquor in co-culture with *Saccharomyces cerevisiae*

II - Characterization of Bioethanol Production from Hexoses and Xylose by the White Rot Fungus *Trametes versicolor*

III – Adaptability of *Trametes versicolor* to the lignocellulosic inhibitors furfural, HMF, phenol and levulinic acid during ethanol fermentation

IV - *Trametes versicolor* involved in biodegradation and biocatalyzation of lignocellulose in ethanol fermentation (manuscript)

Papers I-III are reproduced with the permission of the publishers.

Abstract

Energy consumption has increased rapidly during the last century due to population growth and greater industrialization. Lignocellulosic-based biofuels are being developed as alternatives to fossil fuels. For many years the question of how 5-carbon sugars in biomass are utilized in nature has been a vexed one. A patent by Sellstedt and Holmgren (2005) showed increased ethanol production, compared with fermentation using only *Saccharomyces cerevisiae*, through the use of a fungal mix from a degraded wood sample found in a forest.

The aim of the work presented in this thesis was to find a fungus that naturally utilizes 5-carbon sugars and metabolizes these sugars to ethanol; elucidate the capacity of this fungus to utilize 5-carbon and 6-carbon sugars simultaneously; assess the ability of the fungus to metabolize the inhibitors produced when lignocellulose is used; and find out whether this fungus could be used for biological pretreatment of lignocellulose to replace industrially produced enzymes.

The results showed that the fungal mix grew well on glucose, xylose, hemicellulose and cellulose. In addition, we were able to identify the fungi present, by using PCR-amplification and sequencing of DNA, as *Chalara parvispora*, *Xylaria sp* and *Trametes hirsuta / Trametes versicolor*. In a reconstitution study, the fungi so identified were shown to produce an amount of ethanol equal to that of the fungal mix. We were also able to show that *C. parvispora* could produce ethanol from xylose.

T. versicolor could be grown in culture, under hypoxic conditions, with various mixtures of hexoses and xylose and with xylose alone. After 354 h of culture we found very strong correlations between ethanol fermentation (alcohol dehydrogenase activity and ethanol production), sugar consumption and xylose catabolism (xylose reductase, xylitol dehydrogenase and xylulokinase activities) in the cultures. In a medium containing a 1:1 glucose/xylose ratio, the efficiency of fermentation of total sugars into ethanol was 80 %.

A variety of inhibitors are formed during pretreatment procedures; they include, for example, phenolics, levulinic acid, HMF and furfural. These inhibitors were used in this study in order to reveal their effects on the growth of cells as well as on sugar utilization, enzyme activities and ethanol production by the white-rot fungus *T. versicolor*. The inhibitors had a positive effect on fresh weight, the largest increase being observed with the

inhibitor furfural. *T. versicolor* metabolized all the inhibitors during 15 days of experimentation.

It is known that fungi can degrade cellulose, hemicellulose and lignin through a series of enzymatic reactions. Is it possible to eliminate chemical pretreatment and instead use a biological pretreatment? If *T. versicolor* could serve as both a biodegrader and a biocatalyst it would lead to reductions in the costs of ethanol production and lower costs for pretreatments for other renewable fuels too. Experiments with different pretreatments applied to *Salix viminalis* and *Populus tremula* were conducted with and without fungi, as well as with enzymes, to evaluate whether *T. versicolor* was suitable as a biodegrader. The results showed that *T. versicolor* was able to degrade lignocellulose to glucose, and thus is suitable as a biodegrader and in addition has xylanase and beta-glucosidase enzymes that are related to similar enzymes in other fungi.

Abbreviations

ADH – Alcohol dehydrogenase

ALDH – Acetaldehyde dehydrogenase

ATP – Adenosine 5'-triphosphate

CBS – Centraalbureau voor Schimmelkultures, Netherlands

DNA – Deoxyribonucleic acid

HMF – 5 –Hydroxymethylfurfural

MBMC – Modified Base Medium Composition

NAD^+ - Nicotinamide adenine dinucleotide

PCR – Polymerase chain reaction

PDC – Pyruvate dehydrogenase

XDH – Xylitol dehydrogenase

XK – Xylulokinase

XR – Xylose reductase

Populärvetenskaplig sammanfattning

Med en växande industri och en ökande befolkning ökar också energianvändningen på jorden. Det fossila bränsleanvändandet måste minska i och med dess klimatpåverkan. Som ett alternativ till fossila bränslen utvecklas bränslen från lignocellulosa. I många år har det funderas över och forskats om hur naturen har löst nedbrytningen av 5-kols socker som är en av sockerarterna i biomassa. Ett patent av Sellstedt och Holmgren (2005) visade på en ökad etanolproduktion från en naturligt förekommande svampmix tillsammans med bagerijäst.

Målsättningen med avhandlingen har varit att hitta en naturligt förekommande svamp som kan använda 5-kols socker för fermentering till etanol, att utröna svampens förmåga att samtidigt använda 5- och 6-kols socker, att analysera svampens förmåga att metabolisera inhibitorer som bildas när lignocellulosa förbehandlas samt att utröna om svampen kan användas som biologisk förbehandling av lignocellulosa så att man kan ersätta industriellt producerade kostsamma enzymer.

Resultaten visade att svampmixen växte bra på glukos, xylos, hemicellulosa och cellulosa. Via amplifiering av DNA med hjälp av PCR identifierades svampmixen, den bestod av *Chalara parvispora*, *Xylaria sp* och *Trametes hirsuta*/*Trametes versicolor*. Under ett rekonstruktionsexperiment visade det sig att de identifierade svamparna producerade lika mycket etanol som svampmixen. Vi kunde även visa att *C. parvispora* kunde producera etanol från xylos.

T. versicolor kunde också växa under syrefattiga, förhållanden med olika mixer av sex-kolssocker och xylos samt enbart med xylos. Efter 354 timmar såg vi en stark korrelation mellan fermenteringen till etanol (aktivtet av alkohol dehydrogenas och produktion av etanol) och konsumtionen av socker, med hjälp av enzymer (xylos reduktas, xylitol dehydrogenas och xylokinas aktivitet) i svamp kulturerna. I ett medium som innehåller ett 1:1 glukos/xylos, var fermenteringseffektiviteten av total mängd socker till etanol 80 %.

En mängd olika inhibitorer bildas när biomassa behandlas t.ex. fenoler, levulin syra, HMF och furfural. Dessa inhibitorer har studerats närmare i denna avhandling för att se hur de påverkade rötsvampen *T. versicolors*

celltillväxt, sockeranvändning, enzymaktivitet och etanolproduktion. Friskvikten påverkades i positiv bemärkelse av inhibitorerna och ökade mest med enbart furfural. Under etanolproduktionen metaboliserade *T. versicolor* alla inhibitorer.

Det är känt att en del svampar kan via en rad enzymatiska reaktioner bryta ner cellulosa, hemicellulosa och lignin. Är det möjligt att utesluta kemisk förbehandling och i stället använda sig av biologisk förbehandling av biomassa? Om *T. versicolor* skulle kunna fungera både som bio-nedbrytare och bio-katalysator skulle det leda till lägre kostnader inom etanolproduktionen samt förbehandling av andra biobränslen. Olika förbehandlingar med och utan svamp samt enzymer testades med biomassa från *Salix viminalis* och *Populus tremula* för att utvärdera om denna svamp kan fungera som ”bionedbrytare”. Resultaten visade att *T. versicolor* kunde metabolizera lignocellulosa till glukos och att svampen dessutom har xylanase och beta-glucosidase enzymer som är närbesläktade med liknande enzymer hos andra svampar.

Introduction

The world is in need of a change in the ways in which we use it and its resources. By 2030 the world's energy demand is expected to rise by around 60 % (2) and this will necessitate substantial and sustainable reductions in greenhouse gas emissions in order to limit climate change (3). To be able to convert from fossil fuels to renewable fuels we have to look at all possibilities and use energy more efficiently so that less primary energy is used. By 2050 Europe is aiming to achieve a low carbon scenario, with a reduction of at least 80 % compared to the 1990 level (4).

Climate change and bioenergy

The climate, including ocean warming, temperature extremes, wind patterns and continental-average temperatures, is discernibly influenced by humans (2). Concentrations of CO_2, N_2O and CH_4 have increased rapidly in the atmosphere as a result of human activity (2). Around 80 % of the energy used in Europe is still produced from fossil fuels and we are consuming more and more electricity (5).

Petroleum is being exploited at a faster rate than that of its natural regeneration through the planet's C cycle. That the end of the cheap fossil fuel era is just around the corner is a common and increasing perception, and crude oil, transportation fuels and petroleum-derived chemicals will become more expensive in years to come (6). Changes in all components of the climate system are anticipated, together with further warming caused by continued emissions of greenhouse gases (3).

Shifting to renewable energy resources can mitigate global warming and also those resources are, and, from a socio-economic perspective, these resources are also more evenly distributed across the world than are fossil fuels. (6). One approach is circular economy (in which no waste and no pollution are produced), which could enable the functions of resources in the economy to be transformed (7). Because it places no unsustainable pressure on natural resources and does not breach the limits of the environment, circular economy could help some countries to develop and other, developed, countries to increase wellbeing (7).

Biomass sources of lignocellulose are very promising, since they are cheaply available and widespread in most countries. Because biomass can be used in a variety of applications (shown in Fig. 1) it is important, from

environmental, economic and resource perspectives, to choose the most promising options (6).

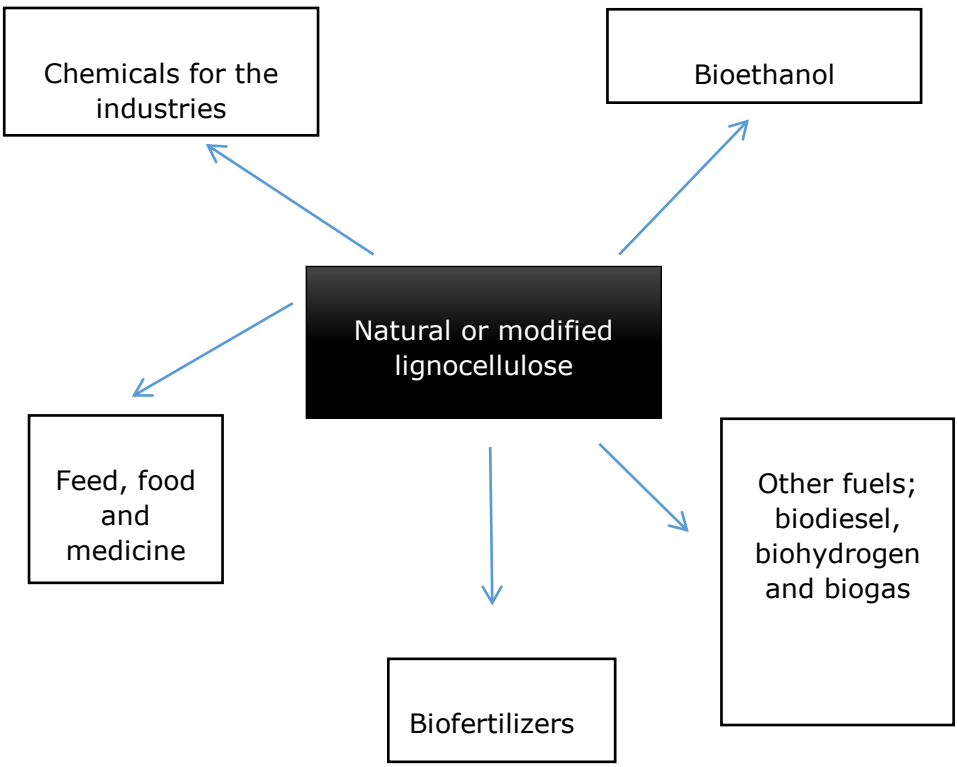


Figure 1. A scheme for the usage of lignocellulose (Redrawn from (8)).

Biofuels - ethanol

The most abundant biofuel currently on the market is ethanol. The Model T Ford was originally designed to use ethanol as its fuel, with the idea that the ethanol could be produced from biomass.

Biofuels produced primarily by fermentation and esterification of food crops such as maize, soy and palm are called first generation biofuels (9). Generally second-generation biofuels are made from non-edible lignocellulosic biomass (10). Third-generation biofuels are currently produced from algal biomass and in extent utilized from CO_2 (9). The second and third generation biofuels are also referred to as next generation biofuels (11).

The raw material to be used determines which process is needed for ethanol production, and the process is commonly carried out in three steps (10): production of fermentable sugars in solution, conversion of the sugars into ethanol by fermentation, and separation and purification of the ethanol.

Sugarcane and corn are still the main feedstocks used for producing ethanol. Increasing demand for food sources and, at the same time, for ethanol means that there is an urgent requirement for other feedstocks (10). According to the International Energy Agency, biofuels could supply 5-15 % of transportation energy by 2035 (12). Lignocellulose is abundantly available and opens up the possibility of producing ethanol in a sustainable and economical manner (13). When using lignocellulose as the raw material for ethanol production there are three different ways of processing the material: biochemically, hydrothermally, thermochemically and biologically (13).

Lignocellulosic material

About half of the biomass produced worldwide by photosynthesis is lignocellulose (14,15). Lignocellulose is a very abundant resource made up mainly of plant cell walls consisting of three polymers: cellulose, hemicellulose and lignin. These polymers are strongly intermeshed and chemically bonded by non-covalent forces and covalent cross-linkages (14). Pectins (mainly found in the primary cell wall), proteins, extractives and minerals are also components of the cell wall (12). The lignin content is generally higher in softwoods than in hardwoods and the former therefore contain a lesser amount of holocellulose (the sum of cellulose and hemicellulose) and extractives (16). There are many limitations and challenges when it comes to converting biomass into biofuel. The

recalcitrance of biomass to degradation by enzymes into sugars is one of these challenges, as is converting all the different sugars into useful end products (17).

The structure of the cell wall

The plant cell has a primary and a secondary cell wall. During cell division the middle lamella and primary cell walls are formed. The shape and size of a cell are determined by the primary wall, which consists of cellulose, hemicelluloses, pectin, structural proteins and phenolic compounds. Even though cellulose is the most abundant polysaccharide on earth, it is not completely understood (18). The synthesis of cellulose is mediated in the plasma membrane by hexagonal-shaped complexes, rosettes that contain 36 cellulose synthase modules. A lattice of microfibrils, which assembles and links by means of hydrogen bonds and van der Waals forces, forms crystalline cellulose (18). Hemicelluloses and pectins are dispositioned by the scaffold that microfibrils constitute (18). The microfibrils make the cell wall stable and strong. Hemicellulose and pectin synthesis occurs in the Golgi bodies. The heterogeneous and complex hemicellulosic polysaccharide molecules crosslink the neighbouring cellulose microfibrils.

In most cell walls secondary wall deposition occurs, accounting for most of the cell wall mass (18). The secondary cell wall contains cellulose, hemicelluloses (mostly xylans), and lignin which is a product of oxidative combinatorial coupling of 4-hydroxyphenylproanoids. In the secondary cell wall, hemicelluloses are crosslinked with lignin and cellulose, and due to this interaction it is stronger than the primary cell wall (18).

Between cells, and accumulating in the middle lamella, pectin provides adhesion by filling in the cellulose-hemicellulose matrix in primary cell walls (12). The main minerals present are silicon, potassium, calcium, sulphur and chlorine, and they are, for example, found in the ash produced when lignocellulosic material is burned (12).

Lignocellulose is usually present in all parts of the plant; it occurs in roots and stalks as well as leaves (16). Depending on the type, species and source of biomass the lignocellulosic proportions vary substantially (19). Within a single plant the composition varies with age, growth stage and other factors (14,12). How and why the amount and composition of cell walls is regulated is still not fully known (12). Figure 2 shows an overview of the cell wall structure and table 1 shows the proportions of the major polymers in different lignocellulosic materials, some nature-derived and others wastes from industry (20).

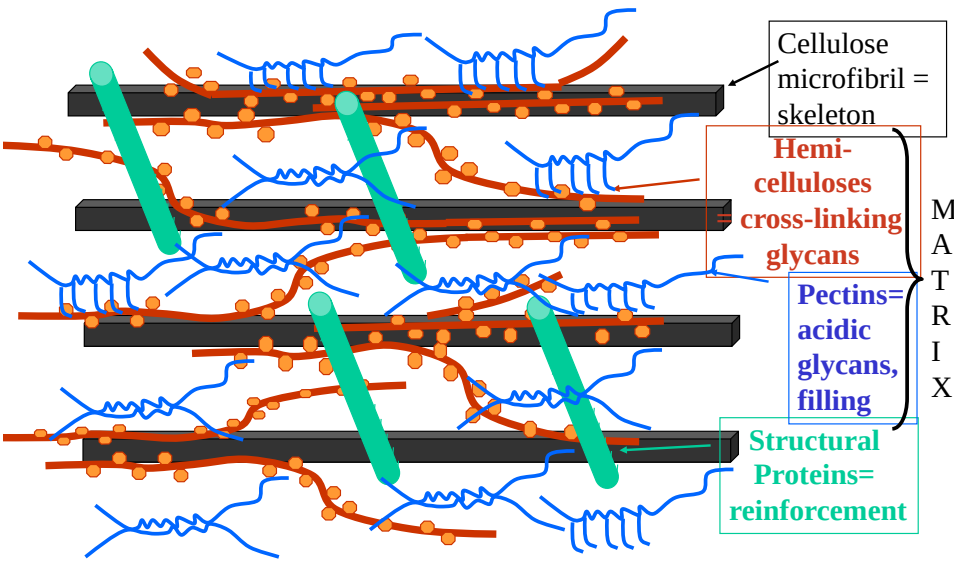


Figure 2. Architecture of the plant cell wall. Picture reproduced with the courtesy of Prof. Ewa Mellerowicz, UPSC, SLU, Umeå.

Table 1. The polymer contents of different lignocellulosic materials. NA – not available (Adapted from ref. 20).

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Wheat straw	30	50	15
Waste papers from chemical pulps	60-70	10-20	5-10
Switch grass	45	31.4	12.0
Swine waste	6.0	28	NA
Sorted refuse	60	20	20
Solid cattle manure	1.6 – 4.7	1.4 - 3.3	2.7 – 5.7
Softwood stems	40-50	25-35	25-35
Primary wastewater solids	8-15	NA	24-29
Paper	85-99	0	0-15
Nut shells	25-30	25-30	30-40
Newspaper	40-55	25-40	18-30
Leaves	15-20	80-85	0
Hardwood stems	40-55	24-40	18-25
Grasses	25-40	35-50	10-30
Cotton seed hairs	80-95	5-20	0
Corn cobs	45	35	15
Coastal Bermuda grass	25	35.7	6.4

Cellulose

The chemical formula for cellulose $(C_6H_{10}O_5)_n$ indicates that it is a polymer of glucose, a polysaccharide that can consist of from several hundred up to over ten thousand β-1,4 linked D-glucose units (21,22). The degree of polymerization (DP), i.e. the number of glucose units that make up one polymer molecule, is correlated with the many properties of cellulose (21). The polymer's arrangement of long straight chains is due to the nature of the bond between the glucose molecules (β-1,4 glucosidic) (21). The long straight chains and evenly distributed hydroxides on both sides of the monomers allow for the formation of hydrogen bonds between cellulose molecules

(14,21). The parallel glucose chains and the successive residues of glucose rotate through 180 degrees forming cellobiose, a repeating disaccharide unit (12).

The structure of cellulose is found in both crystalline and non-crystalline forms (14,21). Microfibrils consists of several polymer chains, which, when united, in their turn form fibres and lead to the crystalline form of cellulose (21). Hydrogen bonds and Van der Waals forces hold the crystalline structure together, allowing the formation of microfibrils (12). In spite of the fact that cellulose is relatively hygroscopic, it is insoluble in water due to its high molecular weight and crystalline structure. The same applies at low temperatures in dilute acid solutions. The polymer's solubility is strongly related to the degree of hydrolysis that can be achieved (21).

Hemicellulose

Hemicellulose, $(C_5H_8O_4)_m$, consists of different polysaccharides such as xylan, galactan, mannan which are β-1,4-linked, or a backbone of glucomannan with single or longer glycosyl residues forming branches (12,21). Mixed-linkage β-1,4: β-1,3 glucans can be also found in some species (12). The structure and composition of hemicellulose differs depending on their source and how they are extracted (21). Xylan, the most common polysaccharide in the hemicellulose category, has 1->4 linkages of xylosyl units with arabinosyl, xylosyl or glucoronic acid substituents resulting in a branched polymer chain (12,19,21).

Hemicelluloses lack crystalline structure due to their highly branched chains and the presence of acetyl groups. (14,21).

Hemicellulose has a high degree of polydispersity, polydiversity and polymolecularity when it is extracted from plants (Harmsen et al. 2010). This means that it has a broad range of size, shape and mass characteristics. In water and at low temperatures hemicellulose is insoluble, but at elevated temperatures hydrolysis starts to occur and it becomes soluble (21). The presence of acid improves its solubility.

A key element for the efficient fermentation of lignocellulose to bioethanol is the disruption of the hemicellulose matrix (21). This represents a large portion of the fermentable sugars, and disruption enables the cellulose lattice to become accessible to the enzymes involved in saccharification and fermentation (21). The hemicellulose is a mixture of both 5-carbon sugars and 6-carbon sugars compared to cellulose that only consists of 6-carbon sugars. Thus the mixture of sugars hemicellulose is easier to hydrolyse than

cellulose, since hemicellulose has shorter chains and the main chains are branched. Hemicellulose is more susceptible to enzymatic hydrolysis than cellulose since cellulose form packed crystalline structures (23). Another important factor is the degree of acetylation, since lignin and acetyl groups attached to the matrix may hinder the breakdown of polysaccharides (24,25).

Lignin

Lignin, $(C_9H_{10}O_3(OCH_3)_{0,9\text{-}1,7})_x$, is the most complex natural polymer, although it consists solely of the elements carbon, hydrogen and oxygen (26). The polymer is amorphous and three-dimensional, with phenylpropane units as its predominant building blocks. The most commonly encountered such units are p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (21), Lignin from softwood consists of more than 90 % coniferyl alcohol. The linkages β-O-4, β-5, and β-1 are the main ones, and they are constructed between monomers and monomers, oligomers and monomers and oligomers and oligomers (26).

Depending on the type of biomass, the linkages can vary, but the ether bond generally constitutes more than two-thirds of the total bonds (26). Under normal conditions lignin is very difficult to breakdown due to these bonds, and this is why woody tissue is so persistent in the environment (12).

Our knowledge about the structure of lignin is limited because it has been obtained from extracted lignin, which differs, to a greater or lesser extent, from the natural lignin in lignocellulose. Lignin structure and the amount of functional groups in extracted lignin have been accepted as being closely related to extraction methods (26). Lignin is characterized by a high degree of polydispersity, and different branching and bonding patterns are encountered. (21). Lignin is important for the plant cell's longevity and development; it is crucial in the transport of water, metabolites and nutrients. The structure creates a composite material that offers remarkable protection against impact, compression and bending (21). Elevated temperatures soften lignin thermally, allowing depolymerization. Solvents that dissolve lignin to a significant extent have also been identified (21). Lignin hinders the access of enzymes to cellulose and hemicellulose, and this reduces the efficiency of hydrolysis (24). The rate of enzymatic hydrolysis can be improved by efficient delignification processes (24).

Pectin

A major component of the primary cell wall is pectin, which is also present in the secondary cell wall to some extent (17). However, the pectin fraction in

secondary cell walls is negligible and it is not considered to be an important source of sugar in, for example, the production of biofuels (12). There are at least four subclasses of polysaccharide found in pectin: homogalacturonan (HG), rhamnogalacturonan-I (RG-I), RG-II and xylogalacturonan (XGA), the structure of pectin is shown in figure 3 (17). Pectins are water-soluble, heterogeneous polymers and some are highly branched and structurally very complex due to their high content of methylated and acidic sugars (12). They chelate calcium and can form gels. In waste residues such as sugar beet pulp, citrus waste, apple pomace and potato pulp, pectins are abundant (12).

To use raw biomass feedstocks for the efficient production of biofuels, the methods used for extracting and degrading pectins need to be optimized (17). It has been suggested that pectin masks cellulose and/or hemicellulose, blocking access by degradative enzymes, though the sugars in pectin themselves also represent captured photosynthetic energy (17).

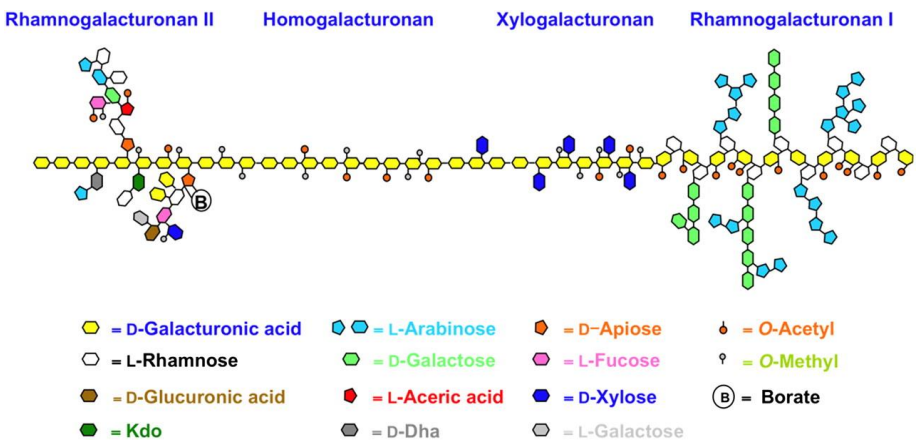


Figure 3. An overview of the different subclasses of pectin. HG and RGI are abundant, more abundant compared with the other components (Harholt *et al*. 2010 Plant Physiol. 153:384-395, reproduced with the permission of the publisher).

In nature pectins are one of the most structurally complex classes of molecule. Pectin consists of both 5-carbon (D-xylose and L-arabinose) and 6-carbon sugars. Depending on the type of feedstock, process and desired

end product, pectins can be viewed as either an impediment to the degradation of biomass or a source of fermentable sugars (17).

Extractable material and ashes

Lignocellulose from straw and wood contains extractives and ashes. The content of ashes differs depending on the lignocellulosic material, and extractives can include fatty acids, resin acids, lipids, phenolic compounds, steryl esters, waxes, sterols, terpenoids and proteins (27, 28). There is interest in removing extractives, either in order to avoid interference during the processing of hemicellulose, cellulose and lignin 'or to exploit them commercially (27). Wood ashes can be of interest for use as fertilizer in forests (29). The production of purer cellulose and lignin will in general be enabled and it may also have a positive effect on enzymatic digestibility in comparison with cases in which extractives have not been removed (27).

Lignocellulosic recalcitrance

Plants consist of the epidermal tissue system, the vascular tissue system and the ground tissue system. The epidermal system is divided into the epidermis and periderm; in vascular tissue there is the xylem and the phloem; and the ground tissue is divided into parenchyma, collenchyma and sclerenchyma. Epidermal tissue has a primary function, due to the hydrophobic skin layer and peripheral skeleton wax of which it consists, which is to protect the internal tissues against microorganisms, and to prevent loss of nutrients and evaporation of moisture (28). The ground tissue surrounds the vascular system, through which nutrients and water are transferred. The ground tissue system is the site of storage for nutrients which are then transferred through the plasmodesmata and pits between adjacent cells (28)

The various components of the cell wall contribute differently to its recalcitrance. Cellulose adds to recalcitrance because its tightly packed glucose units form a crystalline structure (22). Moreover, cellulose is a polymer that requires endo- and exos as well beta-glucosidases in order to be converted to glucose by enzymatic degradation (30). Lignin also contributes to recalcitrance by acting as a cementing material in the cell wall, and its abundance makes digestibility more difficult (31).

Hemicelluloses contribute to closer packing of cellulose and lignin. Removal of hemicelluloses can be achieved by dilute acid pretreatment (22) (See "Chemical pretreatments").

In the industry the properties of recalcitrance conferred by lignocellulose have been widely used, since it contributes to resistance against microbial degradation in the surrounding natural environment (22). To reduce the cost of enzymes and to improve enzymatic hydrolysis the recalcitrance of lignocellulose needs to be analyzed, to make it possible to find efficient deconstruction technologies (22). The meaning of the term recalcitrant, which originated in 1843, is “kicking back”; it was first used to refer to the resistance of plant material to degradation around 1990 (22). A technical report published in 2005 defined the specific meaning of lignocellulose recalcitrance, summarized as describing the lignocellulose biomass (22). The aim of pretreating native lignocellulosic material is to remove barriers, both physical and chemical, and convert it into a form that can be hydrolysed effectively (32, 33).

In summary, it is the complex structure of the plant cell wall that makes the lignocellulose recalcitrant, and the composition and physicochemical nature of the matrix, and the compactness and the strength of the material, which cause the technical and major economic problems inherent in producing biofuel from lignocellulose (34, 35).

Pretreatments

Different technologies are available for the pretreatment of lignocellulosic biomass. These technologies achieve the liberation of cellulose and hemicellulose from lignin, and the production of free sugars by depolymerization (13). It is important to choose and work with sustainable methods of pretreatment (36). The ones used during the work presented in this thesis will be described in more detail.

Physicochemical pretreatments

To reduce particle size and to reduce the crystallinity of cellulose for better hydrolysis, biomass is comminuted by chipping, grinding and/or milling. A combination of different methods (heat, moisture, pressure, chemicals) is then applied in order to make cellulose more accessible. (36). These pretreatments require a lot of energy and special equipment (36).

Chemical pretreatments

For the last 100 years there have been studies on the process of acid hydrolysis for the conversion of cellulose to glucose (36). Hydrolysis mostly involves either acids or bases applied to hydrolyse the biomass. To treat the biomass with acid, concentrated or diluted H_2SO_4 or HCl is used. Concentrated acid hydrolysis has been increasingly replaced by dilute acid hydrolysis because dilute acid is easier to handle (13). The method consists

of two chemical reactions; one that converts the material into sugars and another that converts the sugars into chemicals for industrial applications (37). Even though dilute acid has many advantages, this method results in the formation of furfural, which causes inhibition during the fermentation process (13). Bases used in alkaline hydrolysis are NH_4OH and NaOH, but this is an expensive pretreatment and it is difficult to recover and recycle the bases from the system (13). Chemical pretreatment can also involve thA disadvantage of using chemicals is that some of the methods are expensive and corrosive to the equipment (13,36).

A disadvantage of using chemicals is that some of the methods are not only expensive but also corrosive to the equipment (13,36).

Biological pretreatments

Biological pretreatment is (considered to be) a green and environmentally friendly method that does not need expensive equipment, chemicals or any additional energy (38). It can consist of enzymatic hydrolysis or fungal pretreatment (13). Different microorganisms have been studied for pretreatment methods; they include white rot fungi, brown rot fungi, soft rot fungi, microfungi, bacteria and actinomycetes. The most effective fungi when it comes to converting lignocellulosic biomass are the white rot fungi (13). In this thesis it is mainly white rot fungi that will be discussed.

White rot fungi are able to degrade the structure of wood completely due to the fact that they can depolymerize lignin (39). Among the white rot fungi there is great variation in the rate with which they degrade carbohydrates and lignin in lignocellulosic materials (39). Several studies have shown that after a biological pretreatment enzymatic hydrolysis is enhanced, but it has not yet been shown how fungal delignification affects the physical properties of the substrate (39). One suggestion that has been made is that the cellulase-substrate interaction is affected by an alteration in the properties of the substrate leading to an enhancement of the enzymatic hydrolysis (39). One study showed that the increase in enzymatic hydrolysis after biological pretreatment was related to a decrease in the irreversible adsorption of cellulase to lignocelluloses, a result which may be a consequence of the alteration in the lignin structure (39). A disadvantage of using biological pretreatment is that the rate of hydrolysis is low, and there can also be a loss of dry matter since fungi use carbohydrates for growth (13,36).

Biodegraders and Biocatalysts

Since lignocellulose is chemically complex, both biodegraders and biocatalysts must be able to accelerate different chemical reactions and be as

capable as industrially produced enzymes of carrying out the degradation and utilization of natural lignocellulose (12,40).

Enzymes involving breakdown of lignocellulose

The primary role of biomass-converting enzymes is to degrade polymeric cellulose or hemicellulose to saccharides (sugars), i.e. into simpler forms which can then be fermented by microorganisms to, or serve as platform molecules for, the synthesis of valuable fuels or chemicals (40).

Fungal pretreatment has been used prior to mild physical and chemical pretreatment, showing improved synergism with respect to cellulose digestibility and conferring advantages similar to those of the biopulping process. Researchers have also reported the production of xylanolytic enzymes from white-rot fungi. All the enzymes needed for degrading lignocellulosic components are possessed by white-rot fungi. The hydrolytic system, which is an extracellular enzymatic system, produces hydrolases responsible for degradation of polysaccharides. (41)

The enzymes involved are exoglucanases, endoglucanases and cellobiase, which are responsible for cellulose hydrolysis. Endoxylanases and glucuronidases are enzymes active in xylan hydrolysis. Covalent associations between the components of lignocellulose can be broken down by white-rot fungi. These fungi change the biochemical and physical characteristic of biomass by depolymerizing lignin and decreasing the crystallinity of cellulose. One should not overlook their ability to degrade hemicellulose during the process, as this thesis shows, although white-rots are mostly investigated for their ability to selectively remove lignin. (41)

Depending on tissue type the degree of polymerization can vary from 500 to 15 000 glucose molecules (12). Depolymerization into cellobiose and glucose can be done by the action of endo- and exoglucanases, glucosidases and polysaccharide mono-oxygenases (12); these enzymes act only on microfibrils. The process is rather slow and the reaction needs more time than is required for comparable quantities of starch (12). Cellulose microfibrils may have amorphous regions where no crystalline structure exists, because it was not formed during synthesis or it has been disrupted (12). How this amorphous cellulose arises is not yet known (12).

The modularity of lignocellulose-degrading enzymes is a distinct structural feature; it equips them with vast versatility. Many of these enzymes have, in addition to the catalytic core, a noncatalytic, but functional and important, domain. It can include carbohydrate-binding modules, immunoglobulin-like domains, or functionally unknown “X” domains. These

allow for anchoring, or direct the enzymes containing them to targeted carbohydrate substrates (40).

Hydrolytic reactions, mainly acting on hemicellulose, and oxidoreduction reactions, mainly acting on lignin, are employed by many lignocellulose-degrading enzymes to convert lignocellulose (40).

Known biodegraders

Many studies have been made of biomass-active enzymes and many of them appear promising for industrial use (40), but fungal plant cell wall-degrading enzymes are not yet fully understood (42). Here some of the known biodegraders will be discussed.

Fungi have been used in pretreatment experiments with beech wood chips; white rot fungi such as *Ceriporiopsis subvermispora*, *Dichomitus squalens*, *Pleurotus ostreatus* and *Coriolus versicolor* have been applied prior to ethanol production with *S. cerevisiae* and *Trichoderma viride* (43). *Trichoderma reesei* is known to be the most efficient producer of cellulases and hemi-cellulases for industrial use, but for unknown reasons it has fewer genes encoding cellulolytic enzymes in comparison to related Ascomycetes (42). A promising fungus for the processing of biomass is *Phanerochaete chrysosporium*, with its physico-chemical ability to break down lignin recalcitrant material and at the same time liberate hemicellulose and cellulose (44). Other promising microorganisms include *Ceriporiopsis subvermispora*, *Phlebia subserialis* and *Pleurotus ostreatus*, which are able to metabolize lignin from different lignocellulosics (45). Studies have been made with the ligninolytic enzymes laccase, lignin peroxidase, manganese peroxidase and versatile peroxidase, enzymes that enhance the efficiency of applications in the biorefinery industry (45).

Biocatalysts participating in sugar metabolism and ethanol fermentation

Biological methods where microorganisms such as fungi are used are of great interest. However, the most commonly used fungus when it comes to ethanol production is *Saccharomyces cerevisiae*, and this fungus can only ferment mono- and disaccharides (such as glucose, fructose, maltose and sucrose) into ethanol. *S. cerevisiae* cannot naturally convert pentoses into ethanol, and pentoses are one of the major components of lignocellulosic material (46). When using microorganisms, biocatalysts that can convert both hexoses and pentoses into ethanol would be more efficient. There are several microorganisms (fungi) that have been reported to be able to convert hexoses and pentoses in lignocellulosic biomass into ethanol, e.g. *Phanerochaete chrysosporium, Pichia stipitis, Candida shehatae, Mucor indicus* and *Pachysolen tannophilus* (46,47,48). But their rate of conversion

is low, lower than the rate of glucose conversion when *S. cerevisiae* is used (46).

There are a number of important enzymes involved in the conversion of pentoses into ethanol. The pentose sugars arabinose and xylose are converted to xylulose-5-phosphate before entering central carbon catabolism. Xylose is first reduced by xylose reductase (XR) to xylitol, which is then oxidized to xylulose by xylitol dehydrogenase (XDH). Finally xylulose is phosphorylated to xylulose-5-phosphate by xylulokinase (XK) (49). As a consequence of the decarboxylation of pyruvate, which yields acetaldehyde, and subsequent catalysis by alcohol dehydrogenase (ADH), ethanol is produced (50).

Plants generally have the ability to metabolize both aerobically and anaerobically, together with adaptive regulatory mechanisms, but some possess only the aerobic pathway (51). However, microorganisms only ferment substrates anaerobically, while oxygen is required for growth by xylose-fermenting microorganisms irrespective of the carbon source employed; the requirement for oxygen may indicate that this substrate does not give rise to sufficient ATP (7). As a result ethanol is used as a carbon source for the formation of cell mass, CO_2 and acetate, and this occurs even if there is a substantial concentration of xylose remaining (51).

Inhibitors and their effects

Inhibitors, which are released during hydrolysis and pretreatments, can be divided into four major groups: furan derivatives (furfural and 5-hydroxymethylfurfural, 5-HMF), phenolic compounds, weak organic acids (levulinic, formic and acetic acid) and heavy metal ions (e.g. nickel and aluminum) (33,51).

The microorganism's physiology is affected by inhibitors: its viability decreases, the yield of metabolites becomes lower and the productivity of biofuel is also diminished (51).

When lignocellulosic material is hydrolysed and the sugars are degraded, xylose is converted into furfural, which can further degrade into formic acid (52). Degradation of mannose, galactose and glucose forms 5-hydroxymethylfurfural (HMF) and when HMF degrades it too can form formic acid and also levulinic acid. (52). During partial degradation of lignin, phenolic compounds are formed and they can also be produced during the degradation of carbohydrates (52). The inhibitors differ depending on the type of material that is being hydrolysed.

It has been shown that when hemicellulosic hydrolysates are pretreated with the enzymes peroxidase and laccase obtained from *T. versicolor* there is a threefold increase in productivity (53). Treatment with laccase removes phenolic monomers and phenolic acid (52). Laccase from *Trichoderma reesei* was shown to increase ethanol production even more, but the detoxification mechanisms were different in this case and removed acetic acid, benzoic acid and furfural derivatives (52).

Physiology of *Trametes versicolor*

Trametes versicolor (also known as Turkey Tail) belongs to the class of Agaricomycetes and to the Basidiomycota division, the order Polyporales and the family Polyporaceae (54). The fungus grows on tree trunks all over the world and in different climates (54). Thus *T. versicolor* is a white rot fungus, and it secretes enzymes that take part in the transformation of aromatic compounds, such as phenol oxidase, laccase and peroxidase (55, 56). In addtion, this fungus is able to degrade cellulose, hemicellulose and lignin simultaneously (57). It is one of the fungi that produce extracellular ligninolytic enzymes, which are nonspecific and efficient, especially lignin peroxidases (LiPs), manganese peroxidases (MnPs) and laccase (Lac) and these enzymes have high levels of activity towards secondary metabolites under optimum growth conditions (55). MnP and Lac are produced by almost all white rot fungi but LiP is only produced by some of them (45).

Laccase from *T. versicolor* has been shown to make ethanol fermentation occur faster when using *S. cerevisae* as a biocatalyst *(57)*. The consumption of glucose was found to be more rapid and the ethanol productivity increased when samples were treated with laccase (55). *T. versicolor* is a fungus with potential for treating soil, air and water pollution as well as for production of bioethanol and for bioremediation (55).

Objectives

From the broad perspective, this thesis focuses on ecological and physiological engineering, i.e. using nature's own solutions for biodegradation and biocatalysis; in detail the focus is on physiological aspects of ethanol fermentation by the biocatalyst *T. versicolor*. Studies on ethanol production, enzyme activities, and cellulose and inhibitor degradation were performed to shed light in this subject.

The starting point of this work was a patent describing a fungal mix that could increase ethanol fermentation (1). When this work began the unicellular fungus *S. cerevisiae* was the main biocatalyst used. It was known that lignocellulose could be used for this purpose, but that several inhibitors were formed during the process. It was also known that through a series of enzymatic reactions, fungi could degrade cellulose, hemicellulose and lignin and produce ethanol but at a low rate.

The goals for this thesis were to find fungi that naturally utilize 5-carbon sugars and metabolize these sugars to ethanol. A further aim was to investigate the capacity of *T. versicolor* to utilize 5-carbon and 6-carbon sugars simultaneously, which is very important from an applied perspective. It was also intended to analyze the ability of *T. versicolor* to metabolize inhibitors produced when lignocellulose is used. Finally, the goal was to see whether *T. versicolor* can be used as a pretreatment for lignocellulose instead of industrially manufactured enzymes.

Material and methods

Identification and isolation

Samples from field-collected partially decomposed pieces of wood were put on agar plates with the carbon source xylose (25 g l-1). After 3 days, outgrowths of hyphae were observed, and these were repeatedly transferred onto new plates as well as being transferred into liquid culture in order to measure growth with a variety of carbon sources.

To identify the fungi present, DNA was extracted and primers designed (58, 59) for use in PCR reactions. The sequences obtained were shown to correspond to *C. parvispora*, *T. hirsuta*, *T. versicolor* and *Xylaria sp*.

In addition, *T. versicolor* was ordered from the CBS culture collection (The Netherlands), and grown in BMC medium (48) complemented with 27 g/l mannose, 11 g/l xylose and 9.7 glucose, to mimic the sugar composition of spent sulfite liquid (Domsjö Fabriker AB, Örnsköldsvik, Sweden). For each sugar experiment in paper II, fungal cells were transferred from the stock culture to a medium with only glycerol (1.2 g/l) as carbon source for 7 days, in order to equalize the initial enzyme activities.

Ethanol production

Fermentation of ethanol by *S. cerevisiae* with and without the fungal mix was carried out using yeast at 0.8 g l-1 and fungi at 2 g l-1. Amounts of ethanol and carbohydrates were evaluated. Spent sulfite liquor was obtained from Domsjö Fabriker AB, Örnsköldsvik, Sweden) and analyzed at MoRe Research Lab (MoRe AB, Örnsköldsvik, Sweden). Three g/l of stock fungal culture was tested in different growth media (paper III) under hypoxic and anoxic conditions by flushing argon through an inlet needle for 5 min, with a second needle used as an outlet to get rid of excess pressure. The compositions of the media varied from 0-11 g/L of glucose, 0-27 g/L of mannose and 11-50 g/L of xylose.

Samples were randomly collected from 0 to 354 h of cultivation to determine contents of xylose, mannose and glucose. The neutral sugars were analyzed with myo-inositol as an internal standard. Before analysis the neutral sugars were converted to alditol acetates and they were then analyzed using a GC system equipped with a flame ionization detector (60).

Enzyme assays and protein determination

Fungal hyphae grown in media containing different sugar combinations, as described in Paper II, were used to determine their XR (xylose reductase),

XDH (xylose dehydrogenase), XK (xylose kinase) and ADH (alcohol dehydrogenase) activities after up to 354 h of incubation. Crude protein was extracted from homogenized tissue and enzyme activities of the resulting supernatants were determined in 96 well plates by spectrophotometry. Protein concentrations of extracts of fungal hyphae were determined according to (61).

Analysis of inhibitors

In order to test the fungus for its capacity to decompose inhibitors, different concentrations of phenol, HMF, furfural and levulinic acid were added during the growth of hyphae, either separately or all together, to evaluate the effect of the combination (Paper III). An HPLC equipped with an Aminex HPX-7H column with guard column was used to analyze the levulinic acid, HMF and furfural contents. A different column, Reprosil-Pur C18-AQ, and a UV-detector were used to analyze the phenolics at the MoRe Research Lab (MoRe AB, Örnsköldsvik, Sweden).

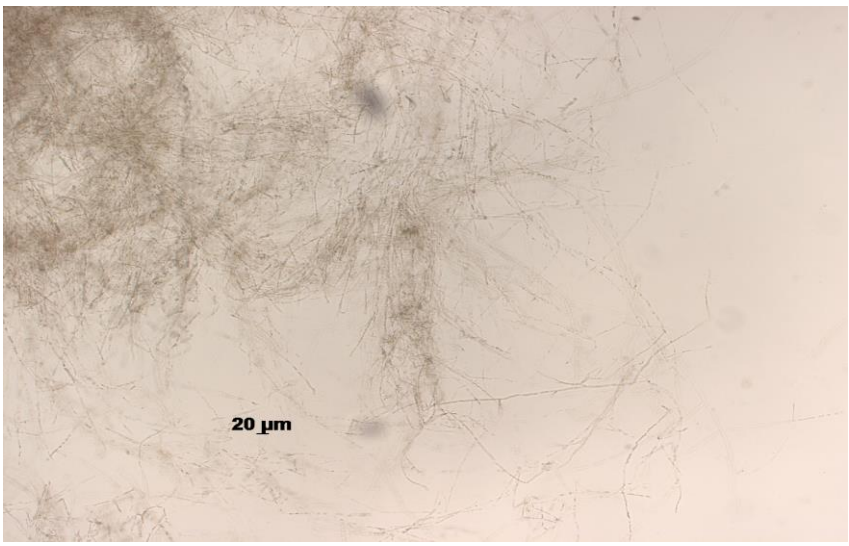
To measure ALDH and PDC activity, the reduction of NAD in ALDH was monitored at 340 nm for 2.5 min of incubation at 30°C. For PDC, it was firstly reduced to ADH, after which coupled NADH oxidation was monitored as described earlier.

Laccase, manganese-1 peroxidase (MN1P), phenol oxidase and peroxidase were monitored at various spectral points (Paper III) in order to record lignin degradation and determine whether the fungus is able to biodegrade lignocellulose.

Pretreatments and analysis

In Paper IV, the methods developed in Paper I-III, as well as additional methods, were used to determine the role of *T. versicolor* as a biodegrader of lignocellulosic material.

Dried samples of *Salix viminalis* and *Populus tremula* were biologically pretreated with *T. versicolor* and compared to the control pretreatment (only sterile water), the enzymatic pretreatment (β-glucosidase, xylanase and cellulase), the chemical pretreatment (1 % sulphuric acid) and different combinations of these pretreatments.



Micrograph of *T. versicolor* isolated in this thesis. Picture with courtesy of PhD student J. C. Nzaysenga, UPSC, Dept. of Plant physiology, Umeå university, Umeå. (Zeiss Axioplan 2 imaging microscope with a camera and axiovision software. Image taken at 10x with bright field imaging)

Analysis of sugars was performed in-house (Cell Wall Lab, UPSC, Umeå, Sweden). The method used involved reduction to alditols and detection was carried out by gas chromatography as described in Paper II.

Analysis of ethanol fermentation by *T. versicolor* was measured as described in Paper I, and calculations of the theoretical amounts of ethanol from hexose and pentose according to the experiments in Paper IV were performed as follows:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2C$$

Ethanol production = 0,51 g ethanol/g glucose

$$3C_5H_{10}O_5 \rightarrow 5C_2H_5OH + 5C$$

Ethanol production = 0,51 g ethanol/g xylose

PCR

DNA was extracted from *T. versicolor* hyphal cells and as described in PaperIV and designed primers was used as also described in PaperIV.

ClustalW analysis

Alignments of protein sequences from five microorganisms, *T. orientale*, *T. reesei*, *T. versicolor*, *A. niger* and *C. cinerea*, were analyzed using ClustalW algorithms. These algorithms employ a progressive alignment technique that aligns pairs of sequences, starting from the most similar sequences and continuing until all sequences contribute to the alignment. The proteins that were analyzed were the beta-glycosidase, beta-xylosidase and xylanase proteins. The phylogenetic trees were designed and exported using Trex-online software, http://www.trex.uqam.ca**.**

Results and discussion

Isolation and identification of rot fungi for ethanol production from lignocellulose

An interesting question that triggered the work in this thesis is whether there are naturally occurring fungi that can utilize 5-carbon sugars. It is a drawback that even now, few fungi are known to use 5-carbon sugars for growth and/or for ethanol production from lignocellulose. We wanted to elucidate the question of whether there are biochemical and/or physiological ways of utilizing biotechnology to solve this scientific problem. We therefore collected wood pieces from the field (Paper I), put them on agar plates with 5-carbon sugars and isolated hyphae growing out of the wood, to obtain the so called fungal mix, which did grow on the 5-carbon sugars, though only after a 36 h lag phase. The fungal mix was also successfully grown on xylan and avicel (an industrially purified cellulose). Identification of fungi was performed using ITS primers in PCR reactions, and three fungi were identified as *C. parvispora* (100 % similarity*), Xylaria sp*. (73 % similarity) and *T. hirsuta*/*T. versicolor* (95 % similarity). *Xylaria sp*. did not produce ethanol and was therefore not used further. The finding that fungi can grow on xylose is of the utmost importance if lignocellulosic materials are to be used as feedstock, since 5-carbon sugars are a major component of this material (62), so our results are of great potential and significance. Interestingly, it had already been shown in 1953 by Beckman et al. that *Chalara sp*. preferred xylose for growth, rather than glucose, and this is in accordance with our findings (63). Recently, another fungus, *Fusarium*

oxysporum, has been shown to grow on xylose. The use of genetically modified microorganisms for utilizing 5-carbon sugars has been discussed extensively, and in 2003 Pretorius et al. (64) focused on the need for modification of *Saccharomyces cerevisiae* for use in ethanol production in order to achieve more efficient use of pentoses in wood as well in other hemicellulosic materials. Genetically modified fungi of different species, e.g. *S. cerevisiae*, *Zygosaccharomyces* sp., *Pichia stipites* and *Zymomonas mobilis*, have been demonstrated to utilize 5-carbon sugars (e.g. 65; 66; 67; 68). Öhgren *et al.* 2006; Sedlak and Ho 2004; and Suez *et al.* 2005 (48, 69, 70) are some of the researchers who have described the utilization of glucose and xylose in different combinations, showing that only recombinant strains utilize xylose to a great extent. However, it is a common occurrence that undesired by-products decrease ethanol production (67, 71). In 2005, Zaldivar *et al.* (72) showed that ethanol yield from a xylose-fermenting *S. cerevisiae* was low due to production of the by-products xylitol and glycerol. A genetically modified *S. cerevisiae* strain containing three genes coding for xylose-metabolizing enzymes was reported to have 72.6 % efficiency in ethanol fermentation from a hydrolysate with 6.9 g/L glucose and 26.9 g/L xylose (70). Toivola et al. (1984) and Skory *et al.* (1997) showed that *Brettanomyces naardenensis*, *Candida sp.* and Rhizopus *sp.* were able to produced ethanol from xylose (73, 74). However, no previous study has shown the fermentation of xylose-containing substrates to ethanol by *T. versicolor*.

Ethanol production in spent sulfite liquor increased three-fold when the fungal mix was added, showing that ethanol production from lignocellulose waste can be increased by using additional fungi (Paper I). Neither *C. parvispora* nor *T. versicolor*, two of the fungi in the fungal mixture, have been previously reported to produce ethanol. However, *T. versicolor* has been reported to be active in decomposition of inhibitory substances during ethanol production (53). A higher level of ethanol production was clearly shown in a reconstitution experiment in which both fungi were used together with *S. cerevisiae*, and it was also shown that the fungi can produce ethanol themselves, as much as 14.00 ±1.61 g l-1, in spent sulfite liquor. Interestingly, Sues et al. (2005) published results on the naturally occurring fungus *Mucor indicus*, which could produce ethanol at high rates (48).

Twenty-four hours before the start of experimentation the fungal mix was pre-cultivated in spent sulfite liquor, an indication that the mixture is adaptable. Utilizing additional fungi for ethanol fermentation has been reported previously by Worall *et al.* (1997) and more recently, e.g. by Paschos *et al.* (2015), who used the fungus *Fusarium oxysporum* in

combination with *S. cerevisiae* and showed that fermentation from pretreated wheat increased by 11 % (75,76).

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Characterization of enzymes in *Trametes versicolor* involved in 5- and 6-carbon sugars

The aim of this study was to further characterize the ability of one fungus from the fungal mix, namely *T. versicolor*, to produce ethanol from mixtures of glucose and xylose. We therefore examined the activities of relevant enzymes, such as alcohol dehydrogenase (ADH), xylose reductase (XR), xylose dehydrogenase (XDH) and xylose kinase (XK). The isolation and identification of *T. versicolor* from field-collected decomposing wood samples was presented in Paper I and the identity of the fungus was confirmed by using ITS1-F and ITS4 primers in the study presented in Paper II. The PCR fragments obtained were shown to have up to 99 % sequence identity with *T. versicolor* (FJ810146.1). In the experiments in this study, this white rot fungus grew exponentially, in different combinations of sugars. However, under anoxic conditions *T. versicolor* did not grow in any of the media during the 354 h of incubation. It has been shown in a previous study, in which liquid culture bottle headspaces of *T. versicolor* cultures were used, that a daily flush giving 95 % replacement of the atmospheric gases was needed to promote growth (77), and in a study by Skoog and Hahn-Hägerdahl (1990) it was shown that oxygenation was necessary for xylose utilization by the fungus *P. stipites* (78). It has also been shown that, for another fungus (*P. tannophilus*), after periodic additions of glucose the fungal growth and the fermentation of xylose to ethanol improved, but there was no improvement in xylose utilization under anaerobic conditions (79).

A typical pattern of growth was shown in both media; throughout the 354 h of hypoxic conditions the total biomass was significantly higher in MBMC

(containing a variety of sugars) than in XY11 (containing only xylose) (Fig. 1b, Paper II). An explanation of why *T. versicolor* thrives in a range of ecosystems is that it has the ability to grow fast in different carbohydrate sources which include 6-carbon sugars (glucose and mannose) and 5-carbon sugars (xylose), and, like most *Trametes* species, it produces generative filamentous hyphae (Fig. 1a, Paper II) (80,81)). This offers advantages for industrial bio-refineries, such as easy handling, stability, versatility and fast multiplication of the fungi (82). It is not only the bio-fuel generation industry which has an interest in the use of such fungi; there are also possible applications in the biosensor, synthetic chemistry, pulp and paper, food, effluent treatment, bioremediation, textile and cosmetic industries.

T. versicolor was cultivated hypoxically and anoxically in different combinations of glucose and xylose, ranging from 11 to 0 g/L of glucose and 50 to 0 g/L of xylose (Table 1, Paper II). No biomass production was detected under anoxic conditions. Under hypoxia there was a slight difference in the production of biomass after 354 h between the three different media (shown in Fig. 3a). Earlier findings (83) showing that efficient aeration of *Rhizopus oryzae* liquid cultures is important for the rapid assimilation of hexoses and pentoses are supported by our results.

Earlier studies have shown that xylose-fermenting yeast, genetically engineered strains could exhibit either the promotion or the inhibition of xylose (84) when glucose is utilized in mixed sugar fermentations, while the rate of glucose fermentation in *P. tannophilus* cultures doubled during aeration (79). To examine the ability of *T. versicolor* to utilize sugars in mixtures of hexoses and xylose during hypoxic conditions, the uptake of sugars at 27°C was analysed for up to 354 h of growth. It was shown that *T. versicolor* could simultaneously use mannose, glucose and xylose under hypoxic conditions during this time. Compared with other microorganisms that use hexoses at higher rates than pentoses during the early growth stages, *T. versicolor* has unique physiological characteristics (67,85). *T. versicolor* consumed mannose, glucose and xylose simultaneously during the experiment; 32 %, 44 %, and 36 % respectively were utilized within 66 h. This study indicates that *T. versicolor* can use effectively use xylose as a source of carbohydrate, and 90% of the xylose was utilized after 354 h.

In this study *T. versicolor* showed a preference for glucose among the hexoses, though with concomitant consumption of xylose, for the first 66 h of cultivation. In the latter growth period around half of the mannose, glucose and xylose were utilized. Analysis of sugars indicated that there was no hexose co-substrate inhibition of xylose use like that shown in a previous report by Govindaswamy and Vane (2007) concerning genetically

engineered xylose-fermenting *S. cerevisiae* (84). This bakers' yeast has non-specific hexose transporters that take up xylose by diffusion, and xylose has a lower affinity than glucose (86). This explains why in *S. cerevisiae* transport of xylose will be inhibited by glucose and why xylose will only be consumed after the glucose is depleted (86). We therefore conclude that the results shown in Paper II strongly indicate that both hexoses and pentoses are taken up simultaneously by the same transporter system. Furthermore, *T. versicolor* also continuously supplies reactants for efficient glycolysis and the same time to facilitate the efficient operation of the pentose phosphate pathway.

We further showed that over the same period of time the activities of the enzymes were correlated, indicating that this fungus catabolizes xylose efficiently without xylose transport being subject to any inhibition by hexoses. When mixtures of glucose/xylose media were tested there was no significant difference in activity, which confirmed that xylose was being utilized. Under oxygen limitation, activation of ethanol fermentation is required so that NAD+ can be recycled for maintenance of glycolysis. The activities of ADH and PDC are important in maintaining the efficiency of fermentation. ADH was analyzed using mycelia grown in the different media used for the experiments and the activity was found to increase continuously during the time period and to be correlated with ethanol production. There were differences with respect to ethanol production in the different media but, interestingly, not when it came to the ADH activity; the activity and trends were similar throughout the whole 354 h. After 66 h there was a rapid increase in ADH activity in all three mixtures of glucose and xylose, and this increase was correlated with ethanol production, biomass production and the activities of XR, XDH and XK. This proves that during the production of ethanol, as well as during active growth, *T. versicolor* expresses xylose catabolic pathways making it possible for it to use both hexoses and xylose.

The ability to produce ethanol was tested and the maximum amount was found to be 20.0 g/l during hypoxic conditions after 354 hours. To be able to use *T. versicolor* in industry, the rate of fermentation needs to be increased and this could be achieved by increasing the initial amount of inoculum and through optimization of biotic factors such as establishing the exact optimal growth phase of the fungi.

Physiological adaptability of T. *versicolor* to the lignocellulosic inhibitors furfural, HMF, phenols and levulinic acid

During the pretreatment of lignocellulosic materials there is always production of different compounds that are often referred to as inhibitors (87). The aim of the work in Paper III was to elucidate the capacity of *T. versicolor* to metabolize such inhibitors. We decided to use the most commonly occurring inhibitors: levulinic acid, phenol, HMF (hydroxyl methyl furfural) and furfural.

When HMF degrades, levulinic acid can be formed; this is a weak acid that can uncouple energy conservation by passing through cell membranes in an un-dissociated form, binding protons in the cellular environment and impairing the proton motive force across the membranes (87,88,89). Via ATP hydrolysis, protons can be pumped and the cells can thus counteract this effect so that demand for more ATP can be met up to a certain level. After that, however, the ethanol production falls.

This study showed that the uptake of mannose and xylose were affected by levulinic acid, possibly due to the associated reductions in ATP availability. Degradation of mannose, galactose and glucose forms HMF. *S. cerevisiae* is known to be able to metabolize HMF (90,91), but the compound prolongs the lag-phase of fermentation since it converts more slowly than furfural (90,92). The growth of *S. cerevisiae* is however, reported to be completely inhibited when both furfural and HMF are present (91). Interestingly, the biocatalyst yeast can grow in the presence of inhibitors, and degrade them, but this requires large amount of inoculum, since the initial cells may be killed, if ethanol production is to continue (93).

Phenolic compounds are generated from decomposing lignin and degrading carbohydrates (87,94,95). Previous reports have shown that numerous phenolics impair the fermentation of lignocellulosic material and generally the most toxic phenolics are those with the lowest molecular weights (52). By partitioning into biological membranes and changing the protein-to-lipid ratios, they affect the ability of the membranes to serve as selective barriers and enzyme matrices (96). Synergistic effects on bacteria and fungi when acids and furan aldehydes or different phenolic compounds are combined have also been shown (87,96,97,98). The fungus *Coniochaeta ligniaria* was found to be able to eliminate furfural and 5-HMF when using hydrolysates from corn stover (99). It was shown that in presence of all inhibitors, combined or singly, the fresh weight of the fungus increased significantly. However, it was shown that while after two days the fresh

weight was highest in samples cultivated without the inhibitors, it was lowest in these samples after 15 days.

In our study, ethanol fermentation decreased 29-41 percent in response to treatment with the inhibitors, singly or together. The enzymes of ethanol fermentation, ADH and PDC, were also affected. The highest activity of ADH was shown when all the inhibitors were present. The activity of ADH rose early in the presence of the inhibitors and the highest activity was shown in the presence of HMF and levulinic acid. The activity of PDC was similar irrespective of whether or not phenol was present (Fig 2). In the presence of the other inhibitors, PDC activity was lower. PDC and ADH showed no severe reduction in activity in the presence of the inhibitors. The activity of ALDH showed a sharp increase with a resulting reduction in ethanol production in favour of converting acetaldehyde into acetate. The associated metabolic switch is unknown, and this requires further investigation.

The rates of glucose consumption as well as ethanol fermentation were increased by treating samples of wood hydrolysates with laccase obtained from *T. versicolor* (53). In our study, the activity of laccase was highest when all the inhibitors were present during day 2 and 5. The same was the case for peroxidase and phenol oxidase, and the highest activity for Mn1 peroxidase was found in the treatment with phenol at day 2. All the inhibitors tested in our study were digested by *T. versicolor*, which is known for its ability to degrade lignin (99) by secreting peroxidase and laccase and oxidizing phenols into radicals that form larger molecules which are less toxic to fermenting microbes (53).

It can be concluded that *T. versicolor* is able to grow in the presence of inhibitors after a short lag phase. Thus this fungus could be used as a biodegrader of inhibitors.

Trametes versicolor involved in biodegradation and biocatalysis in ethanol prodcution - manuspript

How does the white rot fungus *T. versicolor* compare to enzymes used in the industry? This was a question that we wanted to explore further in the final study in this thesis. Is *T. versicolor* acting both as a biodegrader and biocatalyst simultaneously, thereby being of both economic and environmental interest?

Both *Salix viminalis* and *Populus tremula* were pretreated in five different ways and incubated for 48-96 hours on a thermoshaker. The results of sugar analysis showed variation in the amounts of monosugars. Glucose was the main sugar, followed by mannose, galactose and xylose. The pretreatment with dilute acid showed the highest amounts of sugars (1659 µg/ml glucose) followed by the enzymatic pretreatment. The enzymatic pretreatment gained between 784 and 1024 ug/ml of glucose. Using 10 g/L of *T. versicolor* as a biodegrading agent increased the amounts of monosugars for *Populus spp*. as plant residue but not for *S. viminalis*, resulting in app. 633 µg/ml glucose. Pretreatment with dilute acid gave in total approximately 3400 ug/mL of sugars for *Salix spp*. and 4279 ug/mL for *Populus spp*, while biodegradation resulted in app. 800 ug/mL.

Filamentous fungi commonly produce a multiplicity of beta-xylosidases, which exhibit particular specificities that lead to the degradation of xylan. Another enzyme, beta-glucosidase, which is known to decompose cellulose, is also produced by filamentous fungi. A previous study done by Skyba *et al.* (100) showed that the enzymatic system of *T. versicolor* depolymerizes lignin and influences on breaking down lignocellulose; our results are in accordance with this. In Kudahettige *et al.* 2016 (Paper III) we found high laccase activity in *T. versicolor*, and this was also observed by Asiegbu *et al.* 1996 and Zhu *et al.* 2011, in studies showing that white-rot fungi used as a pretreatment of biomass lead to the degradation of lignin since the fungi produce lignin peroxidases and laccase which are lignocellulolytic enzymes (101,102,103).

A ClustalW analysis was performed for the enzymes beta-glucosidase, xylanase and beta-xylosidase (104). Beta-glucosidase is a cellulase enzyme and xylanase and beta-xylosidase belong to the hemicellulase family. Using primers designed for *Trichoderma reesei* genes, the expression of beta-xylosidase and xylanase in *T. versicolor* was studied. The analysis showed that *T. versicolor* xylanase belongs to the same protein cluster as that of *T. orientale*, with a similarity of 40 %. When it came to the beta-xylosidase protein, *T. versicolor* showed almost 60 % similarity to the cluster

containing that of *C. cinerea*. The beta-glucosidase sequence had a similarity of almost 50 % to the *A. niger* cluster (104,105,106). The clustering is shown in Paper IV.

Pretreatment with dilute acid gave the highest amount of sugars as well as the highest amount of ethanol, approximately 11 g/L utilizied sugars from *Populus spp*. and up to 17 g/L for sugars utilized from *Salix spp*. Where only *T. versicolor* were used as pretreatment the ethanol yield was low, and there was also a low yield following enzymatic pretreatment. Irrespective of which pretreatment was used, *Salix spp*. hydrolysates gave higher amounts of ethanol.

Skyba *et al.* 2013 (100) showed that after pretreatments with *T. versicolor,* glucose amounted to almost 53 % of the total amount of monosugars, which is consistent with our findings. One of the best ways of to remove lignin in, for example, birch and spruce wood is by using *T. versicolor*, according to a study made by Kuijk *et al.* 2015 (36). In our study we saw an increase in monosugars due to lignocellulosic degradation.

Different amounts of fresh *T. versicolor* cells were used in the pretreatment, and the lower amounts of cells generated more sugars. This might be because when the amount of cells increases there is competition among the different reactions, such as decomposition of the lignocellulosic matrix, ethanol production and the use of carbohydrates for creation of new cells. In theory, using a larger amount of cells could result in greater ethanol production because only some cells would be affected by inhibitors created during the degradation process, meaning that others would be free to produce ethanol. Our results could not confirm or disprove this hypothesis. *T. versicolor* has the ability to consume all the inhibitors formed during the degradation process, as shown by Kudahettige *et al.* 2016 (101).

Our study has shown that the white rot fungus *T. versicolor* has the ability to decompose lignocellulose through the use of its enzymes cellulase and xylanase. *T. versicolor* has the potential to replace environmentally unfriendly methods even though in this study the yields of ethanol were low. More research needs to be done in order to evaluate and optimize this process, and elucidating the conditions under which *T. versicolor* and/or other white rot, brown rot or soft rot fungi are optimal could result in an increase in the degradation of polysaccharides and lignin generating more sugars and ethanol. *T. versicolor* has the ability to combine pretreatment with fermentation and thus make the ethanol fermentation process more efficient.

Conclusions

This thesis shows that we found fungi that can utilize 5-carbon sugars and metabolize these sugars into ethanol. Our results are of great potential significance, since 5-carbon sugars are a large component of lignocellulosic materials (62). We also showed that when the fungal mix was added to spent sulfite liquor together with *S. cerevisiae*, the yield of ethanol increased three-fold.

We were able to conclude that *T. versicolor*, using the same transporter system that transports hexose, takes up both hexoses and pentoses simultaneously. Moreover, *T. versicolor* supplies reactants continuously for efficient glycolysis, and at the same time facilitates the operation of the pentose phosphate pathway, showing that this fungus expresses xylose catabolic pathways when utilizing hexoses and xylose.

After a short lag phase, *T. versicolor* was able to grow and produce fresh cells in the presence of inhibitors, indicating that the fungus can be used as a biodegrader of inhibitors.

T. versicolor showed ability to decompose lignocellulose resulting in increase in glucose molecules, thus *T. versicolor* has the potential to replace environmentally unfriendly methods and also the ability to combine pretreatment with fermentation, thus making the ethanol fermentation process more efficient.

Future perspective

The development of ethanol as being a second-generation biofuel is greatly needed. Development of biological methods for pretreatment of lignocellulose is of high interest and this thesis is a starting point for this. Furthermore, biological methods for biodegradation of inhibitors formed during handling of lignocellulose are of interest not only scientifically but also industrially to decrease costs for producing bioethanol. Last but not least there is a need for upscaling of the processes.

In detail, biodegrading enzyme activities need to be further investigated to gain a complete understanding of how the different processes operate during biological degradation.

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