Different laccase detoxification strategies for ethanol production from lignocellulosic biomass by the thermotolerant yeast *Kluyveromyces marxianus* CECT 10875

Antonio D. Moreno[1], David Ibarra[2*], José L. Fernández[2], Mercedes Ballesteros[2]

[1]Instituto IMDEA Energía, Biological Processes Unit, Móstoles, Madrid 28933, Spain.
[2]CIEMAT, Renewable Energy Division, Biofuels Unit, Avda. Complutense 40, Madrid 28040, Spain.

*Corresponding author: CIEMAT, Renewable Energy Division, Biofuels Unit, Avda. Complutense 40, Madrid 28040, Spain. E-mail address: david.ibarra@ciemat.es. Tel. +34 91 346 6388 Ext. 6388; fax: +34 91 346 0939.

This is the peer reviewed version of the following article:
Moreno, A.D.; Ibarra, D.; Fernández-Rojo, J.L.; Ballesteros, M. Different laccase detoxification strategies for ethanol production from lignocellulosic biomass by the thermotolerant yeast *Kluyveromyces marxianus* CECT 10875. Bioresource Technology 2012, 106:101-109

which has been published in final form at:
https://doi.org/10.1016/j.biortech.2011.11.108

Abstract

In this work, laccase enzymes were evaluated to detoxify the whole slurry from steam-exploded wheat straw. For it, two different strategies, laccase treatment before or after enzymatic hydrolysis, were employed. The detoxification efficiency was analyzed on enzymatic hydrolysis and fermentation levels by the thermotolerant yeast *Kluyveromyces marxianus*. Laccases reduced phenolic compounds without affecting weak acids and furan derivates. A lower glucose recovery was observed when laccase treatments were carried out before enzymatic hydrolysis, phenomenon that was not showed after enzymatic hydrolysis. In contrast, both laccase treatment strategies enhanced ethanol concentrations, reducing significantly the lag phase of the yeast and allowing substrate loading increments of saccharification and fermentation broths.

Keywords: Ethanol; *Kluyveromyces marxianus;* Laccase detoxification; Lignocellulose; Steam explosion

1. Introduction

Lignocellulosic biomass, including agriculture residues, forest products or energy crops, represents one of the most abundant and low cost resources for ethanol production. Its use could significantly decrease fossil fuel consumption and contribute to a green house gas emissions reduction (Hahn-Hägerdal et al., 2006). The process is based on the conversion of carbohydrates contained in lignocellulosic materials to their monomer sugars and its fermentation by microorganisms to ethanol.

Among different conversion processes, enzymatic hydrolysis is a promising method. Unfortunately, due to the lignocellulosic complex structure, the cellulose accessibility to enzymes is limited. Thus, a preliminary pretreatment step is required to improve the enzymatic hydrolysis and increase the fermentable sugars yields (Alvira et al., 2010). Steam explosion, a process that combines high pressures and temperatures, is one of the most commonly used pretreatment methods. During this process, extensive lignocellulosic structure alteration is produced. Lignin is redistributed and hemicellulose is partially hydrolyzed and solubilized, making cellulose more accessible to enzymes (Alvira et al., 2010). In contrast, this pretreatment generates some soluble inhibitory compounds, derived from a partial sugars and lignin degradation, which can affect enzymatic hydrolysis as well as fermentation steps (Palmqvist and Hahn-Hägerdal, 2000a; Klinke et al., 2004; Panagiotou and Olsson, 2007). The nature and concentration of these toxic compounds depend on the raw material and the harshness of the pretreatment. They are classified according to their chemical structure and include furan derivates (furfural and 5-hydroxymethylfurfural derived from pentose and hexose sugars degradation, respectively), weak acids (mainly acetic acid) and phenolic compounds from lignin (aromatic acids, alcohols and aldehydes) (Palmqvist and Hahn-Hägerdal, 2000a).

Several methods have been studied to reduce the effects of inhibitory compounds. Usually, the whole slurry obtained after steam explosion process is filtered and washed before enzymatic hydrolysis. However, from an economical and environmental point of view, the filtration and washing steps should be avoided since they increase both operational costs and wastewater. Moreover, the use of the whole slurry can increase the fermentable sugars concentration, obtaining a higher ethanol production (García-Aparicio et al., 2006). For these reasons, other detoxification methods have been explored (Palmqvist and Hahn-Hägerdal, 2000b; Klinke et al., 2004; Parawira and Tekere, 2011), including the use of enzymes such as laccases (Jönsson et

al., 1998; Larsson et al., 1999; Martín et al., 2002; Chandel, et al., 2007; Jurado et al., 2009).

Laccases are multicopper-containing oxidases with phenoloxidase activity, which catalyze the oxidation of substituted phenols, anilines and aromatic thiols, at the expense of molecular oxygen (Parawira and Tekere, 2011). They have been mainly used to detoxify prehydrolysates (effluents from whole slurry filtration step) from different steam-exploded biomasses (Jönsson et al., 1998; Larsson et al., 1999; Chandel, et al., 2007). Compared to other detoxification methods, the use of laccases involves substrate-specific reactions under mild conditions, fewer toxic sub-products and low energy requirements (Parawira and Tekere, 2011).

In spite of the knowledge gained on the use of laccases for prehydrolysate detoxification, little information is available for whole slurry. The present work studies the detoxification of whole slurry from steam-exploded wheat straw by *Pycnoporus cinnabarinus* and *Trametes villosa* laccases. Laccase treatments were carried out before or after saccharification step at different substrate loadings. An identification and quantification of different inhibitory compounds was carried out. The detoxification efficiency was evaluated on enzymatic hydrolysis and fermentation levels by the thermotolerant yeast *Kluyveromyces marxianus*.

2. Methods

2.1 Enzymes

Two laccases were studied: a laccase from *Pycnoporus cinnabarinus*, produced by Beldem (Belgium), and a laccase from *Tratmetes villosa*, produced by Novozymes (Denmark). Laccase activity was determined by measuring the oxidation of 5 mM 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) buffered with 100 mM sodium acetate (pH 5) at 24 ºC. Formation of the ABTS cation radical was monitored at 436 nm (ε_{436} = 29,300 M^{-1} cm^{-1}).

A mixture NS50013 and NS50010, both produced by Novozymes (Denmark), was employed to evaluate the effect of laccase enzymes on the cellulose enzymatic hydrolysis step. NS50013 is a cellulase preparation which presents low β-glucosidase activity; therefore extra supplementation with NS50010, which mainly presents β-glucosidase activity, is typically applied in biochemical transformation processes of lignocellulosic biomass into ethanol. Overall cellulase activity was determined using

filter paper (Whatman No. 1 filter paper strips) and β-glucosidase activity was measured using cellobiose. The enzymatic activities were followed by the release of reducing sugars (Ghose, 1987).

One unit of enzyme activity was defined as the amount of enzyme that transforms 1 μmol of substrate per minute.

2.2. Raw material and steam explosion pretreatment

Wheat straw, supplied by Ecocarburantes de Castilla y León (Salamanca, Spain), was used as raw material. It was milled, using a laboratory hammer mill, in order to obtain a chip size between 2 and 10 mm, and stored at room temperature until it was used.

The raw material was pretreated by steam explosion in a 10 L reactor at 220 ºC, 2.5 min, according to previous optimization studies based on optimal sugars recovery and enzymatic hydrolysis yield (Ballesteros et al., 2006). After that, one portion of recovered whole slurry was vacuum filtered with the aim of obtaining a liquid fraction or prehydrolysate and a solid fraction, which was thoroughly washed with distillated water to obtain the water insoluble (WIS) fraction.

Chemical composition of both raw and pretreated material (WIS fraction) was determined using the standard Laboratory Analytical Procedures for biomass analysis (LAP-002, LAP-003, LAP-004, LAP-017 and LAP-019) provided by the National Renewable Energies Laboratory (NREL, 2007). Sugars and degradation compounds contained in the prehydrolysate were also measured. Most of the sugars present in the prehydrolysate were in oligomeric form, because of that a mild acid hydrolysis (4% (v/v) H_2SO_4, 120 ºC and 30 min) was needed for the purpose of obtaining monomeric sugars for analysis.

2.3. Microorganism and growth conditions

The thermotolerant yeast *Kluyveromyces marxianus* CECT 10875, which was selected in our laboratory (Ballesteros et al., 1991), was used to evaluate the effect of laccase enzymes in the fermentation step. Active cultures for inoculation were obtained in 100 mL Erlenmeyer flasks with 50 mL of growth medium containing 30 g/L glucose, 5 g/L yeast extract, 2 g/L NH_4Cl, 1 g/L KH_2PO_4, and 0.3 g/L $MgSO_4 \cdot 7H_2O$. After 16 h on a rotary shaker at 150 rpm and 42 ºC, the preculture was centrifuged at 9000 rpm for

10 min. Supernatant was discarded and cells were washed once with distillated water and then dilute to obtain an inoculum level of 1 g/L (dry weight).

2.4. Laccase treatment of steam-exploded wheat straw

The detoxification assays were performed on whole slurry. In a first set of experiments, the laccase treatments were carried out before enzymatic hydrolysis of slurry (strategy 1). The slurry was dilute with 50 mM citrate buffer pH 5 to give final concentrations, based on % (w/v) total solid content present in the slurry, of 5%, 6% and 7%. An enzyme loading of 10 IU/g (dry weight sample) of *P. cinnabarinus* or *T. villosa* laccases was added to dilute slurries and then incubated at 30 ºC (*T. villosa* laccase) or 50 ºC (*P. cinnabarinus* laccase), according to their optimal temperatures (Ibarra et al., 2006), in a rotary shaker (150 rpm) for 3 h. With the purpose to evaluate the effect of laccases on inhibitory compounds, laccase treated samples from dilute slurry at 5% (w/v) were centrifuged, and the supernatants were taken and analyzed for identification and quantification of inhibitory compounds.

In a second set of experiments, laccase treatments were carried out under the same conditions but after enzymatic hydrolysis of wheat straw slurry (strategy 2).

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

2.5. Enzymatic hydrolysis of wheat straw slurry and fermentation of the detoxified hydrolysate

According to laccase detoxification strategy 1, dilute slurries treated with laccase enzymes were subsequently subjected to enzymatic hydrolysis at 50 ºC for 72 h in a rotary shaker (150 rpm), adding an enzyme loading of 15 FPU/g (dry weight sample) of NS50013 and 15 IU/g (dry weight sample) of NS50010. In the case of laccase detoxification strategy 2, dilute slurries were subjected to enzymatic hydrolysis, under the same conditions previously mentioned, and the resulting hydrolysates were treated with laccase enzymes as explained above. In both strategies, samples from detoxified hydrolysates were taken, centrifuged and the supernatants analyzed for glucose concentration and identification and quantification of inhibitory compounds. Enzymatic hydrolysis yields were calculated as the ratio of glucose released after 72 h of enzymatic hydrolysis divided by potential glucose (glucose content in WIS fraction and glucose and oligomeric form glucose content in prehydrolysate).

Detoxified hydrolysates at 5%, 6% and 7% (w/v), resulting from both laccase detoxification strategies, were supplemented with 5 g/L yeast extract, 2 g/L NH_4Cl, 1 g/L KH_2PO_4, and 0.3 g/L $MgSO_4 \cdot 7H_2O$. The pH was adjusted to 5.5 with NaOH 10 M, the temperature was reduced to 42 ºC and 1 g/L (dry weight) of inoculum was added. The fermentation was performed in a rotary shaker (150 rpm) for another 72 h under no sterile conditions. Samples were taken after 3, 6, 9, 12, 15, 24, 48 and 72 h, centrifuged and the supernatants analyzed for glucose consumption and ethanol concentration.

In order to compare enzymatic detoxification by laccase enzymes with traditional detoxification by filtration and washing of slurry, WIS fraction at 5.2% (w/v) of substrate loading (WIS content corresponding to slurry at 7% (w/v) of total solid content, based on composition of recovered whole slurry) was submitted to enzymatic hydrolysis and fermentation steps as described above.

2.6. Analytical methods

Ethanol was analyzed by gas chromatography, using a 7890A GC System (Agilent Tecnology) equipped with an Agilent 7683B series injector, a flame ionization detector and a column of Carbowax 20 M at 85 ºC. Injector and detector temperature was maintained at 175 ºC.

Sugar concentration was quantified by high-performance liquid chromatography (HPLC) in a Waters chromatograph equipped with a refractive index detector (Waters, Mildford, MA). A CarboSep CHO-682 carbohydrate analysis column (Transgenomic, San Jose, CA) operated at 80 ºC with ultrapure water as a mobile-phase (0.5 mL/min) was employed for the separation.

Furfural, 5-hydroxymethylfurfural (5-HMF), vanillin and syringaldehyde were analyzed by HPLC (Agilent, Waldbronn, Germany), using an Aminex ion exclusion HPX-87H cation-exchange column (Bio-Rad Labs, Hercules, CA) at 65 ºC equipped with a 1050 photodiode-array detector (Agilent, Waldbronn, Germany). As mobile phase, 89% 5 mM H_2SO_4 and 11% acetonitrile at a flow rate of 0.7 mL/min were used.

Formic acid, acetic acid, *p*-coumaric acid and ferulic acid were also quantified by HPLC (Waters) using a 2414 refractive index detector (Waters) and a Bio-Rad Aminex HPX-87H (Bio-Rad Labs) column maintained at 65 ºC with a mobile phase (5 mmol/L H2SO4) at a flow rate of 0.6 mL/min.

All analytical values were calculated from duplicates or triplicates and average results are shown.

3. Results and discussion

3.1. Pretreatment of raw material

Prior to steam explosion pretreatment, wheat straw was characterized with the following composition (% dry weight): cellulose, 40.5; hemicellulose, 26.1 (xylan, 22.7; arabinan 2.1; and galactan, 1.3); lignin, 18.1; ashes, 5.1; and extractives, 14.6. After pretreatment, slurry with a total solids content of 21.56% (w/v) was recovered. WIS content of the slurry was 16.07% (w/v).

Table 1 summarizes WIS and prehydrolysate composition. Compared to cellulose content of untreated raw material (40.5%), pretreatment increased the cellulose proportion of WIS (66.6 %) due to the extensive solubilization and degradation of the hemicellulose fraction, as reflected the high xylose content (12 g/L) and degradation products recovered in the prehydrolysate. Among these degradation products, furfural, 5-HMF, acetic acid, and formic acid were formed at highest concentration. Furfural and 5-HMF are derived from pentoses (mainly xylose) and hexoses degradation, respectively. Acetic acid is formed by the hydrolysis of acetyl groups in hemicellulose and formic acid derives from furfural and 5-HMF degradation (Oliva et al., 2003). In addition, the prehydrolysate also showed low amounts of some phenolic compounds. These compounds included aldehydes such as vanillin, derived from guaiacyl propane units, and syringaldehyde, released from syringylpropane units present in lignin (García-Aparicio et al., 2006); and acids such as ferulic acid and *p*-coumaric acid, both derived from *p*-hydroxycinnamic acids, characteristic of herbaceous plants forming cross-linkages between lignin and hemicellulose (Sun and Cheng, 2002). Several authors have previously identified the same degradation products in prehydrolysates from steam-exploded wheat straw (Tomás-Pejó et al., 2009; Alvira et al., 2010).

3.2. Effect of laccase treatments on degradation compounds

Preliminary studies were carried out to evaluate the effect of laccases on degradation compounds (Table 2). Compared to control samples, the results showed that laccases reduced strongly the phenolic content of the prehydrolysate (reduction between 88-92%). However, the concentration of weak acids and furan derivates were not altered, in agreement with previous studies (Larsson et al., 1999; Martín et al., 2002; Chandel, et al., 2007). In contrast to laccase action, other detoxification methods have

shown less substrate specificity. For example, anion exchange treatment at pH 10 of dilute acid prehydrolysate from steam-exploded spruce led to more than 80% decrease of total phenolics, all weak acids and 70% of furan derivates (Larsson et al., 1999). In the same study, the effect of adjusting the prehydrolysate to a pH of 10, using overliming (addition of $Ca(OH)_2$) or NaOH, only decreased by 20% furan derivates and total phenolics, whereas weak acids were not affected (Larsson et al., 1999). Wilson et al., (1989) reported a decrease in the concentration of acetic acid, furfural and vanillin by 54%, 100% and 29%, respectively, after roto-evaporation of dilute acid prehydrolysate from steam-exploded aspen. Palmqvist et al., (1997) described the capacity of *Trichoderma reesei* to remove acetic acid, furfural and benzoic acid derivates from the prehydrolysate obtained after steam explosion of willow. Treatment of acid sugarcane bagasse hydrolysate with activated charcoal caused 39%, 57% and 47% reduction in furan derivates, phenolics and acetic acid, respectively (Chandel et al., 2007).

The mechanism behind the effect obtained with laccase enzymes is documented. Laccases catalyze the oxidation of phenolic compounds generating unstable phenoxy radicals that lead to polymerization into less toxic aromatic compounds (Jönsson et al., 1998; Jurado et al., 2009). Among identified phenolic compounds, vanillin was less susceptible to laccase oxidation (Table 2), according to their higher redox potential (Camarero et al., 2005). The electron donor effect of methoxy substituents at the benzenic ring enhances laccase activity due to a decreased redox potential. In this way, vanillin, with one methoxy substituent, is slowly oxidized by laccase than syringaldehyde, which presents two methoxy groups. Moreover, laccase enzymes are more active toward *p*-hydroxycinnamic acids than simple phenols, explaining the complete removal of both ferulic acid and *p*-coumaric acid observed (Table 2). This is due to the presence of conjugated double-bond system in *p*-hydroxycinnamic acids side chains, which facilitates the electron abstraction (Camarero et al., 2008).

3.3. Effect of laccase treatments on enzymatic hydrolysis

An important aspect for ethanol production is the substrate loading. By increasing substrate loading during enzymatic hydrolysis leads to increased sugar content and higher final ethanol concentration after fermentation. This approach could reduce operational cost for hydrolysis and fermentation processes and minimize energy consumption during subsequent distillation and evaporation stages, making

lignocellulosic ethanol production economically feasible (Banerjee et al., 2009). With this purpose, the whole slurry was dilute at 5%, 6% and 7% (w/v) of total solids content and hydrolyzed before or after laccase treatments, as described above. After that, samples from control and detoxified hydrolysates, resulting from both laccase detoxification strategies, were analyzed for glucose concentration and identification and quantification of inhibitory compounds (Table 3 and 4). As expected for control samples, the concentration of glucose increased as a function of substrate concentration, and the highest glucose concentration (32.3 g/L and 31.9 g/L under the strategies 1 and 2, respectively) were obtained after 72 h of enzymatic hydrolysis at 7% (w/v) (Table 3). In contrast, higher amounts of degradation compounds were observed (Table 4). These compounds not only are present in the prehydrolysates, they can also be trapped in the pretreated biomass and be released during its enzymatic hydrolysis or washing (García-Aparicio et al., 2006; Gurram et al., 2011).

The cumulative concentration of degradation compounds can result in a major inhibition effects on enzymatic hydrolysis. Among the different compounds identified herein (Table 4), the phenolic compounds have been described to be an important cause of reduced both rate and yields of cellulose hydrolysis (Ximenes et al., 2010; Ximenes et al., 2011). Vanillin and syringaldehyde inhibit cellulose enzymes, especially β-glucosidases, whereas ferulic acid and *p*-coumaric acid deactivate them (Ximenes et al., 2010; Ximenes et al., 2011). Nevertheless, the rest of identified degradation compounds as well as end-sugars accumulation have been also shown to be inhibitory to cellulose enzymes (García-Aparicio et al., 2006; Panagiotou and Olsson, 2007). In spite of these inhibitory effects reported, the increments of degradation compounds observed at higher substrate loadings did not affect strongly the yields of cellulose hydrolysis, showing yields of 81% and 79% at 5% (w/v) and 7% (w/v) of enzymatic hydrolysis, respectively.

Laccase treatments reduced strongly the phenolic content of the different dilute hydrolysates (Table 4). The highest reduction (93-94%) was achieved when laccase treatments were carried out before enzymatic hydrolysis. However, laccase treatments after enzymatic hydrolysis produced lower removal rates (73-84%). As observed above, in both strategies vanillin was again the less susceptible to laccase action.

The glucose recovery did not improve when laccase treatments were carried out before enzymatic hydrolysis, despite the great phenolic content reduction observed. On the contrary, the laccase treatments resulted in a slightly lower glucose recovery (Table 3). Contradictory results have been reported in this matter. Tabka et al. (2006) and

Jurado et al. (2009) described a lower glucose recovery after enzymatic hydrolysis of steam-exploded wheat straw treated with laccase, phenomenon that attributed to the release of certain phenolic compounds by laccases inhibiting cellulolitic enzymes. Martín et al. (2002) observed a similar phenomenon when steam-exploded sugarcane bagasse was treated with laccase enzymes. Other detoxification methods, such as anion exchange treatment at pH 10 and the use of fungus *Trichoderma reesei*, have also shown a considerable loss of fermentable sugars (Palmqvist et al., 1997; Larsson et al., 1999). On the other hand, laccase treatment of steam-exploded softwood improved the glucose yield obtained during cellulose hydrolysis (Palonen and Viikari, 2004), enhancement that could be due to removal of inhibitory compounds or reduction of non-productive binding of cellulolytic enzymes to lignin. Our results together with the information found in the literature make necessary further studies to understand the influence of laccase enzymes on enzymatic hydrolysis.

In contrast to strategy 1, laccase treatment after enzymatic hydrolysis did not affect the glucose content detected in the hydrolysates, attaining the same glucose content in both control and treated laccase samples (Table3).

3.4. Effect of laccase treatments on fermentation

Control and detoxified hydrolysates at 5%, 6% and 7% (w/v) of total solids content, resulting from both laccase detoxification strategies, were submitted to fermentation process using the thermotolerant yeast *Kluyveromyces marxianus* CECT 10875, a strain adapted and selected in our laboratory (Ballesteros et al., 1991). This yeast, together with other strains recently discovered (Kwon et al., 2011), is gaining great significance due to its capability of growing and fermenting at temperature above 40 ºC, close to the optimum temperatures of enzymatic hydrolysis, which can lead to an integration of both enzymatic hydrolysis and fermentation processes. The fermentation step was performed for 72 h, taking samples at different times for glucose consumption and ethanol production analyses.

Time courses for glucose consumption and ethanol production during fermentation process of control and detoxified hydrolysates at different total solids content are illustrated in Fig. 1 (strategy 1) and 2 (strategy 2). As seen in both strategies for control samples, *K. marxianus* showed slower glucose consumption and ethanol production rates with increasing total solids content. In the case of strategy 1, fermentation of control samples at 5% (w/v) consumed glucose and produced ethanol

much faster (12 h) than 6 % (w/v) (24 h). Similar pattern, thought more extended, was observed for strategy 2. This phenomenon is due to the extension of the yeast lag phase, period that the microorganism needs to adapt to fermentation conditions and which duration is related with the inhibitory compounds type and their concentrations (Palmqvist and Hahn-Hägerdal, 2000a; Klinke et al., 2004). At 7% (w/v) of substrate loading the yeast was practically inhibited in both strategies and neither glucose consumption nor ethanol production was observed after 72 h of fermentation. Moreover, the ethanol yields decreased, from 0.36 g/g at 5% (w/v) to 0.32 g/g at 6% (w/v) for strategy 1 and from 0.36 g/g at 5% (w/v) to 0.30 g/g at 6% (w/v) for strategy 2; and barely ethanol production was observed at 7% (w/v) (Table 3). All these negative effects could be explained by the increments of degradation compounds at higher substrate loadings (Table 4), which could affect negatively the yeast fermentation performance by cumulative concentration of them. Nevertheless, in previous experiments the inhibitory compounds concentrations found herein did not show negative effects on *K. marxianus* (Oliva et al., 2003). For this reason, probably inhibition by synergistic effects between different degradation compounds could also occur (Palmqvist and Hahn-Hägerdal, 2000a; Klinke et al., 2004; Panagiotou and Olsson, 2007; da Cunha-Pereira et al., 2011).

The inhibitory effects observed, together with reduction in the specific yeast growth rate and decrease in biomass production, have been reported on different fermenting microorganisms (Palmqvist and Hahn-Hägerdal, 2000a; Klinke et al., 2004). Regarding *K. marxianus*, several negative effects have been attributed to different degradation compounds, either individually (Oliva et al., 2003) or combined (Oliva et al., 2006). Furfural showed a strong inhibition of growth and ethanol production (Oliva et al., 2003; Oliva et al., 2006), due to a direct inhibition of both glycolytic and non-glycolytic enzymes of the yeast (Palmqvist and Hahn-Hägerdal, 2000a; Klinke et al., 2004). On the other hand, *K. marxianus* was resistant to HMF (Oliva et al., 2003). Weak acids, such as acetic acid and formic acid, produced negative effects mainly on biomass production (Oliva et al., 2003; Oliva et al., 2006), as a result of a disruption of intracellular pH by its accumulation in the yeast cells (Palmqvist and Hahn-Hägerdal, 2000a; Klinke et al., 2004). Nevertheless, fermentation pH conditions used in this study (pH 5.5) reduce considerably the toxicity of these acids (Oliva et al., 2003). Vanillin and syringaldehyde showed similar inhibitory effects than furfural (Oliva et al., 2003; Oliva et al., 2006), although different inhibition mechanisms. They produce cell damage

and direct interferences with biological membranes, which affect their ability to serve as selective barriers and enzymes matrices (Palmqvist and Hahn-Hägerdal, 2000a; Klinke et al., 2004). Finally, inhibitory effects of *p*-coumaric acid and ferulic acid have been described on *Saccharomyces cerevisiae* (Almeida et al., 2007), but not on *K. marxianus*. Similar to vanillin and syringaldehyde, they may act on biological membranes, causing loss of integrity.

Both laccase detoxification strategies enhanced the fermentation performance of *K. marxianus*, showing better fermentation parameters when laccase treatments were carried out before enzymatic hydrolysis. The barley phenolic content of laccase detoxified hydrolysates resulted in a shortening of *K. marxianus* lag phase. It can be inferred from faster glucose consumption and ethanol production rates obtained, as illustrated for different tested substrate loadings in Fig. 1 and 2, and higher ethanol volumetric productivity values achieved (Table 3). Moreover, laccase treatments enhanced the ethanol yields (Table 3). At 5% (w/v) of total solids content this improvement was very slight, from 0.36 g/g for control samples to 0.37-0.38 g/g for laccase detoxified samples. However, at 6% (w/v) the yield increases were more pronounced, from 0.32 g/g for control samples to 0.37-0.38 g/g for laccase detoxified samples in strategy 1; and from 0.30 g/g for control samples to 0.35 g/g for laccase detoxified samples in strategy 2. Finally, at 7% (w/v) of substrate loading laccase treatments triggered the fermentation of hydrolysates by *K. marxianus*, and highest ethanol concentrations, close to 11 g/L (Table 3), were obtained. These results are comparable to fermentation parameters of yeast when hydrolysate from WIS fraction at 5.2% (w/v) of substrate loading was used, obtaining both ethanol yield and concentration in the same range (Table 3). Nevertheless, faster glucose consumption and ethanol production rates were observed (Fig. 3) and higher ethanol volumetric productivity values achieved (Table 3) with the filtration and washing of slurry.

Similar enhancements of fermentation performances have been described with other microorganisms on different laccase detoxified materials types. Jönsson et al. (1998) and Larsson et al. (1999) reported higher glucose consumption rates, ethanol volumetric productivities, and ethanol yields when prehydrolysates from steam-exploded wood were submitted to laccase treatments and fermented with *S. cerevisiae*. In the same way, Martín et al. (2002) described similar improvements when enzymatic hydrolysates from steam-exploded sugarcane bagasse were treated with laccase enzymes and fermented using a strain recombinant xylose-utilising of *S. cerevisiae*; and

Chandel et al. (2007) when acid hydrolysates from sugarcane bagasse were detoxified by laccases and fermented with *Candida sehatae*.

Only detoxification by anion exchange treatment at pH 10, overliming and activated charcoal have resulted in higher ethanol yields and productivities than laccase treatment (Larsson et al., 1999; Martín et al., 2002; Chandel et al., 2007). However, these methods need additional operations that increase the processing costs. In contrast, laccases offer the possibility to be used directly, before or after enzymatic saccharification, in the fermentation vessel prior to fermentation, as shown in this study. Nevertheless, the integration level should proceed towards a one single step that integrates the laccase detoxification, saccharification and fermentation processes. With this purpose, new studies are ongoing.

4. Conclusions

A substantial removal of phenolic compounds by laccases reduced the inhibitory effects of slurry from steam-exploded wheat straw. It led to improve the fermentation performance of *K. marxianus* strain used, shortening its lag phase and enhancing the ethanol yields, and increase the substrate loadings of saccharification and fermentation broths. These enhancements were more significant when laccase treatment was carried out before enzymatic hydrolysis, in spite of decreased glucose recovery. According to this study, detoxification by laccases could reduce costs of lignocellulosic ethanol process through the use of partially detoxified whole slurry and increasing higher fermentation rates and ethanol yields.

Acknowledgements

This work has been supported by the RESTOENE project (Consejería de Educación-Comunidad de Madrid, Spain). A.D.M. acknowledges a CPI contract (Consejería de Educación-Comunidad de Madrid, Spain) and D.I. acknowledges a "Juan de la Cierva" contract (Ministerio de Ciencia e Innovación, Spain).

References

Almeida, J.R.M., Modig, T., Petersson, A., Hahn-Hägerdal, B., Liden, G., Gorwa-Grauslund, M.F., 2007. Increased tolerance and conversion of inhibitors in lignocellulose hydrolysates by *Saccharomyces cerevisiae*. J. Chem. Technol. Biotechnol. 82. 340−349.

447 Alvira, P., Tomás-Pejó, E., Ballesteros, M., Negro, M.J., 2010. Pretreatment technologies for an
448 efficient bioethanol production process based on enzymatic hydrolysis: A review. Bioresour.
449 Technol. 101, 4851−4861.

450 Ballesteros, I., Ballesteros, M., Cabañas, A., Carrasco, J., Martín, C., Negro, M.J., Saez, F.,
451 Saez, R., 1991. Selection of thermotolerant yeasts for simultaneous saccharification and
452 fermentation (SSF) of cellulose to ethanol. Appl. Biochem. Biotechnol. 28, 307–315.

453 Ballesteros, I., Negro, M.J., Oliva, J.M., Cabañas, A., Manzanares, P., Ballesteros, M., 2006.
454 Ethanol production from steam-explosion pretreated wheat straw. Appl. Biochem.
455 Biotechnol. 129, 496–508.

456 Banerjee, S., Mudliar, S., Sen, R., Giri, B., Staputi, D., Chakrrabarti, T et al., 2009.
457 Commercializing lignocellulosic bioethanol: technology bottlenecks and possible
458 remedies. Biofuels Bioprod. Bioref. 4, 77–93.

459 Camarero, S., Ibarra, D., Martínez, M.J., Martínez, A., 2005. Lignin-derived compounds as
460 efficient laccase mediators for decolorization of different types of recalcitrant dyes.
461 Appl. Environ. Microbiol. 71, 1775–1784.

462 Camarero, S., Cañas, A.I., Nousiainen, P., Record, E., Lomascolo, A., Martínez, M.J., Martínez
463 A., 2008. *p*-Hydroxycinnamic acids as natural mediators for laccase detoxification of
464 recalcitrant compounds. Environ. Sci. Technol. 42, 6703–6709.

465 da Cunha-Pereira, F., Hickert, L.R., Sehnem, N, T., de Souza-Cruz, P.B., Rosa, C.A., Ayub,
466 M.A.Z., 2011. Conversion of sugars present in rice hull hydrolysates into ethanol by
467 *Spathaspora arborariae*, *Saccharomyces cerevisiae*, and their con-fermentations.
468 Bioresour. Technol. 102, 4218–4225.

469 Chandel, A.K., Kapoor, R.K., Singh, A., Kuhad, R.C., 2007. Detoxification of sugarcane
470 bagasse hydrolysate improves ethanol production by *Candida shehatae* NCIM 3501.
471 Bioresour. Technol. 98. 1947−1950.

472 García-Aparicio, M.P., Ballesteros, I., González, A., Oliva, J.M., Ballesteros, M., Negro, M.J.,
473 2006. Effect of inhibitors released during steam-explosion pretreatment of barley straw
474 on enzymatic hydrolysis. Appl. Biochem. Biotechnol. 129, 278−288.

475 Ghose, T.K., 1987. Measurement of cellulase activity. Pure Appl. Chem. 59, 257–68.

476 Gurram, R.N., Datta, S., Lin, Y.J., Snyder, S.W., Menkhaus, T.J., 2011. Removal of enzymatic
477 and fermentation inhibitory compounds from biomass slurries for enhanced biorefinery
478 process efficiencies. Bioresour. Technol. 102, 7850−7859.

479 Han-Hägerdal, B., Galbe, M., Gorwa-Grauslund, M.F., Liden, G., Zacchi, G., 2006. Bio-ethanol
480 – the fuel of tomorrow from the residues of today. Trends Biotechnol. 24, 549−556.

481 Ibarra, D., Romero, J., Martínez, M.J., Martínez, A.T., Camarero, S., 2006. Exploring the
482 enzymatic parameters for optimal delignification of eucalypt pulp by laccase-mediator.
483 Enzyme Microbiol. Technol. 39, 1319−1327.

484 Jurado, M., Prieto, A., Martínez-Alcalá, A., Martínez, A.T., Martínez, M.J. 2009. Laccase
485 detoxification of steam-exploded wheat straw for second generation bioethanol.
486 Bioresour. Technol. 100, 6378−6384.

487 Jönsson, J.L., Palmqvist, E., Nilvebrant, N.-O., Hahn-Hägerdal, B., 1998. Detoxification of
488 wood hydrolysates with laccase and peroxidase from the white-rot fungus *Trametes*
489 *versicolor*. Appl. Microbiol. Biotechnol. 49, 691−697.

490 Kwon Y-J., Ma, A-Z., Li, Q., Wang, F, Zhuang, G-Q., Liu, C-Z., 2011. Effect of lignocellulosic
491 inhibitory compounds on growth and ethanol fermentation of newly-isolated
492 thermotolerant *Issatchenkia orientalis*. Bioresour. Thecnol. 102, 8099−8104.

493 Klinke, H.B., Thomsen, A.B., Ahring, B.K., 2004. Inhibition of ethanol-producing yeast and
494 bacteria by degradation products produced during pretreatment of biomass. Appl.
495 Microbiol. Biotechnol. 66, 10−26.

496 Larsson, S., Reimann, A., Nilvebrant, N.-O., Jönsson, J.L., 1999. Comparison of different
497 methods for the detoxification of lignocellulose hydrolyzates of spruce. Appl. Microbiol.
498 Biotechnol. 77−79, 91−103.

499 Martín, C., Galbe, M., Wahlbom, C.F., Hahn-Hägerdal, B., Jönsson, J.L., 2002. Ethanol
500 production from enzymatic hydrolysates of sugarcane bagasse using recombinant
501 xylose-utilising *Saccharomyces cerevisae*. Enzyme Microbiol. Technol. 31, 274−282.

502 NREL, 2007. Chemical Analysis and Testing Laboratory Analytical Procedures. National
503 Renewable Energy Laboratory, Golden, CO.

504 Oliva, J.M., Saez, F., Ballesteros, I., González, A., Negro, M.J., Manzanares, P, et al., 2003.
505 Effect of lignocellulosic degradation compounds from steam explosion pretreatment on

ethanol fermentation by thermotolerant yeast *Kluyveromyces marxianus*. Appl. Biochem. Biotechnol. 105, 141−154.

Oliva, J.M., Negro, M.J., Sáez, F., Ballesteros, I., Manzanares, P., González, A., Ballesteros, M., 2006. Effects of acetic acic, furfural and catechol combinations on ethanol fermentation of *Kluyveromyces marxianus*. Process Biochem. 41, 1223−1228.

Palmqvist, E., Hahn-Hägerdal, B., Szengyel, Z., Zacchi, G., Reczey, K., 1997. Simultaneous detoxification and enzyme production of hemicellulose hydrolysates obtained after stem pretreatment. Enzyme Microbiol. Technol. 20, 286−293.

Palmqvist, E., Hahn-Hagerdal, B., 2000a. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanism of inhibition. Bioresour. Technol. 74, 25–33.

Palmqvist, E., Hahn-Hagerdal, B., 2000b. Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification. Bioresour. Technol. 74, 17–24.

Palonen, H., Viikari, L., 2004. Role of oxidative enzymatic treatments on enzymatic hydrolysis of softwood. Biotechnol. Bioeng. 86, 550–557.

Panagiotou, G., Olsson, L., 2007. Effect of compounds released during pre-treatment of wheat straw on microbial growth and enzymatic hydrolysis rates. Biotechnol. Bioeng. 96, 250−258.

Parawira, W., Tekere, M., 2011. Biotechnological strategies to overcome inhibitors in lignocellulose hydrolysates for ethanol production: review. Critical Reviews Biotechnol. 31, 20–31.

Sun, Y., Cheng, J., 2002. Hydrolysis of lignocellulosic materials for ethanol production: A review. Bioresource Technol. 83, 1–11.

Tabka, M.G., Herpoel-Gimbert, I., Monod, F., Asther, M., Sigoillot, J.C., 2006. Enzymatic saccharification of wheat straw for bioethanol production by a combined cellulase, xylanase and feruloyl esterase treatment. Enzyme Microb. Technol. 39, 897–902.

Tomás-Pejó, E., Oliva, J.M., González, A., Ballesteros, I., Ballesteros, M., 2009. Bioethanol production from wheat straw by the thermotolerant yeast *Kluyveromyces marxianus* CECT 10875 in a simultaneous saccharification and fermentation fed-batch process. Fuel. 88, 2142−2147.

Wilson, J.J., Deschatelets, L., Nishikawa, N.K., 1989. Comparative fermentability of enzymatic and acid hydrolysates of steam-pretreated aspenwood hemicellulose by *Pichia stipitis* CBS 5776. Appl. Microbiol. Biotechnol. 31, 592−596.

Ximenes, E., Kim, Y., Mosier, N., Dien, B., Ladisch, M., 2010. Inhibition of cellulases by phenols. Enzyme Microb. Technol. 46, 170−176.

Ximenes, E., Kim, Y., Mosier, N., Dien, B., Ladisch, M., 2011. Deactivation of cellulases by phenols. Enzyme Microb. Technol. 48, 54−60.

Table captions

Table 1. Composition of steam-exploded wheat straw.

Table 2. Inhibitory compounds composition of samples resulting from control and laccase treated 5% (w/v) slurries.

Table 3. Summary of hydrolysis and fermentation assays after different laccase detoxification strategies.

Table 4. Inhibitory compounds composition of hydrolysates resulting from control and laccase treated dilute slurries.

Figure captions

Figure 1. Time course for ethanol production (filled symbols and continuous lines) and glucose consumption (open symbols and discontinuous lines) during fermentation of hydrolysates resulting from detoxification strategy 1. Symbols used: *P. cinnabarinus* (▲, △); *T. villosa* (●, ○); and control samples (■, □). Dilute slurry at 5% (A), 6% (B) and 7% (w/v) (C) of substrate loading.

Figure 2. Time course for ethanol production (filled symbols and continuous lines) and glucose consumption (open symbols and discontinuous lines) during fermentation of hydrolysates resulting from detoxification strategy 2. Symbols used: *P. cinnabarinus* (▲, △); *T. villosa* (●, ○); and control samples (■, □). Dilute slurry at 5% (A), 6% (B) and 7% (w/v) (C) of substrate loading.

Figure 3. Time course for ethanol production (filled symbols and continuous line) and glucose consumption (open symbols and discontinuous lines) during fermentation of hydrolysates from WIS fraction at 5.2% (w/v) of substrate loading.

Table 1

Table 1. Composition of steam-exploded wheat straw.

WIS		**Prehydrolysate**				
Component	**% Dry weight**	**Monosaccharides**	**Monomeric form (g/L)**	**Oligomeric form (g/L)**	**Inhibitors**	**g/L**
Cellulose	66.6	Glucose	0.98	3.49	Furfural	3.5
Hemicellulose	1.95	Xylose	3.94	8.07	5-HMF	1.1
Lignin	36.7	Arabinose	0.27	0.20	Acetic acid	11.9
		Galactose	0.31	0.19	Formic acid	9.3
		Mannose	0.09	0.38	Vanillin	0.05
					Syringaldehyde	0.03
					p-Coumaric acid	0.02
					Ferulic acid	0.03

Table 2

Table 2. Inhibitory compounds composition of samples resulting from control and laccase treated 5% (w/v) slurries.

Inhibitors	**Slurry 5% (w/v)**		
	C	**PC**	**TV**
Furfural	427	395	395
5-HMF	158	125	124
Acetic acid	2024	1852	1830
Formic acid	1508	nq	1309
Vanillin	21	9	6
Syringaldehyde	10	0	0
p-Coumaric acid	17	0	0
Ferulic acid	26	0	0

nq, not quantified; C, control samples; PC, *P. cinnabarinus* laccase treated samples; TV, *T. villosa* laccase treated samples; concentrations expressed as mg/L.

Table 3

Table 3. Summary of hydrolysis and fermentation assays after different laccase detoxification strategies.

Empty Cell	**Substrate loading (w/v)**	**Sample**	**$EtOH_M$ (g/L)**	**Glu (g/L)**	**$Y_{E/G}$ (g/g)**	**Q_E (g/L h)**
S1	5%	C	8.1	22.6	0.36	0.47¤
		PC	8.0	21.4	0.37	0.89¤
		TV	8.1	21.5	0.38	0.89¤
	6%	C	8.8	27.0	0.32	0.13†
		PC	8.9	23.4	0.38	0.71†
		TV	9.5	25.8	0.37	0.77†
	7%	C	1.3	32.3	0.04	0.05‡
		PC	9.8	26.6	0.37	0.40‡
		TV	10.8	30.4	0.36	0.45‡
S2	5%	C	8.1	22.4	0.36	0.16†
		PC	8.3	22.8	0.37	0.69†
		TV	8.5	22.5	0.38	0.45†
	6%	C	8.2	27.5	0.30	0.05‡
		PC	9.5	27.4	0.35	0.40‡
		TV	9.5	27.4	0.35	0.39‡
	7%	C	0.8	31.9	0.03	0.02§
		PC	10.4	31.7	0.33	0.22§
		TV	10.9	30.9	0.35	0.23§
WIS			10.7	29.2	0.36	1.69*

S1, strategy 1; S2, strategy 2; C, control samples; PC, *P. cinnabarinus* laccase treated samples; TV, *T. villosa* laccase treated samples; $EtOH_M$, maximum ethanol concentration; Glu, glucose produced after 72 h of enzymatic hydrolysis; $Y_{E/G}$, ethanol yield based on the glucose produced after 72 h of enzymatic hydrolysis; Q_E, volumetric ethanol productivity based on time when maximum ethanol concentration is achieved: 6 h (*), 9 h (¤), 12 h (†), 24 h (‡) and 48 h (§); WIS fraction at 5.2% (w/v) of substrate loading (WIS content corresponding to slurry at 7% (w/v) of total solid content, based on composition of recovered whole slurry).

604 Table 4

605

606 Table 4. Inhibitory compounds composition of hydrolysates resulting from control and laccase treated
607 dilute slurries.

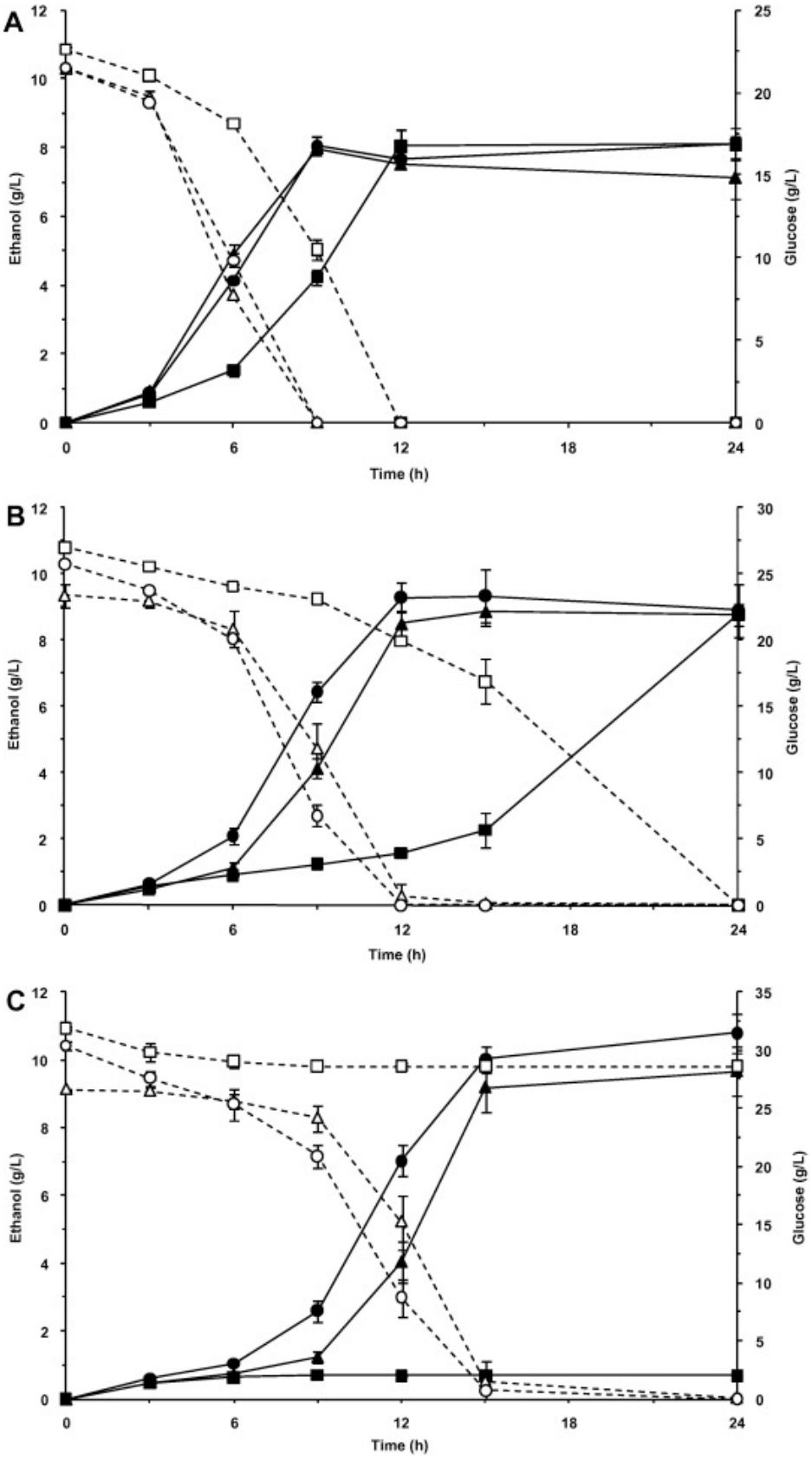
Empty Cell	**Inhibitors**	**Slurry 5% (w/v)**			**Slurry 6% (w/v)**			**Slurry 7% (w/v)**		
		C	**PC**	**TV**	**C**	**PC**	**TV**	**C**	**PC**	**TV**
S1	Furfural	534	448	511	621	617	536	631	600	537
	5-HMF	168	152	161	201	177	189	233	206	217
	Acetic acid	2474	2355	2334	2947	2913	2896	3538	3445	3418
	Formic acid	1454	nq	1370	1764	nq	1709	2093	nq	2038
	Vanillin	26	3	3	27	4	3	29	4	4
	Syringaldehyde	12	0	0	13	0	0	14	0	0
	p-Coumaric acid	24	0	0	26	0	0	28	0	0
	Ferulic acid	46	0	0	48	0	0	49	0	0
S2	Furfural	670	608	624	833	778	793	911	876	846
	5-HMF	168	166	164	206	201	199	235	230	231
	Acetic acid	2692	2605	2612	3368	3247	3270	3863	3786	3769
	Formic acid	1500	nq	1446	1879	nq	1730	2171	nq	2107
	Vanillin	22	16	9	28	15	11	31	14	10
	Syringaldehyde	10	0	0	15	0	0	16	0	0
	p-Coumaric acid	27	0	0	30	0	0	38	0	0
	Ferulic acid	35	0	0	61	0	0	66	0	0

608 S1, strategy 1; S2, strategy 2; C, control samples; PC, *P. cinnabarinus* laccase treated samples; TV, *T.*
609 *villosa* laccase treated samples; *nq*, not quantified; concentrations expressed as mg/L.
610

611

612

613 Figure 1

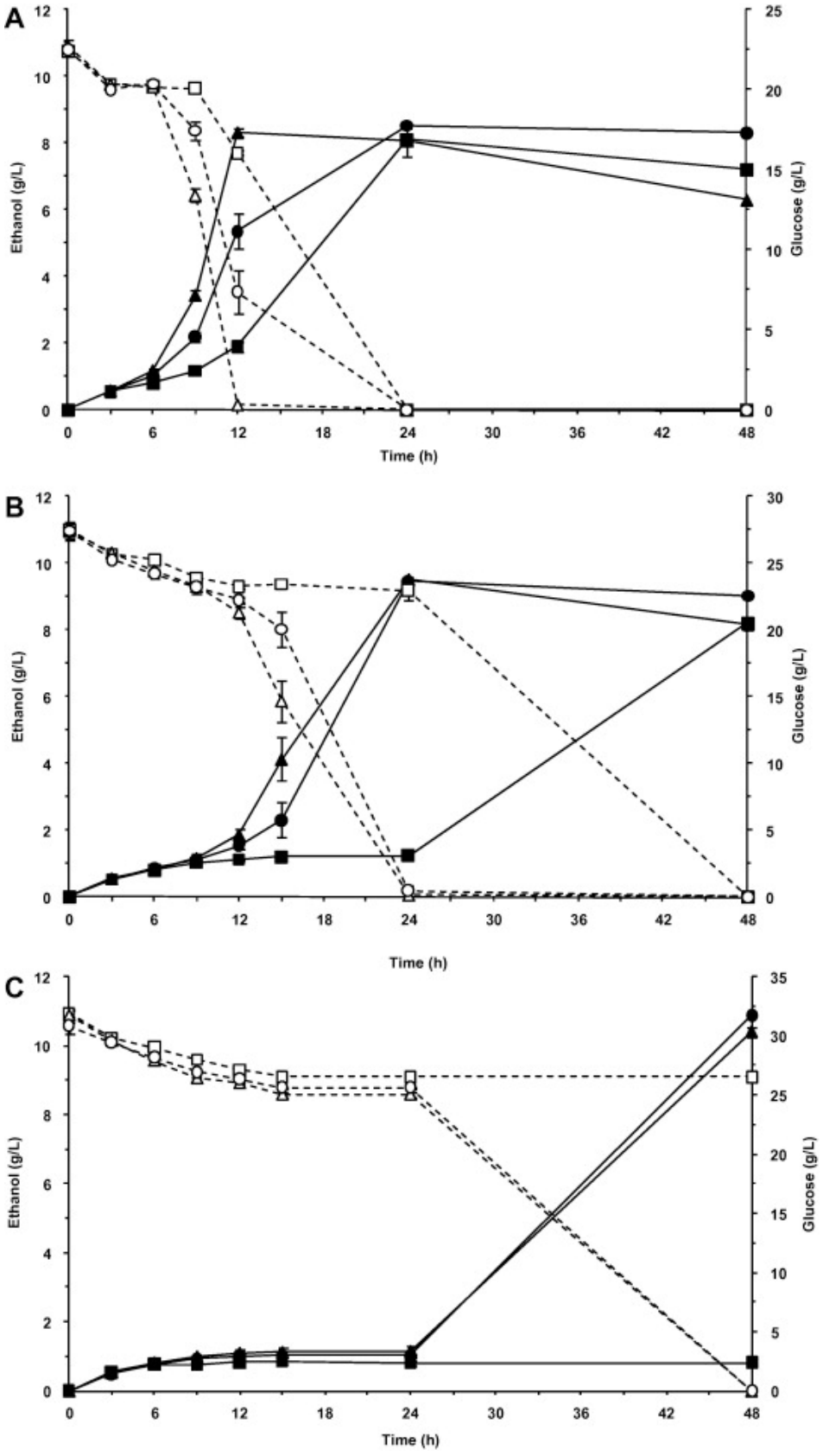


614

615

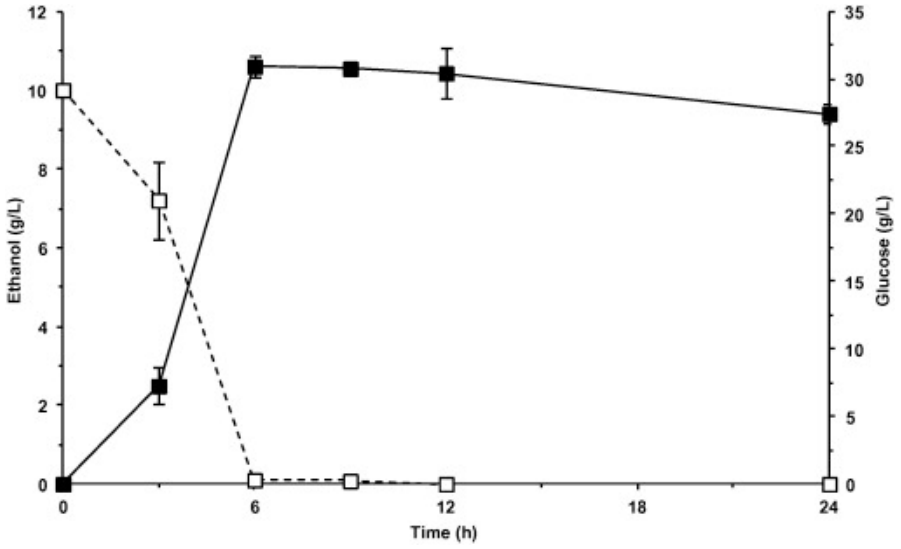
616

617 Figure 2



618
619
620

621 Figure 3



622

623