Bioresource Technology 100 (2009) 6378–6384

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Laccase detoxification of steam-exploded wheat straw for second generation bioethanol

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ARTICLE INFO

Article history:
Received 3 June 2009
Received in revised form 16 July 2009
Accepted 17 July 2009
Available online 14 August 2009

Keywords:
Laccase
Phenols
Steam-explosion
Bioethanol
Wheat straw

ABSTRACT

In this work we compared the efficiency of a laccase treatment performed on steam-exploded wheat straw pretreated under soft conditions (water impregnation) or harsh conditions (impregnation with diluted acid). The effect of several enzymatic treatment parameters (pH, time of incubation, laccase origin and loading) was analysed. The results obtained indicated that severity conditions applied during steam explosion have an influence on the efficiency of detoxification. A reduction of the toxic effect of phenolic compounds by laccase polymerization of free phenols was demonstrated. Laccase treatment of steam-exploded wheat straw reduced sugar recovery after enzymatic hydrolysis, and it should be better performed after hydrolysis with cellulases. The fermentability of hydrolysates was greatly improved by the laccase treatment in all the samples. Our results demonstrate the action of phenolic compounds as fermentation inhibitors, and the advantages of a laccase treatment to increase the ethanol production from steam-exploded wheat straw.

1. Introduction

Lignocellulosic materials, including non-food cultures or wastes from agriculture or forestry, are considered as a sustainable environmental alternative to produce bioethanol. They are a suitable low-cost source, do not compete with food chains, and can contribute to reduce the use of fossil fuel, reducing simultaneously carbon dioxide emission and global warming.

The two main polysaccharides in plant biomass are cellulose and hemicellulose, being closely linked to lignin, a recalcitrant polymer that acts as cementing agent between cellulose fibers and protect the plants from microbial attack (Kirk and Farrell, 1987; Shimada and Higuchi, 1991). The hydrolysis of these polysaccharides is hampered by the presence of lignin and the compact architecture of the cell-wall which makes them much more difficult than starch to be enzymatically degraded to fermentable sugars. Therefore, a pretreatment of this material is necessary to remove or alter the lignin in order to improve the rate of enzymatic hydrolysis of cellulose or hemicellulose and increase the yields of fermentable sugars (Wyman et al., 2005).

Steam-explosion is one of the most commonly used method for lignocellulose pretreatment since partially degrades and solubilises lignin and hemicellulose due to the high pressure and temperature conditions used in the process (McMillan, 1994). The liquid effluent or prehydrolysate, with hemicelluloses partially degraded, is separated from the pretreated biomass, and then the cellulose is enzymatically hydrolyzed to glucose to be fermented by *Saccharomyces cerevisiae* into ethanol. In order to be cost competitive with grain-derived ethanol, the presence of glucose and non-glucose sugars in the feedstock must be considered to obtain a more efficient fermentation process, as well as the use of lignin to be burnt to provide heat and electricity for the process or to obtain valuable co-products (Hahn-Hägerdal et al., 2007). Usually the addition of an acidic catalyst, such as H_2SO_4 or SO_2, is a prerequisite to increase the recovery of hemicellulose sugars in the liquid phase (prehydrolysate), and the enzymatic hydrolysis of the solid fractions, where cellulose is the major polysaccharide. However, this process also affects the non-sugar fraction forming compounds that contaminate the prehydrolysate and can be also embedded in the biomass, producing adverse effects on the downstream processes (Lloyd and Wyman, 2005; García-Aparicio et al., 2006). The inhibiting compounds derived of the pretreatment of steam-exploded raw material are classified according to their chemical structure and include weak acids, furan derivatives, phenolic and inorganic compounds (Hahn-Hagerdal et al., 2006). Several procedures for the removal of these compounds have been assayed, including biological, physical and chemical methods (Palmqvist and Hahn-Hägerdal, 2000a,b). Among them, laccases have been applied in a few cases to remove specifically phenolic compounds in steam-exploded biomass from wood and sugarcane bagasse (Jönsson et al., 1998; Palonen and Viikari, 2004; Chandel et al., 2007).

Laccase is a copper-containing blue oxidase that catalyzes the oxidation of phenolic units in lignin and a number of phenolic

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doi:10.1016/j.biortech.2009.07.049

compounds and aromatic amines to radicals, with molecular oxygen as the electron acceptor, that is reduced to water (Saparrat et al., 2002). Laccases have been used to decrease the toxicity of industrial mill effluents with high content of phenolic compounds since the enzyme generates unstable phenoxy radicals that lead to polymerization into high-molecular-mass products (Casa et al., 2003; Jaouani et al., 2005). Although extensive work has been performed on identification and detoxification of inhibitors from different biomasses, such as hardwoods, corn stover and sugarcane bagasse, relatively little information is available for wheat straw. In the present work we have analysed detoxification of steam-exploded wheat straw with laccases, using samples previously impregnated either with water (soft pretreatment conditions) or diluted acid (harsh pretreatment conditions). The aim was to evaluate the effect of a laccase treatment of steam-exploded wheat straw on the enzymatic hydrolysis of cellulose and fermentation of reducing sugars for bioethanol production.

2. Methods

2.1. Raw materials

Two samples of steam-exploded wheat straw previously impregnated either with water or H_2SO_4 (1% w/w) were obtained at 190 °C for 10 min and 12 bars pressure as previously described (Tucker et al., 2003). The biomass was not washed after steam-explosion pretreatment. The pH of water-impregnated wheat straw (WWS) was 3.8, and the pH of acid-impregnated wheat straw (AWS) was 1.5.

2.2. Laccase treatment of steam-exploded wheat straw

The treatments were performed using laccases from *Coriolopsis rigida* and *Trametes villosa*. *C. rigida* (CECT 20449) was used to produce laccase in a C-limited-yeast extract medium supplemented with peptone (5 g L^{-1}) and 150 μM $CuSO_4$ (Saparrat et al., 2002). The liquid from 15-day-old cultures was separated from the mycelium by centrifugation at 20,000*g*, and concentrated and dialyzed against 10 mM sodium acetate (pH 5) by ultrafiltration (Filtron; 5-kDa cut-off membrane). This crude enzyme was used for treatment of steam-exploded wheat straw since only laccase activity was present and other ligninolytic enzymes, such as manganese-oxidase peroxidase or lignin peroxidase were not detected, as it has been previously reported (Saparrat et al., 2002). The commercial laccase from *T. villosa* was supplied by Novozymes (Bagsvaerd, Denmark).

Preliminary assays were performed to optimize the conditions (pH, time of incubation, laccase origin and dosage) of the detoxification treatments. In a first stage, the assays were performed for 6 h at 28 °C in a rotary shaker (150 rpm) using 1 U mL^{-1} of laccase from *C. rigida* or *T. villosa*. The assays were carried out in 125 mL Erlenmeyer flasks containing 2.5 g (dry weight) of either WWS or AWS in 50 mL of 0.1 M sodium citrate buffer (pH 3, 4 and 5). Samples were periodically taken and the supernatants analysed for total phenols as described below. After 5 h of treatment, samples treated at pH 3 and 4 were adjusted to pH 5 with NaOH 10 M, and then incubated for one more hour to evaluate the effect of pH on the solubility of the free phenolic compounds.

The effect of enzyme dosage (0.1, 0.25, 0.5, 1, 5 and 10 U mL^{-1}) was evaluated in a second stage of optimization by treating the AWS and WWS samples with laccases from *C. rigida* at pH 5 for 2 h. The above pH, laccase and time of incubation were chosen based on the results obtained in the first optimization.

After preliminary assays for optimization of detoxification parameters, definitive enzymatic treatments of WWS and AWS were carried out using 0.5 U mL^{-1} of laccase from *C. rigida* for 2 h. These assays were performed at 28 °C in a rotary shaker (150 rpm) in 125 mL Erlenmeyer flasks containing 2.5 g (dry weight) of either WWS or AWS, in 50 mL of 0.1 M sodium citrate buffer, pH 5. Additional assays were performed under the same conditions but after enzymatic hydrolysis of WWS and AWS with cellulases.

In all cases, control assays were performed under the same conditions but without addition of laccase. All the experiments were carried out by triplicate.

2.3. Enzymatic hydrolysis of biomass and fermentation of the hydrolysate

Samples of WWS and AWS treated with laccase in 0.1 M citrate buffer (pH 5) at 5% (w/v) consistency were centrifuged for 15 min at 1000 rpm, and the supernatants (prehydrolysate) were separated from the solid biomass. The solid fraction was then used as a substrate for hydrolysis experiments. Enzymatic hydrolysis tests were performed in autoclaved 250 mL flasks, each containing 50 mL of 0.1 M sterile citrate buffer (pH 5) at 5% (w/v) steam-exploded wheat straw, at 50 °C for 72 h in a rotary shaker (150 rpm). An enzyme loading of 15 FPU/g (dry substrate) of Celluclast 1.5 L and 15 IU/g (dry substrate) of Novozyme 188 was employed. Enzymes were a gift from Novozymes (Bagsvaerd, Denmark). All the experiments were carried out by triplicate.

To evaluate the effect of laccase detoxification on yeast growth, the hydrolysate was fermented with compressed baker's yeast (Fermentis LPA 3035). Hydrolysate samples (7 mL) were aseptically inoculated with 1400 CFU (colony forming units) of *S. cerevisiae* in 12 mL glass tubes. The tubes were sealed with rubber plugs and incubated at 28 °C in a rotary shaker (150 rpm) for 24, 48 and 72 h. All the experiments were carried out by triplicate.

The hydrolysate was pelleted (15 min and 4000 rpm) and the supernatant was analysed for phenols, reducing sugars and ethanol content as described below. The pellet was resuspended in an equivalent volume of sterilized saline buffer (1% NaCl) and colony counts after the different fermentation times were obtained by plating the samples on YMA (yeast medium agar) containing 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, and 2% agar. Plates were incubated for 2 days at 28 °C.

2.4. Phenol and reducing sugars contents and enzyme activities

The total phenolic content was analysed in the prehydrolysate as well as in the supernatants after enzymatic hydrolysis according to the Folin–Ciocalteau method (Singleton and Rossi, 1965). Diluted samples (200 μL) were added to distilled water (800 μL) followed by Folin–Ciocalteau reagent (500 μL). After 3 min, sodium carbonate (2.5 mL, 20% w/v) was added. Samples were left in the dark for 30 min before absorbance was measured at 725 nm using a Perkin–Elmer Lambda bio 20 UV–vis spectrometer. Results were expressed as grams of catechol equivalents (CE) per litre of liquid phase.

Reducing sugars were analysed in the supernatants after enzymatic hydrolysis and during subsequent fermentation, by the Somogyi and Nelson method (Somogyi, 1945), using glucose as the standard.

Laccase activity was measured using 5 mM 2,6-dimethoxyphenol (DMP) in 100 mM sodium citrate buffer (pH 5.0; ε_{469} = 27,500 M^{-1} cm^{-1}, referred to DMP concentration). Peroxidase activity was assayed as laccase activity in the presence of 0.1 mM H_2O_2. Manganese peroxidase activity was estimated by measuring the formation of Mn^{+3}-tartrate complex (ε_{238} = 6500 M^{-1} cm^{-1}) during the oxidation of 0.1 mM $MnSO_4$ in 100 mM sodium tartrate

buffer (pH 5) in the presence of 0.1 mM H_2O_2. International enzymatic units (micromoles per minute) were used.

2.5. Molecular-mass distribution

Gel filtration on Sephadex G-75 was used to analyse the changes in molecular-mass distribution of WWS after treatment with laccase from *C. rigida*. Controls incubated under the same conditions (without laccase) were also analysed. Samples (2 mL) were filtered and placed on a Sephadex coarse G-50 column (3 × 50 cm) previously equilibrated with NaOH 0.05 M, LiCl 0.025 M. The flow rate was adjusted to 0.33 mL min^{-1}. Spectrophotometrical measurements were performed at 280 nm.

2.6. Sugar and ethanol analysis by gas chromatography (GC)

GC analyses were performed on an Autosystem instrument (Perkin–Elmer) equipped with a flame ionization detector, using a TR-CN100 capillary column (30 m × 0.25 mm, 0.2 μm film thickness) and helium as the carrier gas. Peaks were identified on the basis of sample coincidence with relative retention times of commercial standards, and quantified using peak areas and the corresponding response factors.

Sugar content was determined in the prehydrolysate as well as in the hydrolysate after enzymatic hydrolysis of the biomass. Inositol (1 mg) was added as internal standard. The products were converted into their corresponding alditol acetates (Laine et al., 1972) and analysed. The GC oven was programmed from 210 °C (1 min) to 240 °C (at 15 °C min^{-1}, final temperature 7 min). Injection was performed in the split mode (split ratio 50:1). Injector and detector were kept at 250 °C, and helium pressure at 30 psi.

The hydrolysate was incubated with *S. cerevisiae* as described above, and ethanol content was determined in the supernatants after 72 h fermentation. The fermentation broth (5 mL) was extracted with chloroform (0.5 mL), and 0.5% methanol was added as internal standard. Ethanol content in the extracts was determined isotermically at 30 °C. Injector and detector were maintained at 200 °C and helium pressure at 25 psi.

2.7. Statistical analysis

Analysis of variance (ANOVA) followed by the multiple range test at 95% confidence level (Statgraphics v. 5.0) was applied to evaluate the statistical significance of the phenolic content estimations.

Statistical analysis regarding sugar contents, and ethanol and yeast concentration were fulfilled by the *t* test at 95% confidence level (Statgraphics v. 5.0).

3. Results and discussion

3.1. Effect of laccase treatment on phenolic compounds

Preliminary studies were carried out at different pH conditions to evaluate the effect of laccases (1 U mL^{-1}) from *C. rigida* and *T. villosa* on the phenolic content of the prehydrolysates from WWS and AWS. The results are summarized in Fig. 1.

The phenolic content in the untreated samples was higher ($p \leqslant 0.5$) in WWS than in AWS at pH 4 and 5. The phenolic content released to the aqueous phase tends to be higher as pretreatment severity conditions increase in straw pre-impregnated with water (Hongqiang and Hongzhang, 2008; Zhang et al., 2008). However, some reports have shown that if the steam-explosion is performed with diluted acid, lignin is less soluble as severity conditions increase (Ballesteros et al., 2006; Kabel et al., 2007). This would explain the lower phenolic content found in AWS samples, where sulphuric acid used as a catalyst would reduce phenol solubility at harsh conditions.

In the case of WWS, solubilisation of phenolic compounds in control samples was higher ($p \leqslant 0.5$) as the pH increases. In the AWS controls, pH conditions did not affect phenolic compounds solubilisation ($p \leqslant 0.5$). This suggests that phenolic compounds

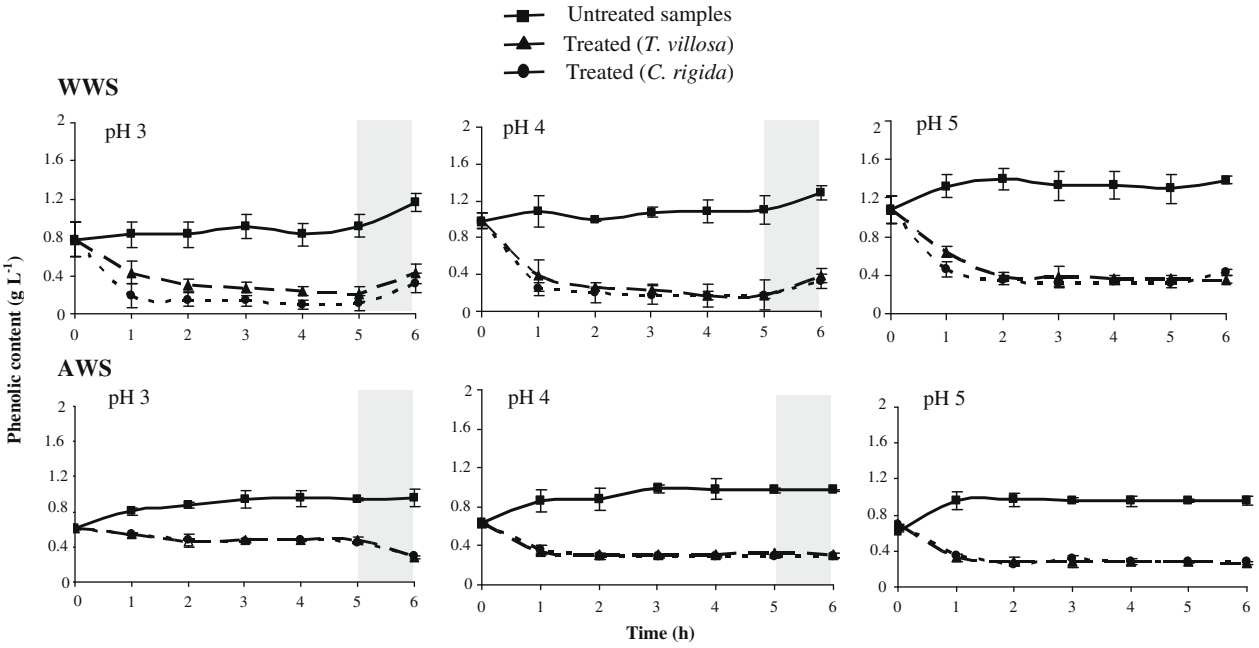

Fig. 1. Time course of phenolic content of WWS and AWS prehydrolysate during pretreatment with laccases from *T. villosa* and *C. rigida*, at different pHs. Untreated control (without laccase) is also shown. Shadowed area indicates adjustment to pH 5. Error bars indicate standard deviations from mean values.

during AWS steam-explosion are totally solubilised at pH 3, whereas in the case of WWS solubilisation of phenolic compounds improves as pH increases.

Laccase treatment removed about 75% of the phenolic compounds in WWS and about 70% in AWS at all the pH conditions tested. Statistical analysis did not show significant differences between treatments performed with laccases from *C. rigida* or *T. villosa*. In the WWS treated samples, the average phenolic content increased ($p \leqslant 0.5$) with pH. When pH was adjusted to 5, in samples previously treated for 5 h at pH 3 and 4, and these samples were incubated for one more hour, the phenolic content was not significantly different ($p \leqslant 0.5$). This would indicate that the lower phenolic content on WWS samples treated with laccases at pH 3 and 4 is due to a lower solubilisation of phenols, more than to an increased performance of laccases.

In the case of AWS treated with laccases, no statistically significant differences were found on average phenolic compounds among samples treated at pH 4 and 5, but the contents were higher when samples were treated at pH 3 ($p \leqslant 0.5$). When pH was adjusted to 5 in samples previously treated at pH 3 and 4, and incubated for one more hour, there was no effect on the phenolic content of samples incubated at pH 4, although a statistically significant decrease ($p \leqslant 0.5$) was observed in samples treated with laccases at pH 3. The higher phenolic content in samples treated at pH 3, and the decrease of phenols after adjustment to pH 5, could not be due to a lower solubility at pH 5, since phenolic content did not change after adjustment of control samples to pH 5. Therefore, polymerization of phenols seems to be inhibited at pH 3 in AWS samples. Although laccases from *C. rigida* and *T. villosa* were active at pH 3 (data not shown), the lower reduction of phenolic content at this pH in WWS samples could indicate an inhibitory effect on laccase activity mediated by pH inactivation of the enzyme. Therefore, reduced polymerization could be due to the presence of some compounds in AWS samples which would inhibit laccase activity or would interfere in polymerization of phenols at pH 3.

Experiments were performed to determine the effect of laccase dosage on reduction of phenolic compounds. Treatments were carried out with laccase from *C. rigida* with an incubation time of 2 h, since longer incubation times did not result in higher detoxification. Since further enzymatic hydrolysis with cellulases is performed at pH 5, the same pH conditions were used for detoxification to avoid readjustment of pH conditions. The results showed that 0.5 U mL^{-1} of laccase was the minimum activity required to detoxify both WWS and AWS samples at pH 5. The increase of laccase doses did not result in higher detoxification (data not shown). Therefore, low laccase concentrations are required for detoxification of steam-exploded wheat straw, which is an important aspect for the application of enzymes at industrial scale.

After these preliminary assays, WWS and AWS samples were treated with laccases under optimized conditions (0.5 U mL^{-1} of laccase from *C. rigida* at pH 5 for 2 h) before or after enzymatic hydrolysis with cellulases. Phenolic content was analysed in the prehydrolysate, as well as in the supernatants after enzymatic hydrolysis. Table 1 summarizes the results obtained, showing that prior enzymatic hydrolysis had no effect on the reduction of phenolic content.

To check the efficiency of laccases in the degradation of free phenolic compounds present in the pretreated material, the supernatants (prehydrolysate) of samples of WWS were analysed by gel filtration. Oxidation of monomeric phenolic compounds in the different WWS fractions treated with laccase could produce radicals leading to polymerization. This phenomenon was evidenced in the sample treated with laccase since the main peak decreased and a new peak with higher molecular mass appeared

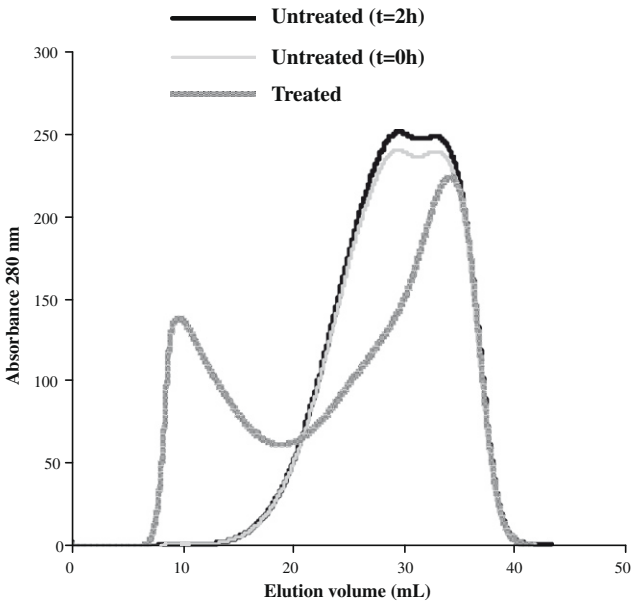

Fig. 2. Size exclusion chromatography profiles ($A_{280\ nm}$) of the prehydrolysate before and after treatment with *C. rigida* laccase.

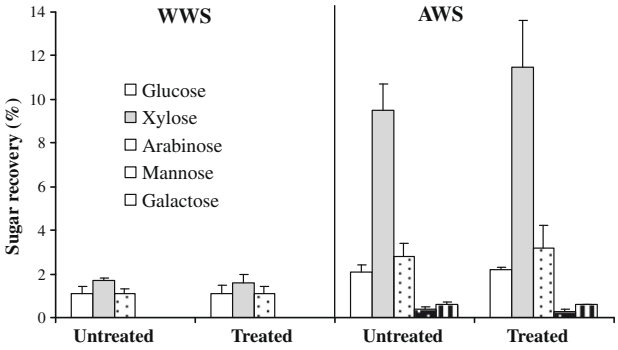

Fig. 3. Monosaccharide recovery (%) from prehydrolysates of WWS and AWS, treated and untreated with *C. rigida* laccase. Error bars indicate standard deviations from mean values.

Table 1
Phenolic content (g L^{-1}) in prehydrolysate and hydrolysate of WWS and AWS samples of steam-exploded wheat straw treated with *C. rigida* laccase before and after the enzymatic hydrolysis (and the untreated controls).

	Prehydrolysate		Hydrolysate	
	WWS	AWS	WWS	AWS
Untreated control	1.386 ± 0.034	0.967 ± 0.07	0.579 ± 0.013	0.198 ± 0.003
Treatment before hydrolysis	0.356 ± 0.035	0.281 ± 0.046	0.153 ± 0.025	0.055 ± 0.01
Treatment after hydrolysis	0.349 ± 0.028	0.277 ± 0.054	0.151 ± 0.011	0.058 ± 0.009

(Fig. 2). These results agree with those previously reported by Jaouani et al. (2005) for *Pycnoporus coccineus* laccase treatment of an effluent from olive oil production with a high content of free phenols.

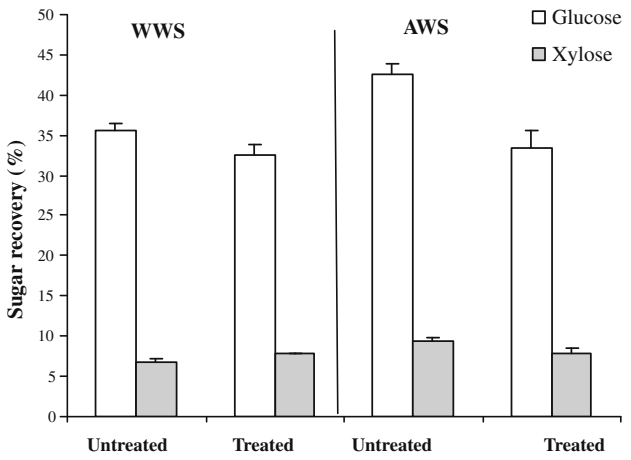

Fig. 4. Monosaccharide recovery (%) from hydrolysates of WWS and AWS, treated and untreated with *C. rigida* laccase. Error bars indicate standard deviations from mean values.

3.2. Effect of laccase treatment on free sugars and enzymatic hydrolysis

Samples of WWS and AWS were treated with laccases from *C. rigida* (0.5 U mL^{-1}) for 2 h at pH 5, as described above. After laccase treatment, samples were centrifuged, and the liquid fraction (prehydrolysate) containing hemicellulose-derived sugars released during steam-explosion, was analysed for monosaccharide contents (Fig. 3). The results showed that laccase treatment did not influence the free sugars detected on the prehydrolysate. However, glucose, xylose and arabinose recovery was much higher in AWS than in WWS ($p \leqslant 0.5$), and galactose and mannose were not detected in the WWS prehydrolysate. These results agree with those reporting that more severe conditions during steam-explosion (higher temperature, time of residence and acidic conditions) increase hemicellulose solubilisation in the prehydrolysate (Ballesteros et al., 2006; Kabel et al., 2007).

Enzymatic hydrolysis was carried out using the solid fraction as substrate. The highest glucose recovery was obtained in AWS samples after 72 h hydrolysis with cellulases (Fig. 4). As previously

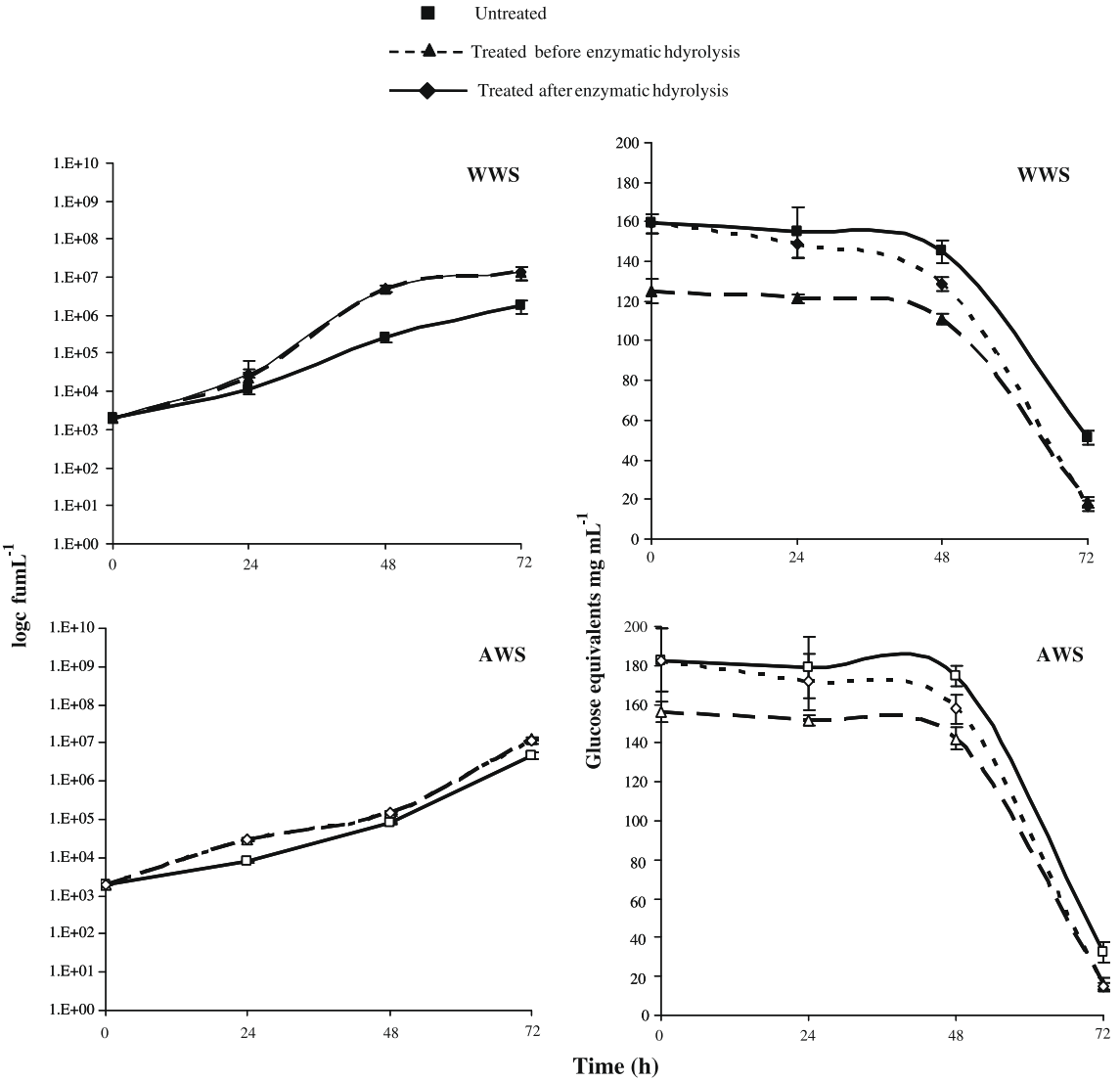

Fig. 5. Time course of reducing sugars consumption and yeast growth (log CFU mL^{-1}) on WWS and AWS hydrolysate treated and untreated with *C. rigida* laccase. Error bars indicate standard deviations from mean values.

mentioned, these harsh conditions dissolved a greater fraction of hemicellulose, increasing accessibility to cellulose and as consequence a higher glucose recovery was produced after the enzymatic hydrolysis (Yang and Wyman, 2004; Saha et al., 2005; Ballesteros et al., 2006).

Previous reports mention the use laccases for detoxification of steam-exploded biomass (sugarcane bagasse and wood), but most of them were performed only after cellulose hydrolysis. However, a matter of major interest is to analyse the possible effect of treatment with laccases on cellulolytic hydrolysis, in order to evaluate the effects of detoxification on cellulose hydrolysis and sugar fermentation separately. The results obtained in this work showed that laccase treatments performed before enzymatic hydrolysis of cellulose resulted in a lower glucose recovery in both WWS and AWS samples at a significant level, the reduction being higher in AWS (Fig. 4). Contradictory results have been reported in this matter. Inhibition of steam-exploded wheat straw hydrolysis (using cocktails of cellulase, xylanase and feruloyl esterase) by laccase treatment has been previously reported (Tabka et al., 2006). The lower glucose recovery after laccase treatment has been explained by the release of phenolic compounds which can inhibit cell wall-degrading enzymes (Akin et al., 1996; Gamble et al., 2000). However, hydrolysis of cellulose was improved by laccase treatment of steam-exploded softwood, and a decrease in the unproductive binding of cellulases to lignin after laccase treatment has been suggested (Palonen and Viikari, 2004). In this work, the higher reduction on glucose recovery after laccase treatment in AWS samples compared to WWS samples indicates an influence on the severity conditions during steam-explosion. Further work is necessary to explore these findings since in addition to the chemical composition and structure of lignocellulosic biomass, other parameters could have importance in the treatment, such as the hydrolysis conditions or the source the enzymes.

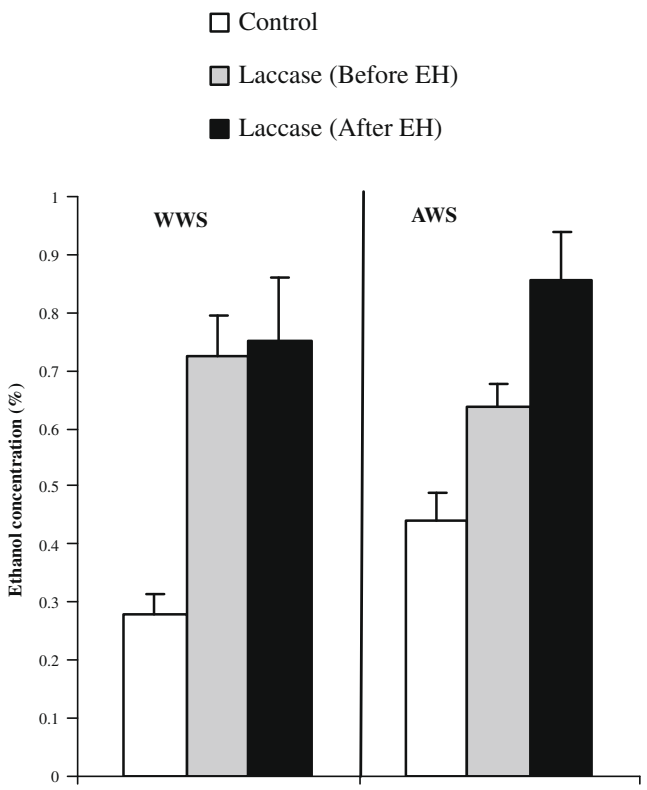

Fig. 6. Ethanol concentration (%) after three days of fermentation in WWS and AWS hydrolysate treated and untreated with *C. rigida* laccase. Error bars indicate standard deviations from mean values.

3.3. Effect of laccase treatment on yeast growth and ethanol production

The effect of enzyme treatment of AWS and WWS on yeast growth (Fig. 5) and ethanol production (Fig. 6) was evaluated. Fermentation was greatly improved after detoxification with laccases. After 3 days of incubation with *S. cerevisiae*, the results showed that yeast concentration, sugar consumption and ethanol yield were higher in samples treated with laccases than in control samples ($p \leqslant 0.5$). The effect of laccase, which removed phenols specifically, demonstrates the inhibitory effect of the phenolic compounds from steam-exploded wheat straw on yeast growth and fermentation of sugars to ethanol, as previously shown in other lignocellulosic materials (Jönsson et al., 1998; Palonen and Viikari, 2004; Martín et al., 2007). Laccase oxidation would decrease the toxic effect of phenolic compounds derived from lignin degradation, such as 4-hydroxy-3,5-dimethoxybenzoic (siringic) acid, 4-hydroxy-3-methoxybenzaldehide (vanillin), and 4-hydroxy-3-methoxybenzoic (vanillic) acid, which have been proven to be important fermentation inhibitors (Jönsson et al., 1998). The inhibition is based on a decrease in the membrane ability as a selective barrier caused by phenols, which reduces both cell growth and sugar assimilation (Palmqvist et al., 1999). The highest ethanol yield was obtained in AWS samples treated with laccase after hydrolysis of cellulose, being explained by both the positive effect of laccase on yeast growth and the higher sugar content in the hydrolysate. In the case of WWS samples, the removal of phenolic compounds increased the ethanol production 2.7 times, whereas in AWS samples the ethanol yield was 2 times higher. These differences would imply that removal of inhibitors may have a different effect depending on the severity conditions used during pretreatment of biomass. These differences could be related to the presence of non phenolic inhibitors in AWS samples, since harsher conditions improve cellulose hydrolysis, but also result in higher concentration of other fermentation inhibitors such as furfural or hydroxyl-methyl-furfurals (Ballesteros et al., 2006; Kabel et al., 2007). It should be also noticed that the removal of phenolic inhibitors seems to influence ethanol conversion more than yeast growth, since yeast counts were not significantly different when comparing detoxified samples of AWS and WWS. Therefore, the results obtained in this work suggest that conditions during pretreatment of biomass should be taken into account to evaluate the efficiency of detoxification methods.

In the present work, only glucose fermentation using *S. cerevisiae* has been considered. However, recently some work has focused in the development of pentose fermenting yeasts (Hahn-Hägerdal et al., 2007). Since inhibitors are mainly hydrosoluble compounds, the prehydrolysate (containing the pentose fraction) is expected to have a higher concentration of toxic compounds, including free phenols. The potential industrial use of strains able to ferment pentoses could also make feasible the use of laccase for detoxification of the prehydrolysates, where the presence of inhibitors could be even more problematic.

4. Conclusions

The removal of free phenols by laccase polymerization reduced the toxic effect on *S. cerevisiae,* resulting in higher yeast growth and improved ethanol production. Detoxification should be better performed after the cellulose hydrolysis in the case of steam-exploded wheat straw, since enzymatic treatment with laccases before enzymatic hydrolysis, slightly decreased glucose recovery in the hydrolysate. This highlights that detoxification methods must be

independently studied for each pretreated material. Some differences were found when comparing the efficiency of detoxification on WWS and AWS samples, which also implies the importance of testing materials pretreated under different conditions when a detoxification method is evaluated.

Acknowledgements

This work was supported by the CENIT project "Ethanol for automation" (Ministerio de Industria-Abengoa Bionergía Nuevas Tecnologías) and the project AGL-2007-66416-C05-02. We thank Dr. M. Ballesteros (CIEMAT) for providing the steam-exploded wheat straw.

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