Application of *Abies grandis* Wood for Technical Use in Biofuel Production

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Abstract

This study focused on effects chemical and physico-chemical pretreatments of *Abies grandis* wood on enzymatic hydrolysis of cellulose into glucose in view of potential applications in biofuel production. Wood particles were treated with phosphoric acid, incubated in a hot water bath at 50°C or they were submitted to microwave irradiation. The highest glucose yields of up to 100% of converted cellulose were reached by using 1 g of wood particles in 8 ml of 80% phosphoric acid irradiated in a microwave for 20 second and applying a combination of 4 U ml^{-1} cellulase, 4 U ml^{-1} xylanase and 4 U ml^{-1} laccase in 5 ml of 50 mM sodium acetate buffer, pH 5.0 for cellulose hydrolysis for 24 hrs. Evidence for the latter was given in studies where glucose yields from cellulosic pellets obtained from physico-chemical pretreatments were dramatically increased when laccase was added to the samples prepared for cellulose hydrolysis: generally, the combination of 4 U ml^{-1} cellulase, 4 U ml^{-1} xylanase and 4 U ml^{-1} laccase was more effective in increasing glucose yields than 4 U ml^{-1} cellulase and 4 U ml^{-1} xylanase alone. Likely, laccase has a potential to decrease inhibitory effects of phenolic compounds present wood extractives on enzymatic hydrolysis. Evidence for this was further given by experiments where wood extractives or specific phenolic compounds as found in wood extractives were added to enzymatic reactions. Water extractives, and *p*-coumaric acid, vanillic acid and ferulic acid in concentration-dependent manner all inhibited the actions of cellulase on carboxymethylcellulose (CMC). Addition of laccases in most instances could partially or fully reverse the inhibitory effects but in the case of ferulic acid. This compound in presence of laccase or its products obtained by laccase-mediated oxidation partially blocks cellulase activity, possibly by chemical interactions (dimerization of cysteines, cross-linking of tyrosines) on the cysteine and tyrosines-rich cellulose-binding-motifs (CBMs) present in distinct cellulases at the outmost C-terminal ends of the enzymes.

***Index Terms*—Biofuel, Chemical pretreatment, Physico-chemical pretreatment, Microwave irradiation, Rate of production of glucose, Kinetic curve of production of glucose, influence of wood extractives, influence of phenolic compound**

INTRODUCTION

Nowadays, much research has been performed and more and more research concentrates on transforming lignocellulose based on enzymatic hydrolysis to produce bioethanol. Most efforts on the first generation of biofuels have been focused on using biological resources that are also used as food and fodder (such as sugarcane, cereals, soybeans, seed from oil plants, animal fat) and this development was induced by the various kinds of the world's demand and supply of energy consumption. However, the first generation biofuel competes directly with nourishing humans and animals. Therefore, these resources are limited in available amounts. According to this problematic [1, 2], the new (second) generation of biofuels is targeted to use non-food and non-fodder materials as substrates in enzymatic hydrolysis [3, 4, 5]. Straw and other agricultural residues as well as certain fast growing grasses (e.g. *Miscanthus*) are established biomaterials for second generation biofuels´ production [6]. Much work has now also been carried out on using e.g. hardwood trees from forests and plantations, such as on wood of poplars and olive trees, respectively [7, 8, 9]. Less work has been invested into using softwood in biofuel production [10].

A. grandis wood is especially attractive because *A. grandis* is a fast growing coniferous tree with a high potential for sustainable wood production and also interesting applications in the wood products industries [11, 12, 13, 14]. Moreover, as shown by the research in this work, the renewable wood biomass from *A. grandis* is also a potential source for enzymatic treatment technologies to unlock the energy stored in its lignocellulose for transferring it into biofuels. In this work, the *A. grandis* softwood has been explored using different pretreatment methods and different combinations of hydrolytic and oxidizing enzymes (cellulase, xylanase, β-glucosidase, laccase) in wood hydrolysis. The different pretreatments resulted in the production of fermentable sugars from the wood cellulose to possibly be used as a bio-base for fermentation processes of bioethanol with e.g. suitable yeast [15, 16]. In the near future, process integration and optimization to transform the whole wood of trees such as from *A. grandis* into value-added and biochemical products, such as ethanol, but also butanol and other bio-based chemicals such as lactic acid, succinic acid etc. could become promising attempts to fulfill the world's energy consumption [17, 18].

The research presented here addresses new enzymatic technologies as required if biofuels by environmentally friendly production are to significantly contribute to planetary energy needs and, with it, to the reduction of greenhouse gas emission [19]. Next to other developing technologies such as creating better yeast strains for sugar conversion [16, 20], efficient hydrolysis protocols for wood have to be established in finally enabling the

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commercial viability of lignocellulosic ethanol [3, 4, 17]. The purpose for this is on the development of approaches and technologies with a significant promise in reducing production cost and in caring for environmentally friendly procedures in order to make lignocellulosic ethanol economically competitive with the first generation biofuel production. This knowledge is thus urgently needed and also knowledge from timely research in how to integrate enzymatic hydrolysis of material such as wood into already existing basic fermentation processes in order to adjust the available biotechnology for biofuel production to the specific challenges meet when transforming cellulose from wood e.g. into ethanol [2, 3, 4].

Material and Method

Wood

Wood particles of *A. grandis* were prepared in cooperation with the work group of Prof. Kharazipour from the Division of Molecular Wood Biology and Technology and Technical Mycology, Büsgen-Institute, Göttingen, Germany, by chipping wood log into wood shaving (length about 1 cm, width about 1 cm and height about 2 cm) in a drum chipper (Klöckner Trommelhacker KTH 120 x 400 H2WT, Klöckner Wood Technology GmbH, Hirtscheid, Germany), flaking these with a knife ring flaker (Condux HS 350, Condux Maschinenbau GmbH. and. Co. KG, Hanau, Germany) into smaller particles and then sieving the wood particles (60-40 mesh), following the methods presented in [21].

Wood extractives

Wood particles (10 g) was extracted with a Soxhlet apparatus in 450 ml boiling water for 6 hrs, following TAPPI Test Method T 204 om-88 (1988). The water extractives were concentrated and evaporated to dryness using a rotatory evaporator (Heidolph, W105/38, V 220, and Hz 50) at 60°C. Wood extractives were dissolved in dimethyl sulfoxide (DMSO) in a concentration of 1 ml DMSO per 1 g extracted wood. Wood extractives were analyzed by GC-MS (Gas Chromatography Mass Spectrometry, Agilent technologies, 6890N, network GC system, USA) to define individual extractive constituents in the solution. The extractives in 0.5 ml DMSO were concentrated to dryness by speed-vacuum-centrifugation (Concentrator 5301, Eppendorf, Wesseling-Berzdorf, Germany) at 45°C. Dry samples were dissolved in 50 µl pyridine (PIERCE Biotechnology, Rockford, USA) and 50 µl bis-N,O-trimethylsilyl trifluoroacetamide (BSTFA: PIERCE Biotechnology, Rockford, USA) for derivatization in order to increase the volatile phase of compounds and give more thermal stability to the samples. The extractives were directly dried and resuspended in 300 µl toluene to be injected into the GC-MS (capillary column, Agilent 122-5532). Compounds were identified by comparing resulting data with standard references in the data program NIST (National Institute of Standards and Technology, Maryland, USA). Concentration of specific compounds were determined by injecting mixtures of specific compounds (2-methoxyphenol, 4-hydroxybenzaldehyde, 1,4-dihydroxybenzene, 3,5-di-tertbut-4-hydroxy-toluene, 3-methoxy-4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, 3-methoxy-4-hydroxybenzoic acid, 4-hydroxy cinnamic acid, and 4-hydroxy-3-methoxy cinnamic acid) in defined amounts (0.025 µg each, 0.05 µg each, 0.1 µg each, 0.25 µg each, 0.5 µg each, 2.5 µg each, and 5.0 µg each) into the GC-MS and creating for comparison a standard curve of abundances for each tested compounds.

Chemical and physico-chemical pretreatment

Chemical and physico-chemical pretreatments followed with modifications the procedure presented by [9]. Wood particles were treated with phosphoric acid (8 ml per 1 g wood in 25 ml glass beaker), then incubated in a hot water bath at 50°C or incubated with microwave irradiation (Intellowave microwave, LG Co., Ltd, Germany). For irradiation of 1 g wood particles in 8 ml phosphoric acid in a 25 ml glass beaker, a rating power 1000 W, an out power 700 W, and working voltage 230 V and 50 Hz was used.

Chemicals and enzymes

All chemicals were reagent grade and purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise noted. A commercial cellulase enzyme (Cellulase "Onozuka R-10" from *Trichoderma viride*, SERVA Electrophoresis GmbH, Heidelberg, Germany), xylanase from *Trichoderma viride* (Fluka), β-glucosidase from almonds and recombinant laccase from *Aspergillus* sp. (Novozyme, EEC No. 420-150-4) and pure laccase V (as purified by [22]) from *C. cinerea* Okayama 7 were used for enzymatic hydrolysis.

Enzymatic cellulose hydrolysis

All enzymatic hydrolysis experiments were carried out at 37°C in 5 ml of 50 mM sodium acetate buffer, pH 5.0 for 100 mg substrate in 50 ml Falcon tubes (SARSTEDT, Nümbrecht, Germany) on a standing shaker (Uniform, Infors AG, Bottmingen, Switzerland) applying 200 rpm for shaking. 500 µl samples were periodically removed and centrifuged with 16,000 g for 5 min. The production of sugar was determined by using a glucose detection GAGO-20 assay, Sigma-Aldrich, Steinheim, Germany through measurements in a spectrophotometer (Spectra max340PC, Molecular Devices, California, USA). In another set of experiments, the influences of wood extractives and specific phenolic compounds on cellulose hydrolysis were determined by analyzing a kinetic curve between production of glucose and incubation time. The experiment was carried out by using 5 mg carboxymethylcellulose (CMC) as a substrate in a 50 ml Falcon tube on a standing shaker 200 rpm, 37°C in 5 ml of 50 mM sodium acetate buffer, pH 5.0. 500 µl samples were periodically collected after 60, 90 and 120 min of incubation, and boiled for 5 min to stop

the enzymatic reaction, and centrifuged for 16,000 g for 5 min. The production of sugar was measured by using the glucose oxidase/peroxidase method of the GAGO-20 assay, Sigma-Aldrich.

Sugar assay

Production of sugar was quantified by using a glucose (GO) assay kit, GAGO-20 (Sigma) following the suppliers directions. An adapted methodology was carried out using a 96-microplate format. Per individual test, 40 µl of centrifuged, diluted sample was mixed with 80 µl glucose reagent and incubated at 37°C on a standing shaker 200 rpm for 30 min. Reactions were stopped at 30-60 seconds by adding 80 µl of 12 N sulphuric acid (H_2SO_4). Absorbance at 540 nm was measured in a microplate spectrophotometer. In order to convert absorbance values to glucose concentration, measured values were interpolated using a glucose calibration curve which kept a linear relationship between 37.04 and 1.37 µg ml^{-1}.

Statistical analysis

Group values for all parameters in the tests were compared by analysis of variance (ANOVA) tests using the Fisher's least significant difference (LSD) and Duncan's test procedure for multiple comparison (SPSS 8.0 for Windows; USA). Relations among the values of samples were compared for each factor to the controls kept under the same conditions than the samples of interest.

RESULT AND DISCUSSION

The effects of different combinations of enzymes in cellulose hydrolysis by using each time 100 mg of cellulosic pellets obtained in an up-scaled pretreatment process in which in 1 hr incubation 5 g of *A. grandis* wood were incubated in 40 ml of 85% phosphoric acid in a 100 ml beaker kept at 50°C in a hot water bath.

Each 100 mg of the cellulosic pellet were hydrolyzed either by a single enzyme or by combinations of enzymes in 50 mM sodium acetate buffer, pH 5.0 at 37°C for one day. In a comparative analysis, single enzyme hydrolysis was followed up, thereby using 4 U ml^{-1} of either cellulase, xylanase, laccase, laccase+mediator (mediator: HBT; 1-hydroxybenzotriazole; 1 mM) or β-glucosidase, respectively (Fig. 1). Of these, only cellulase and xylanase hydrolysis obtained about 60% glucose yields, whereas laccase, laccase+HBT and β-glucosidase obtained glucose yields lower than 10% (Fig. 1) and no glucose yield was obtained by un-hydrolyzed controls. Combinations of enzymes (laccase and/or β-glucosidase together with cellulase and xylanase) in contrast were in all instances more successful in yielding glucose from cellulose degradation (up to 100% glucose yield) but the combination of only cellulase and xylanase (Fig. 1). This combination of only two enzymes reached just about 60% glucose yield, similarly to that what was achieved with the two enzymes in individual digestion (Fig. 1).

Interesting to note is that regardless of whether laccase, laccase with a mediator (HBT), β-glucosidase or combinations of these were added, the yield of glucose was always optimal (Fig. 1). The laccase applied were supplied from Novo, Denmark (Novozyme, laccase EEC No. 420-150-4). To elucidate possible contaminations, 0.12 U of the laccase solved in 30 µl of 50 mM sodium acetate buffer, pH 5.0 were used in final total volumes of 1 ml of 100 mM sodium lactate buffer, pH 5.0 to determine cellulase activity with 4.0% (w/v) CMC and xylanase activity with 1.5% (w/v) with xylan form oat spelts. To determine β-glucosidase activity with 1 mM *p*-nitro phenol (pNP-Glc), 2 U of laccase solved in 500 µl of 100 mM sodium acetate buffer, pH 5.0 were tested for foreign enzymatic activities. In no case, such enzymatic activities were detected as contaminations in the preparations. Accordingly, if these types of enzymes are at all present, they should occur in very minor amounts in the solutions of 4 U ml^{-1} of laccase applied in the *A. grandis* wood pretreatments.

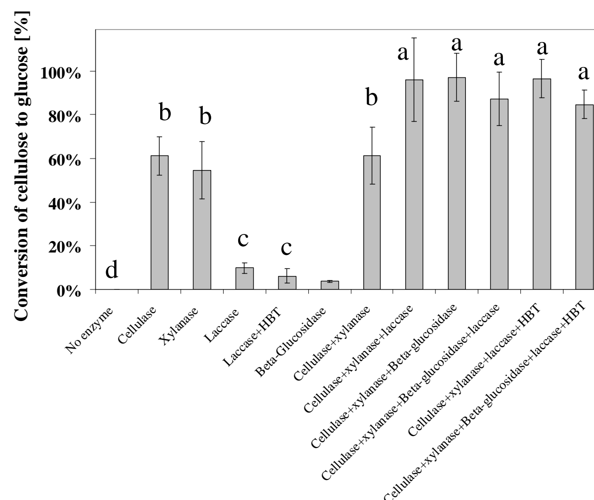

Fig. 1 Conversion of cellulose to glucose in cellulosic pellets of *A. grandis* wood particles pretreated prior to enzymatic hydrolysis with 8 ml of 85% phosphoric acid at 50°C for one hour in a hot water bath, and washed with water. Each time 100 mg of left cellulosic pellet were incubated with enzymes in 50 mM sodium acetate buffer, pH 5.0 for 24 hrs at 37°C under constant shaking. Per different treatment, three samples were incubated in parallel and average values and standard deviations were calculated. The different superscripts on the chart indicate values of glucose yields that differ significantly (p<0.05) between treatments as based on analysis of variance (ANOVA)

Effects of laccase were further tested on *A. grandis* wood that had undergone phosphoric acid-microwave pretreatments by five or 20 sec irradiation in a volume of 8 ml phosphoric acid at the different concentrations of 80%, 40% and 20% acid, respectively. Each time for enzymatic hydrolysis, 100 mg of resulting cellulosic pellets were hydrolyzed by a combination of 4 U ml^{-1} cellulase, 4 U ml^{-1} xylanase and 4 U ml^{-1} laccase in 50 mM sodium acetate buffer, pH 5.0 for 24 hrs. The glucose yields after enzymatic hydrolysis obtained were

highest with about 100% when wood particles were pretreated with 80% phosphoric acid in 20 sec microwave irradiation, whereas a pretreatment with 80% phosphoric acid in 5 sec microwave irradiation resulted in a glucose yield of about 60% (Fig. 2). Application of lower phosphoric acid concentrations in the pretreatment procedures resulted in lower glucose yields (around 40% to 50%) in subsequent enzymatic cellulose conversion (Fig. 2). When applying a scheme of microwave irradiation of 4 rounds of 5 sec irradiation and 5 sec of no irradiation, when higher concentrations of phosphoric acid were applied (80% and 40%), the resulting glucose yields in the subsequent enzymatic hydrolysis were significantly lower as in microwave experiments in which 20 sec in a constant incubation was applied (Fig. 2). In contrast, for the lowest applied phosphoric acid concentration (20%), no differences in the yields were seen regardless of the time of microwaving applied (Fig. 2).

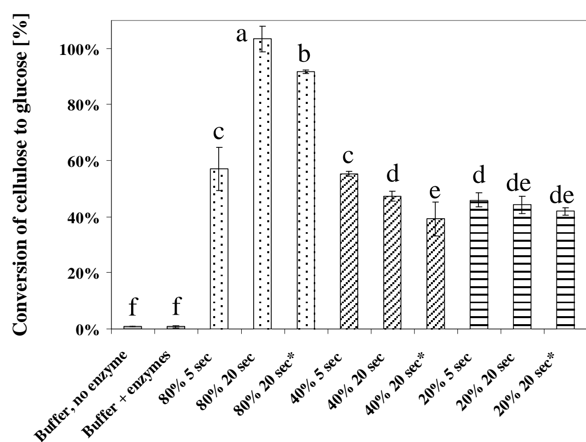

Fig. 2 Enzymatic conversion of cellulose to glucose of *A. grandis* wood particles pretreated prior to enzymatic hydrolysis for 5 or 20 sec in a microwave (Intellowave microwave, LG Co., Ltd, Germany) at a rating power of 1000 W, an out power of 700 W, and a working voltage of 230 V and 50 Hz, with 8 ml of various concentrations of phosphoric acid. Each 100 mg of left cellulosic pellets were incubated with 4 U ml^{-1} of cellulase and 4 U ml^{-1} xylanase and 4 U ml^{-1} laccase in 50 mM sodium acetate buffer, pH 5.0 for 24 hrs at 37°C under constant shaking. Three different samples per pretreatment and three controls with 100 mg original *A. grandis* wood particles were treated in parallel and average values of conversion of cellulose to glucose the experimentally determined amount in the respective samples and standard deviations were calculated. The different superscripts on the chart indicate values of glucose yields that differ significantly ($p<0.05$) between treatments as based on analysis of variance (ANOVA). * Incubation for 5 sec, stop for 5 sec, repeated for 4 cycles; total 20 sec

Another interesting result from the experiments presented in Fig. 2 is, that reasonable yields of glucose were obtained both with application of 40% and of 20% phosphoric acid in the microwave pretreatments but that overall there were no or only marginal differences in the glucose yields. Thus, a combination of very low amounts of phosphoric acid with laccase gives the ecological favorable situation.

As indicated already from the conclusion from the results presented in Fig. 2, wood extractives are one of the possible drawbacks of enzymatic hydrolysis processes since the functions of the enzymes might be inhibited by the wood extractives. Water extractives were subjected to a GC-MS analysis in order to identify their individual compounds (Fig. 3). The different compounds were present in the chromatograms of water extractives with an identification quality higher than 95%: 4-Hydroxy-3-methoxy cinnamic acid (ferulic acid), 4-Hydroxy cinnamic acid (*p*-coumaric acid) and 3-Methoxy-4-hydroxybenzoic acid (vanillic acid) were obtained highest amount in concentration of 17.82, 16.28 and 14.43 µg in 1 g dry wood, respectively (Fig. 3; filled arrows).

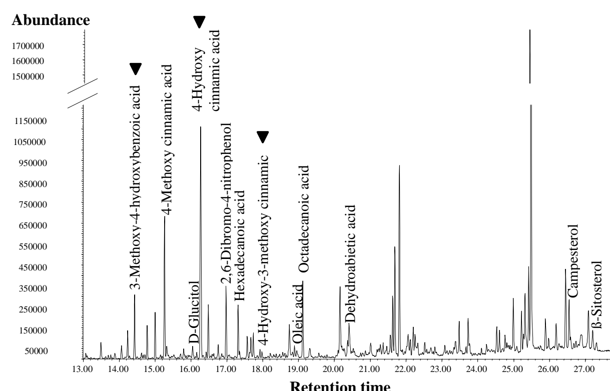

Fig. 3 GC-MS analysis of compounds in water extractives from *A. grandis* wood particles. Filled arrows mark compounds obtained highest amount in concentration (µg) in 1 g dry wood

This section therefore investigates the influences of wood extractives in cellulase hydrolysis carried out in 50 mM sodium acetate buffer, pH 5.0 (Fig. 4). A commercial carboxymethylcellulose (CMC) preparation (product No. 21901, Fluka) was used as a substrate in cellulase hydrolysis at a concentration of 5 mg in 5 ml 50 mM sodium acetate buffer pH 5.0 with either 0.008 U ml^{-1} cellulase (Cellulase "Onozuka R-10" from *Trichoderma viride*, SERVA Electrophoresis GmbH, Heidelberg, Germany) or 0.008 U ml^{-1} cellulase and 0.008 U ml^{-1} laccase (Novozyme, EEC No. 420-150-4) added to the samples for a 2 and a half hour incubation at 37°C. Water extractives were used to simulate the influence of extractives on enzymatic cellulose hydrolysis. Moreover, addition of laccase was also used to possibly improve cellulase hydrolysis under such circumstances.

Fig. 4A shows that water extractives negatively influenced hydrolysis of 5 mg CMC in 50 mM sodium acetate buffer, pH 5.0 by cellulase over the whole time of incubation observed, although the kinetic curve of production of glucose (PG) by CMC hydrolysis through cellulase increased linearly according to the time of incubation (T), (Fig. 4A). At the end of the experiment, the glucose yield was 13 µg ml^{-1} and thus only half as much as achieved with the enzyme without addition of wood extractives (24 µg ml^{-1}; Fig. 4A). However, when adding in addition laccase to the samples, the negative effect by the wood extractives was counteracted and a nearly identical kinetic curve for CMC hydrolysis was

obtained than for CMC+cellulase alone (compare Fig. 4A and 4B).

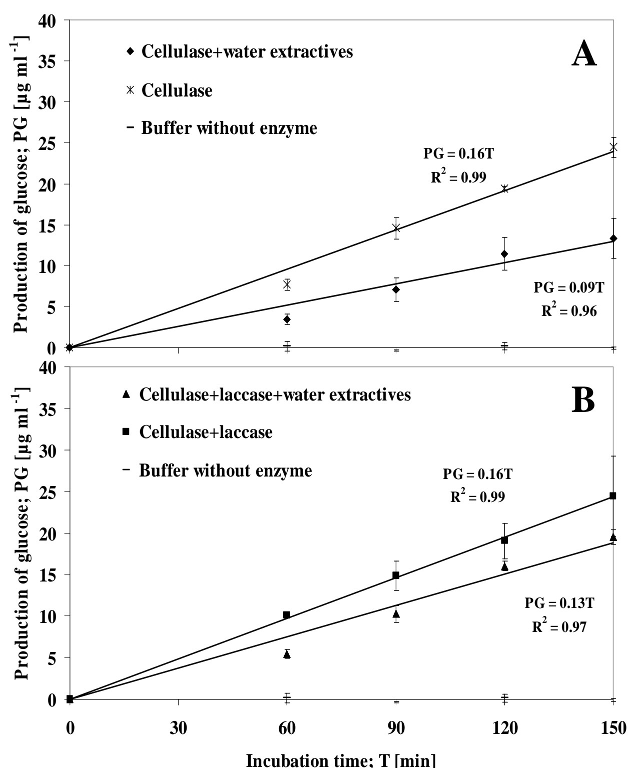

Fig. 4 Influence of water extractives in cellulase hydrolysis **A**) and water extractives in cellulase+laccase hydrolysis **B**) of 5 mg carboxymethylcellulose (CMC) by using 0.008 U ml^{-1} cellulase (cellulase "Onozuka R-10" from *Trichoderma viride*, SERVA Electrophoresis GmbH, Heidelberg, Germany) and 0.008 U ml^{-1} laccase (Novozyme, EEC No. 420-150-4) in 5 ml 50 mM sodium acetate buffer, pH 5.0 at an incubation temperature of 37°C. Water extractives were added portions of 2.5 µl DMSO containing the amount of extractives corresponding to 5 mg of *A. grandis* wood. To all other reactions, 2.5 µl DMSO was added for comparison. Per treatment, each three samples were analyzed. At 60, 90, 120 and 150 min of incubation, 150 µl per sample were taken from the reactions and tested for glucose content using the GAGO. An acceptable positive correlation was present with R^2>0.95 for each kinetic curve

According to previous own results [23], *p*-coumaric acid (4-hydroxy cinnamic acid), vanillic acid (3-methoxy-4-hydroxybenzoic acid) and ferulic acid (4-hydroxy-3-methoxy cinnamic acid) are active in inducing a high laccase production from *Trametes versicolor*. These phenolic compounds (*p*-coumaric acid, vanillic acid and ferulic acid) were also found in the highest amounts within water extractives (Fig. 3). These phenolic compounds in pure commercial form were therefore added in different concentrations (1 mM and 1 µM) to reactions of enzymatic CMC hydrolysis carried out in 50 mM sodium acetate buffer, pH 5.0 (Fig. 5).

In this series of tests with 5 mg of commercial CMC preparation, again 0.008 U ml^{-1} cellulase was used and 0.5 U ml^{-1} of purified laccase V from *Coprinopsis cinerea* Okayama 7. As expected, the purified laccase added into samples with just CMC did not lead to recognizable conversion of cellulose into glucose like in the samples with no added enzyme and unlike in the samples where just cellulase was added to the CMC (Fig. 5 till Fig. 7). Additions of the three different phenolic compounds always lead to a reduction cellulase activity.

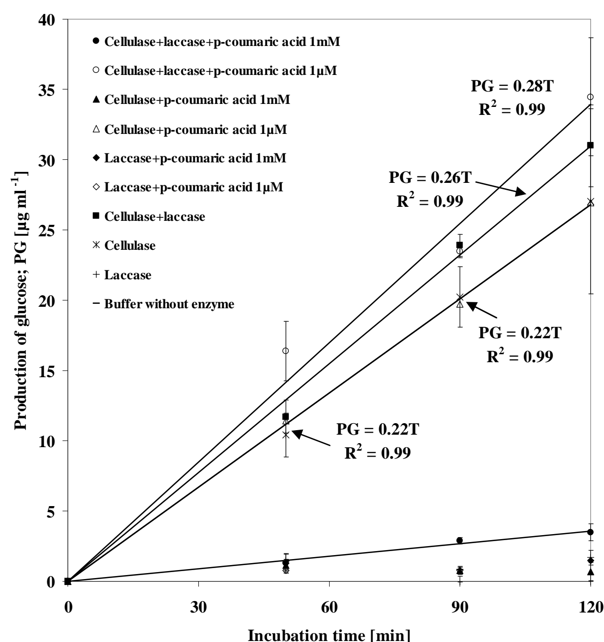

Fig. 5 Influence of addition of *p*-coumaric acid on hydrolysis of 5 mg CMC by cellulase in presence and absence of laccase. 0.008 U ml^{-1} cellulase (Cellulase "Onozuka R-10" from *Trichoderma viride*, SERVA Electrophoresis GmbH, Heidelberg, Germany) and 0.5 U ml^{-1} of purified laccase V of *C. cinerea* Okayama 7 in 5 ml of 50 mM sodium acetate buffer, pH 5.0 were used. *p*-Coumaric acid was dissolved in 100 µl DMSO. To all other samples, for reasons of comparison, 100 µl of pure DMSO was added. Per treatment, three different samples were followed up in glucose formation over the time. At 60, 90, and 120 min of incubation, per sample aliquots of 150 µl were taken and analyzed for glucose content with the GAGO kit

The results in Fig. 5 show that *p*-coumaric acid at a high concentration (1 mM) nearly fully blocked the enzymatic hydrolysis of CMC whereas production of glucose was to a certain level reduced at the lower concentration of 1 µM of *p*-coumaric acid (Fig. 5). At the high concentration of *p*-coumaric acid (1 mM), as in controls without added enzymes, a neglectable rate of production of glucose much below than 0.03 µg ml^{-1} min^{-1} were obtained in comparison to a rate of production of glucose of 0.22 µg ml^{-1} min^{-1} at the low concentration of *p*-coumaric acid. When laccase was added to samples with cellulase and 1 µM *p*-coumaric acid, the hydrolysis activity was fully restored with a highest rate of glucose production of about 0.28 µg ml^{-1} min^{-1}, respectively even increased when compared to rates of production of glucose in samples with cellulase alone (0.22 µg ml^{-1} min^{-1}). Also, some activity was restored upon addition laccase to samples with cellulase and 1 mM *p*-coumaric acid, although the production rate of glucose was very low with a rate of 0.03 µg ml^{-1} min^{-1} (Fig. 5).

Notable is further that the combination of the cellulase + laccase without addition of *p*-coumaric acid had also a better rate of glucose production (0.26 µg ml^{-1} min^{-1}) than cellulase alone (0.22 µg ml^{-1} min^{-1}), (Fig. 5).

Addition of vanillic acid (Fig. 6) followed the same trend in the production of glucose than previously observed with samples with added *p*-coumaric acid (Fig. 5). A high concentration of vanillic acid (1 mM) resulted in a rate of production of glucose of less than 0.12 µg ml^{-1} min^{-1}, whereas in comparison a low concentration of vanillic acid (1 µM) resulted in a rate of production of glucose of 0.20 µg ml^{-1} min^{-1} which was somewhat reduced as compared to the rate of production of 0.22 µg ml^{-1} min^{-1} of glucose in samples with only cellulase and CMC as substrate.

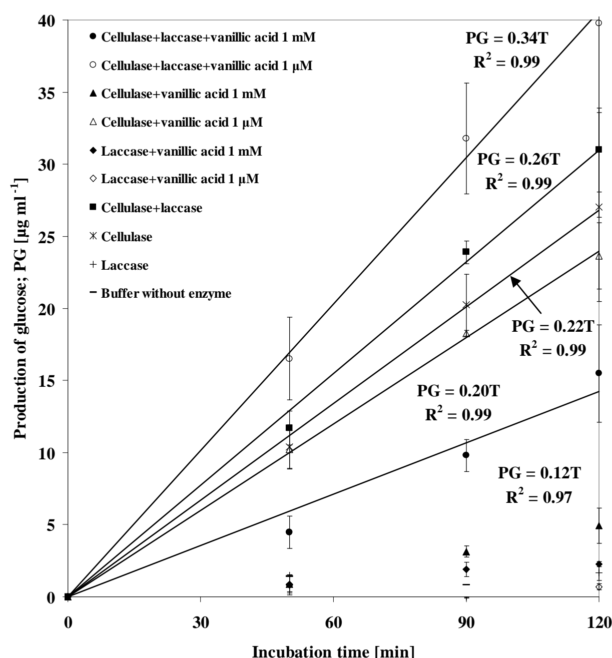

Fig. 6 Influence of addition of vanillic acid on hydrolysis of 5 mg CMC by cellulase in presence and absence of laccase. 0.008 U ml^{-1} cellulase (Cellulase "Onozuka R-10" from *Trichoderma viride*, SERVA Electrophoresis GmbH, Heidelberg, Germany) and 0.5 U ml^{-1} of purified laccase V of *C. cinerea* Okayama 7 in 5 ml of 50 mM sodium acetate buffer, pH 5.0 were used. Vanillic acid was dissolved in 100 µl DMSO. To all other samples, for reasons of comparison, 100 µl of pure DMSO was added. Per treatment, three different samples were followed up in glucose formation over the time. At 60, 90, and 120 min of incubation, per sample aliquots of 150 µl were taken and analyzed for glucose content with the GAGO kit

Upon addition of laccase to samples with cellulase and vanillic acid at a concentration of 1 µM vanillic acid, highest rate of production of glucose with about 0.34 µg ml^{-1} min^{-1} a same trend was measured and at a concentration of 1 mM vanillic acid, a reduced rate of about 0.12 µg ml^{-1} min^{-1}. Thus, rates of production of glucose in presence of laccase in combination with cellulase and a low amount of a phenolic compound (1 µM) and in presence of laccase in combination with cellulase without any added phenolic compound (0.26 µg ml^{-1} min^{-1}) were obtained that were higher than the rate of production of glucose in samples with just cellulase alone.

When testing effects of ferulic acid (Fig. 7), at the high concentration of 1 mM no CMC hydrolysis by cellulase was achieved but a nearly normal rate of production of glucose of less than 0.21 µg ml^{-1} min^{-1} was observed when the low concentration of 1 mM of ferulic acid was added to samples. In comparison, upon addition of laccase to samples with the low concentration of ferulic acid (1 µM) a rate of production of glucose over 0.15 µg ml^{-1} min^{-1} was obtained. These results suggest that by the actions of laccase in combination with ferulic acid, negative actions on the hydrolysis of cellulose to glucose via the applied cellulase occur.

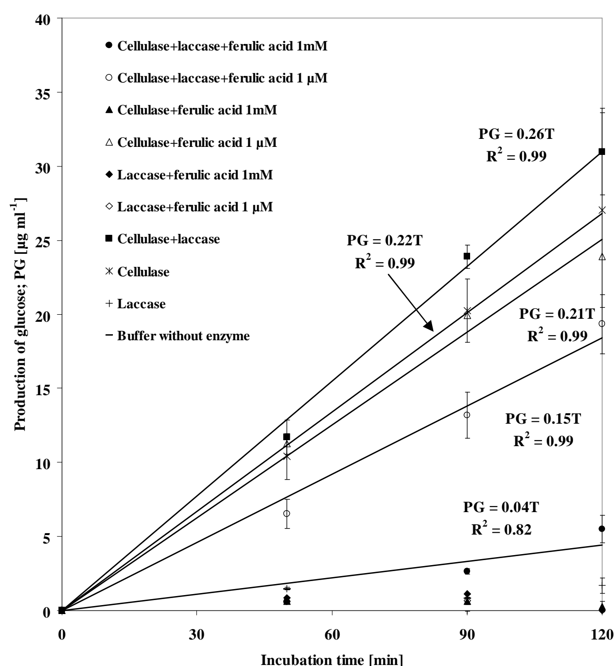

Fig. 7 Influence of addition of ferulic acid on hydrolysis of 5 mg CMC by cellulase in presence and absence of laccase. 0.008 U ml^{-1} cellulase (Cellulase "Onozuka R-10" from *Trichoderma viride*, SERVA Electrophoresis GmbH, Heidelberg, Germany) and 0.5 U ml^{-1} of purified laccase V of *C. cinerea* Okayama 7 in 5 ml of 50 mM sodium acetate buffer, pH 5.0 were used. Ferulic acid was dissolved in 100 µl DMSO. To all other samples, for reasons of comparison, 100 µl of pure DMSO was added. Per treatment, three different samples were followed up in glucose formation over the time. At 60, 90, and 120 min of incubation, per sample aliquots of 150 µl were taken and analyzed for glucose content with the GAGO kit. Note that for the curve calculated for samples with added cellulase, laccase and 1 mM ferulic acid, the R^2 was only low with 0.82, indicating a low reliability of the calculated value for the rate of production of glucose via the applied cellulose occur

Laccases transfer ferulic acids into different radicals and these may form several different ferulic acid dimers and trimers [24, 25, 26]. Moreover, in presence of ferulic acid, laccase may oligomerize peptides containing cysteine and crosslink tyrosine containing peptides [24, 25]. On longer peptides, cross-linking may particularly often occur when tyrosines are present close to the C-terminal end [25]. The Onozuka cellulose R-10 applied in this study is indeed a mixture of various cellulases for

T. viride: six endoglucanases (Endo I to Endo VI), three exoglucanases (Exo I to Exo III) and a β-glucosidase are present in the enzyme mixture [27]. Of these, Exo I and Endo I for example have an outmost C-terminal 30 amino-acid long CBM domain (cellulose binding motif) with four cysteine and four tyrosines ([28]; GenBank accession number ACC59774), offering a good target for ferulic-acid-mediated cross-linking through laccase action, presenting an explanation for then here observed reduction in cellulase activity on CMC by the presence of laccase plus 1 μM ferulic acid (Fig. 7). In contrast, other cellulases of *T. viride* such as Endo II (GenBank BAA 36216) and Endo III (GenBank AAQ 21383) and two other endoglucanases with not yet assigned number (GenBank ADJ10627 and ABQ95572) have an N-terminal CBM domain, offering both protection from ferulic-acid mediated cross-linking by laccase. Accordingly, the low hydrolysis activity (0.15 μg ml^{-1} min^{-1}) on cellulose in samples with added laccase and 1 μM ferulic acid may be due to such cellulase being less accessible from inactivation.

What are the reasons for optimizing the glucose yields from cellulosic pellets in the presence of laccase? Cellulases may bind to lignin and this can hinder their enzymatic actions [29]. In the study here, by addition of wood extractives and by addition of specific phenolic compounds shown to be present in *A. grandis* wood extractives to CMC and cellulase it was shown that such compounds also hinder the cellulolytic enzymatic activities (Fig. 4).

Water extractives, *p*-coumaric acid, vanillic acid and ferulic acid all influenced the production of glucose from cellulose by cellulase hydrolysis, (Fig. 4). The concentration in water extractives of *A. grandis* wood applied in the enzymatic test with CMC (water extractives from 1 mg *A. grandis* wood transferred into 1 ml buffer; Fig. 4) were 0.123 mM of *p*-coumaric acid, 0.137 mM vanillic acid and 0.013 mM ferulic acid and thus in between the two concentrations of 1 mM and 1 μM used in the test for the purified compounds (Fig. 5 to Fig. 7). From the results shown in Fig. 5 to 7, it is to be expected that at the natural concentrations the three compounds will also provide negative effects to cellulase activity, in particular when considering in addition that in the wood extractives the negative actions of the different compounds will likely add up to a stronger effect. Therefore, since addition of laccase to the cellulase overcomes in concentration-dependent manner the negative effects of the phenolic compounds, it can be concluded that laccase has a potential to decrease inhibitors in form of phenolic compounds in wood extractives blocking cellulases in cellulose hydrolysis (Fig. 4). Whilst the data show that laccase can very much improve the enzymatic generation of glucose from cellulosic pellets, the example of ferulic acid shows that in certain situations their might be also some negative effects by addition of laccase. Ferulic acid potentially blocks upon laccase-activation a subrange of cellulase in their activities.

In summary, however, laccase has a good potential to improve the drawbacks of lignin and lignin-related compounds on enzymatic cellulose degradation because laccase is efficient in degrading of lignin and of phenolic compound as present in wood extractives or even somewhat of hemicellulose [30]. Thus, use of laccase in combination of enzymatic hydrolysis can improve the hydrolysis process and gain higher glucose yields, as found in the study by [30] and also in this study.

Future own studies for upcoming applications will concentrate on the predominant wood industries in Thailand, especially in Southern Thailand, that rely economically mainly on rubberwood and palm tree plantations. Knowledge from this work gained by studying *A. grandis* wood will be transferred and adapted to such wood species playing on important role in Thailand. As a result of the Thai wood industries, huge amounts of wood product residues and wood sawdust from the local tree species and fruit bunches of palm trees accumulate. Countries in Southeast Asia including Thailand are the biggest rubberwood producers in the world, making use of the wood for furniture and particleboard production. Surprisingly, only 10% of the total wood end up as products, while about 90% of the material represent wood residues. From this, a ratio of 54% comes from small branches, and a ratio of 32 % wastes from wood sawmills and the 4% rest wood comes from furniture factories. In total, there is an annual availability of rubberwood residues of 1,100,000 tons from rubberwood plantations and sawmills for which optimal economical and ecological uses have to be found [31]. Moreover, a palm oil research unit was established in 2008 in the province of Surat Thani, where the Prince of Songkla University (author work's office) is located, by implementing the province policy to develop in the next few years the Surat Thani province to be a "Palm city" (http://opru.surat.psu.ac.th). Also this development will lead to huge amounts of further waste materials that should economically und ecologically be made use of. According to the official Thai policy for generating sustainable biomass products, producing bioethanol from wastes sources of both tree species are highly interesting challenges, for Thailand in the near future's research, both for industry and for society.

Conclusion

Laccase in combination of enzymatic hydrolysis of cellulose by application of Onozuka R-10 cellulase in combination or in a mixture with also xylanase improved the glucose yields by factors of 60 to 100% as compared to enzymatic hydrolysis reactions performed without laccase. The conversion of cellulose to glucose reached up to 100% when using for 1 g of *A. grandis* wood particles 8 ml of 85% phosphoric acid for one hour incubation at 50°C in a hot water bath and in subsequent enzymatic hydrolysis of 100 mg of left cellulosic pellets each 4 U ml^{-1} of cellulase, of xylanase and of laccase, or each 4 U ml^{-1} of cellulase, of xylanase and of β-glucosidase, or each 4 U ml^{-1} of cellulase, of xylanase, of β-glucosidase and of laccase, or each 4 U ml^{-1} of

cellulase, of xylanase and of laccase plus 1 mM HBT, or each 4 U ml^{-1} of cellulase, of xylanase, of β-glucosidase and of laccase plus 1 mM HBT, respectively, in 50 mM sodium acetate buffer, pH 5.0 for one day incubation, or alternatively, when using 1 g of *A. grandis* wood particles in 8 ml of 80% phosphoric acid pretreatment for incubation with microwave irradiation for 20 sec and in subsequent enzymatic hydrolysis 100 mg of left cellulosic pellets each 4 U ml^{-1} of cellulose, of xylanase and of laccase in 50 mM sodium acetate buffer, pH 5.0 for one day incubation. Water extractives, *p*-coumaric acid, vanillic acid and ferulic acid in concentration-dependent manner all inhibited on cellulase hydrolysis. The negative effects of phenolic compounds overcome in concentration-dependent manner when added laccase into cellulose hydrolysis by cellulase. Laccase has a good potential to decrease inhibitors in form of phenolic compounds in wood extractives blocking cellulases activity.

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