Effects of Laccase in Combination with other Enzymes on Hydrolysis of Cellulose to Glucose in Biofuel Production

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***Abstract**: The chemical pretreatments of Abies grandis wood on enzymatic hydrolysis of cellulose into glucose in view of potential applications in biofuel production were carried out by 85% phosphoric acid, incubated in a hot water bath at 50°C. The highest glucose yields of up to 100% of converted cellulose were reached by using 1 g of wood particles in 8 mL of 85% phosphoric acid incubated in a hot water bath for 1 hr 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 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. Changes in structure of the cellulose during a pretreatment produce of the A. grandis wood and/or production of compounds with inhibitory effects on the hydrolyzing enzymes can be reasons for a failure of glucose production. 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 and vanillic 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.*

Keywords: Biofuel, Kinetic curve of production of glucose, Phenolic compound, Pretreatment, Rate of production of glucose, Wood extractives

1. INTRODUCTION

Bioethanol has been promoted to replace fossil fuel and becomes more and more interesting for this target because of the large growing demand for the world energy consumption, limited resources on petroleum and gas, and global warming also linked to consumption of fossil fuels. Crude-oil resources are rapidly decreasing by using fossil-based energy carriers, thereby increasing also the speed of climate change. Political attention and commitment therefore turned currently towards improved utilization of renewable energy resources. Presently, ethanol is the most important renewable fuel in terms of volume and market value. However, the first generation of biofuels was produced from food and fodder, which competes with human and animal consumption. To overcome this point of disadvantage for the first generation biofuels, the second generation of biofuels was generated from non-food and non-fodder materials. Plant lignocellulosic biomass is the most attractive choice in biofuel production. Biomass represents the most abundant of all bio-materials for renewable energy resources, including for biofuel production and value added chemicals. Wood biomass from forest land and fast growing tree plantations could be supplier of large amounts of raw materials, supporting biofuel production for many regions of the world. Woody biomass is most attractive to use as a feedstock in production of bio-based materials because of its many advantages in terms of production, harvesting, storage, and transportation as well as overall energy content in comparison to herbaceous materials [1, 2, 3, 4, 5, 6, 7].

The research presented here addresses the problem of making the so far little studied softwood better accessible to efficient cellulose hydrolysis. Softwoods have generally not yet been as thoroughly investigated in their potential for biofuel production. *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 [8, 9, 10, 11]. 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 [12, 13]. 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 [14, 15]. 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 [1, 3, 4].

2. MATERIAL AND METHOD

2.1 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 [16].

2.2 Wood extractives

Wood particles (10 g) were 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.

2.3 Chemical and physico-chemical pretreatment

Chemical and physico-chemical pretreatments followed with modifications the procedure presented by [13]. 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.

2.4 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 [17]) from *C. cinerea* Okayama 7 were used for enzymatic hydrolysis.

2.5 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 xg 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 xg for 5 min. The production of sugar was measured by using the

glucose oxidase/peroxidase method of the GAGO-20 assay, Sigma-Aldrich. The conversion of cellulose to glucose was calculated by using the cellulose content of wood as percentage converting to glucose.

2.6 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}.

2.7 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.

3. RESULT AND DISCUSSION

In this experiment, the effects of different amounts of 85% phosphoric acid were tested by incubation of 1 g *A. grandis* wood particles for one hour in different volumes of the acid. Subsequently, each time 100 mg of the respective left cellulosic pellets were hydrolyzed for one day by 4 U ml^{-1} of cellulase and 4 U ml^{-1} of xylanase in 5 ml of 50 mM sodium acetate buffer, pH 5.0 at 37°C (Fig. 1). Glucose yields were found to decrease in a manner in dependence of the increasing amounts of phosphoric acid.

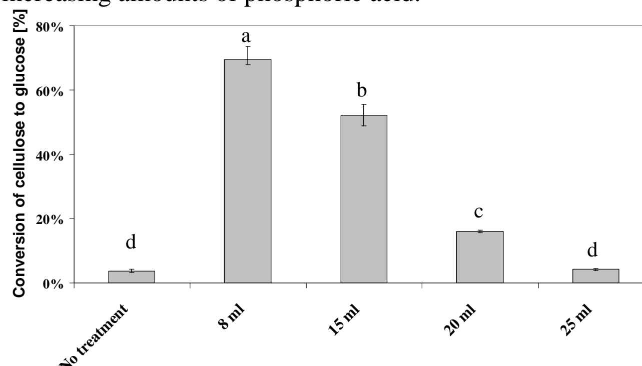

Fig. 1 Enzymatic conversion of cellulose to glucose of *A. grandis* wood particles pretreated prior to enzymatic hydrolysis for one hour at 50°C in a hot water bath with 8, 15, 20, and 25 ml of 85% phosphoric acid, respectively. Upon washing the left material with water, each 100 mg of left cellulosic pellets were incubated with 4 U ml^{-1} of cellulase and 4 U ml^{-1} xylanase 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 with enzymes in parallel and average values of glucose yields and standard deviations were calculated against the cellulose content (respectively the hexose content after treatment with sulfuric acid) experimentally determined in 100 mg of *A. grandis* wood particles, respectively. 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)

The glucose yield obtained by using 8 ml, 15 ml, 20 ml or 25 ml of 85% phosphoric acid in the pretreatment procedure of *A. grandis* wood particles corresponded to yields of 70%, 50%, 15% and 5% of conversion of cellulose in the left cellulosic pellets to glucose, respectively (Fig. 1). Enzymatic degradation depends on the general structure of cellulose, i.e. whether accessible or not by enzymes [18] and the results of poor cellulose conversion upon application of higher phosphoric acid concentrations may point to an un-favored chemical structural stage generated in applications of high phosphoric acid volumes. The 100 mg of the cellulosic pellets of the different samples were treated for 24 hrs with both 4 U ml^{-1} cellulase and 4 U ml^{-1} xylanase in 50 mM sodium acetate buffer, pH 5.0, and, subsequently, glucose yields were determined. Glucose levels in the samples were calculated against the measured cellulose content being present in treated 100 mg samples of cellulosic pellets. The treatment with phosphoric acid drastically increased subsequent yields of glucose from cellulose conversion to values of over 55% up to 70% (% of conversion of the cellulose to glucose that was left in the cellulosic pellets) as compared to the about 5% yields of cellulose to glucose conversion from wood control samples handled without chemical pretreatment (Fig. 2). 56% cellulose conversion into glucose was already reached of cellulosic pellets from *A. grandis* wood particles obtained from 15 min incubation with 85% phosphoric acid as pretreatment whilst prolongation of incubation to 1 hr further increased the glucose yield to 70% conversion of the

cellulose present in the left cellulosic pellet to glucose. Moreover, conversion of cellulose was appears to significantly increase when the wood extractives were extracted from wood comparing to no extracted one.

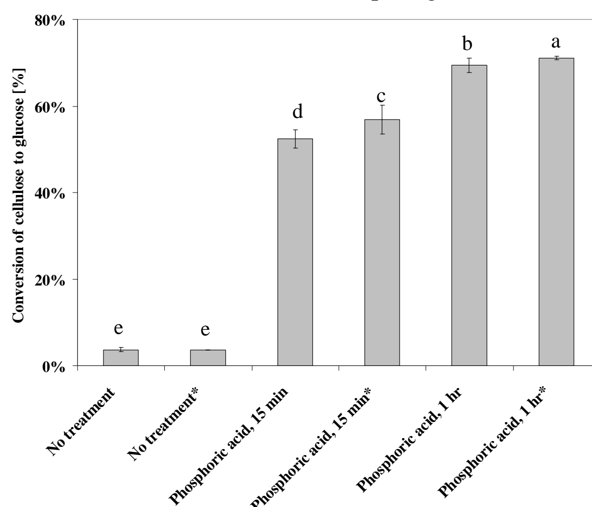

Fig. 2 Enzymatic conversion of cellulose to glucose of 100 mg of cellulosic pellets left from *A. grandis* wood particles pretreated prior to enzymatic hydrolysis with 8 ml liquid acid [of either 85% phosphoric acid during 15 min or 1 hr incubation at 50°C in a hot water bath and, subsequently, the left material was washed with water. For enzymatic hydrolysis, in each three parallel samples the respective materials were incubated with 4 U ml^{-1} of cellulase and 4 U ml^{-1} xylanase in 50 mM sodium acetate buffer, pH 5.0 for 24 hrs at 37°C under constant shaking. In all instances, average yields of glucose and standard deviations were calculated relative to the cellulose content experimentally determined in 100 mg of the wood particles. 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). * Wood after water extraction

Materials were observed under the microscope (Fig. 3). Before pretreatment, cell structures and even entire wood fibers were clearly identified in the *A. grandis* wood particles (Fig. 3A). If only a pretreatment of 8 ml 85% phosphoric acid was done for 1 hr at 50°C by incubating in a hot water bath, the fibril structures loosened, although cellular structures were still recognizable (Fig. 3B) or the structures totally dissolved in case of the 25 ml pretreatment (Fig. 3C).

Fig. 3 *A. grandis* wood particles before any pretreatment **A**), after pretreatment with 8 ml and with 25 ml of 85% phosphoric acid for 1 hr at 50°C in a hot water bath **B**) and **C**), respectively. The samples shown in **B** to **C** correspond to the experiments on glucose production presented in Fig. 1

Looking at the phosphoric acid pretreatment, how stronger the morphological changes (aggregation) and the color changes were, how less the yields in glucose were in subsequent enzymatic hydrolysis. However, just from the appearance of the material after pretreatment it is not possible to do any reliable prediction on possible yields in enzymatic glucose production. Nevertheless, a dark staining of the material and a dissolved aggregation appears to be negative clumping in some part (compare Fig. 3). 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. 4). The different compounds were present in the chromatograms of water extractives with an identification quality higher than 95%: 4-Hydroxy cinnamic acid (*p*-coumaric acid) and 3-Methoxy-4-hydroxybenzoic acid (vanillic acid) were obtained highest amount in concentration of 16.28 and 14.43 µg in 1 g dry wood, respectively (Fig. 4; filled arrows).

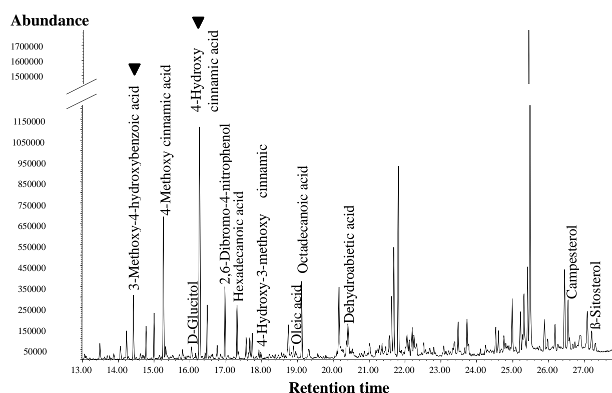

Fig. 4 GC-MS analysis of compounds in water extractives from *A. grandis* wood particles. Filled arrows mark compounds obtained highest amount in concentration (µg) in 1 g dry wood

This section therefore investigates the influences of wood extractives in cellulase hydrolysis carried out in 50 mM sodium acetate buffer, pH 5.0 (Fig. 5). A commercial carboxymethylcellulose (CMC) preparation 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 or 0.008 U mL^{-1} cellulase and 0.008 U mL^{-1} laccase added to the samples for a 2 and a half hour incubation at 37^oC. 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.

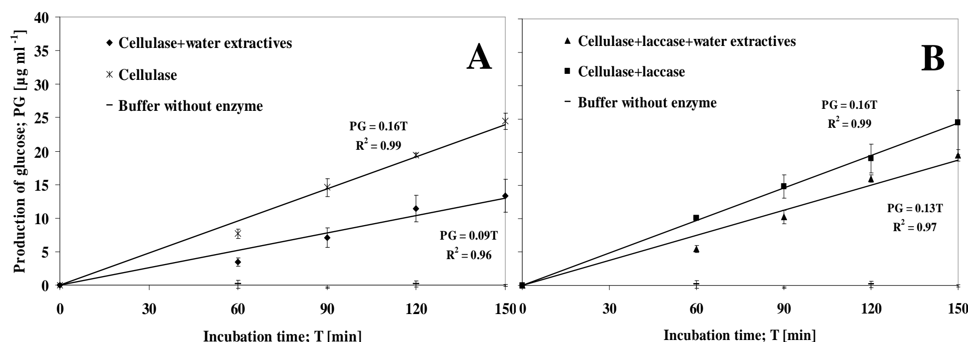

Fig. 5 Influence of water extractives in cellulase hydrolysis **A**) and water extractives in cellulase+laccase hydrolysis **B**) of 5 mg carboxymethylcellulose (CMC) by using 0.008 U mL^{-1} cellulase (cellulase "Onozuka R-10" from *Trichoderma viride*, SERVA Electrophoresis GmbH, Heidelberg, Germany) 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^oC. 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

Fig. 5A 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. 5A). 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. 5A). 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. 5A and 5B). According to previous own results [19], *p*-coumaric acid and vanillic acid are active in inducing a high laccase production from *Trametes versicolor*. These phenolic compounds (*p*-coumaric acid and vanillic acid) were also found in the highest amounts within water extractives (Fig. 4). 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. 6). 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. 6). Additions of the two different phenolic compounds always lead to a reduction cellulase activity. The results in Fig. 6A 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. 6A).

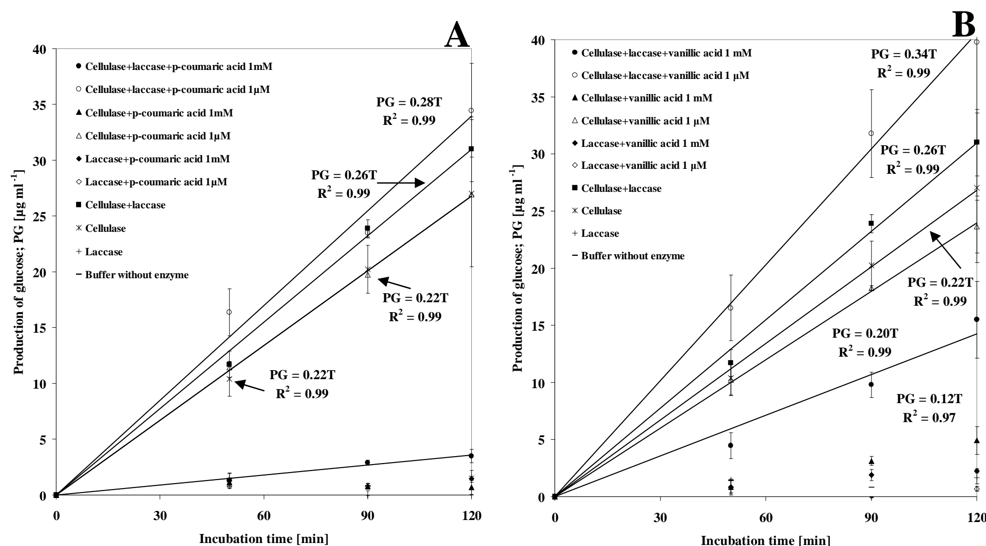

Fig. 6 Influence of addition of *p*-coumaric acid **A**), and vanillic acid **B**), on hydrolysis of 5 mg CMC by cellulase in presence and absence of laccase. 0.008 U mL^{-1} cellulase (Cellulase "Onozuka R-10" from *Trichoderma viride*, SERVA Electrophoresis GmbH, Heidelberg, Germany) and 0.5 U mL^{-1} 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

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. 6A). 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. 6A). Addition of vanillic acid (Fig. 6B) followed the same trend in the production of glucose than previously observed with samples with added *p*-coumaric acid (Fig. 6A). 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. 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. 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 [20]. 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. 5). Water extractives, *p*-coumaric acid and vanillic acid all influenced the production of glucose from cellulose by cellulase hydrolysis, (Fig. 5). 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. 5) were 0.123 mM of *p*-coumaric acid and 0.137 mM vanillic acid and thus in between the two concentrations of 1 mM and 1 µM used in the test for the purified compounds (Fig. 6). From the results shown in Fig. 6, it is to be expected that at the natural concentrations the two 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. 5). Whilst the data show that laccase can very much improve the enzymatic generation of glucose from cellulosic pellets. 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 [21]. 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 [21] and also in this study.

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. 7). 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. 7) 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. 7). 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. 7). 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. 7). 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.

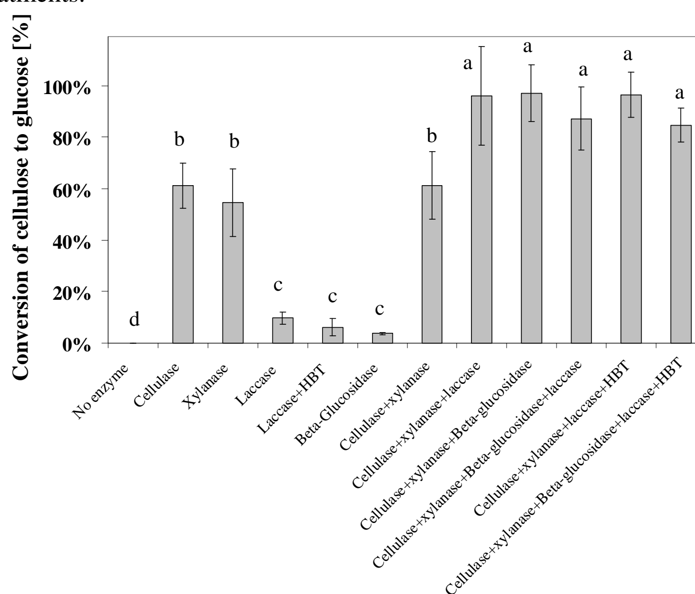

Fig. 7 Conversion of cellulose to glucose in cellulosic pellets of *A. grandis* wood particles pretreated prior to enzymatic hydrolysis with 8 ml of 85% phosphoric acid at 50°C for one hour in a hot water bath, and washed with water. Each time 100 mg of left cellulosic pellet were incubated with enzymes 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)

4. CONCLUSION

The conversion of cellulose to glucose reached highest up to 70% 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. 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, 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. Water extractives, *p*-coumaric acid and vanillic 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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