Improving the fermentation performance of *Saccharomyces cerevisiae* by laccase during ethanol production from steam-exploded wheat straw at high substrate loadings

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Abstract

Operating the saccharification and fermentation processes at high substrate loadings is a key factor for making ethanol production from lignocellulosic biomass economically profitable. However, increasing the substrate loading presents some disadvantages, among them larger generation of inhibitors, which negatively affect fermentation performance. In this study, laccase enzymatic treatment was evaluated as a method to reduce these inhibitory effects. The laccase efficiency was analyzed in a presaccharification and simultaneous saccharification and fermentation process (PSSF) at different high substrate loadings. Water insoluble fraction (WIS) from steam-exploded wheat straw was used as substrate and *Saccharomyces cerevisiae* as fermenting organism. Laccase supplementation reduced strongly the phenolics content in the media, without affecting weak acids and furan derivates. It resulted in an improved yeast performance during simultaneous saccharification and fermentation process, increasing significantly ethanol productivity.

1. Introduction

Greenhouse gas (GHG) effects besides depletion of fossil fuels in a near future have increasing the interest in ethanol as a biofuel, being notably promoted by ambitious mandatory targets for its introduction into current fuel distribution (IEA). Sugar or starch biomass has been traditionally used for ethanol production. However, its sustainability is questionable, particularly because of the conflict with food supply. In contrast, lignocellulosic biomass, including agriculture residues, forest products or energy crops, represents an interesting alternative for its large abundance, low-cost, and not competing with food production (Hahn-Hägerdal et al., 2006). In this context, wheat straw is a readily available candidate for ethanol production, mainly in Europe (Kim and Dale. 2003).

Typical ethanol production from lignocellulosic biomass mainly consists of pretreatment, enzymatic hydrolysis and alcoholic fermentation. Pretreatment of biomass is required to enhance cellulose accessibility to the enzymes and increase the yields of fermentable sugars (Alvira et al., 2010). Steam explosion, a process that combines high pressures and temperatures, is a very appropiate pretreatment method to disrupt the lignocellulose structure (Ballesteros et al., 2006). However, this process leads to a partial sugars and lignin degradation forming some soluble inhibitory compounds, including furan derivates, weak acids and phenolic compounds. Some of them contaminate the liquid phase (prehydrolysate) of the pretreated material, whereas other become embedded in the biomass and are released during the downstream hydrolysis and fermentation steps (García-Aparicio et al., 2006). Besides affecting enzymes in the hydrolysis step, these inhibitors can also retard yeast growth and decrease ethanol productivity and yield during fermentation (Palmqvist and Hahn-Hägerdal, 2000a; Klinke et al., 2004; Panagiotou and Olsson, 2007).

Working at high substrate loadings is a key factor in industrial ethanol production. By increasing substrate loading during enzymatic hydrolysis leads to increased sugar content and higher ethanol concentration after fermentation. This aproach could reduce operational cost for hydrolysis and fermentation processes, as a consequence of lower water consumption and lower downstream processing cost, and minimize energy consumption during subsequent distillation and evaporation stages, making lignocellulosic ethanol production economically feasible (Banerjee et al., 2009). Unfortunately, increasing the substrate loading presents some disadvantages such as inhibition of cellulolytic enzymes by higher glucose and cellobiose accumulation, mixing and mass transfer limitations, and larger amounts of inhibitors generated during pretreatment (Mohagheghi et al., 1992; Rosgaard et al., 2007; Panagiotou and Olsson, 2007).

In a simultaneous saccharification and fermentation (SSF) process, where enzymatic hydrolysis and fermentation are carried out in the same vessel, the glucose concentration is constantly kept low due to its simultaneous conversion to ethanol by fermenting microorganisms. It can minimize the end-product inhibition, allowing higher substrate loadings (Olofsson et al., 2008). Moreover, enzymatic prehydrolysis prior to SSF makes the high substrate media more fluid and easer to handle, faciliting the mixing during subsequent SSF (Öhgren et al., 2007).

Removal of inhibitors generated during pretreatment step is also an important aspect for operating at high substrate loadings. Usually, after steam explosion pretreatment the prehydrolysate is separated by filtration from the solid fraction, which is thoroughly washed prior to enzymatic hydrolysis. Nevertheless, other strategies have been tested for removing these inhibitory compounds (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; Kalyani et al., 2012; Moreno et al., 2012).

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). Their oxidative capabilities towards phenols make them a suitable method for detoxification of industrial wastewaters with high content of phenolic compounds, such as pulp and paper production or olive oil industry (Jurado et al., 2011). This ability have been also used for selective removal of phenolic compounds in whole slurries (Jurado et al., 2009; Kalyani et al., 2012; Moreno et al., 2012), prehydrolysates (Jönsson et al., 1998; Larsson et al., 1999; Chandel et al., 2007), and enzymatic hydrolysates (Martín et al., 2002; Jurado et al., 2009; Moreno et al., 2012) from different steam-exploded biomasses, increasing their saccharification yields or enhancing their fermentabilities to ethanol.

The present work studies the use of *Pycnoporus cinnabarinus* laccase for improving the fermentability to ethanol of steam-exploded wheat straw. High substrate loadings (17–25% solids loading) and two different hydrolytic enzymes mixtures are also evaluated. For it, water insoluble fractions from pretreated material were submitted to presaccharification step, followed by laccase supplementation, and subsequent SSF process. An identification and quantification of inhibitory compounds was carried out. The laccase efficiency was evaluated on the fermentation performance of *Saccharomyces cerevisiae*.

2. Methods

2.1 Enzymes

Pycnoporus cinnabarinus laccase was produced by Beldem (Belgium). Activity was measured by oxidation of 5 mM 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) to its cation radical (e_{436} = 29 300 $M^{-1}cm^{-1}$) in 0.1 M sodium acetate (pH 5) at 24 °C.

A standard mixture NS50013 and NS50010, both produced by Novozymes (Denmark), was employed for lignocellulose saccharification. NS50013 is a cellulase preparation with some xylanase activity, but practically no 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. In addition, a new mixture CTec2 and HTec2, both produced by Novozymes (Denmark), was employed in a second set of lignocellulose saccharification experiments. CTec2 is a cellulase preparation which presents higher β-glucosidase activity with respect to NS50013; therefore no extra supplementation β-glucosidase activity is necessary. HTec2 is a hemicellulase preparation with endoxylanase activity. Overall cellulase activity was determined using filter paper (Whatman No. 1 filter paper strips) and β-glucosidase activity was measured using cellobiose as substrate (Ghose 1987). Xylanase activity was determined using birchwood xylan (Baley et al., 1991).

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 presented 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.

Prior to steam explosion, wheat straw was milled, using a laboratory hammer mill, in order to obtain a chip size between 2 and 10 mm. Then, the raw material was exploded in a 10 L reactor at 210 ºC, 2.5 min. These conditions were selected according to previous optimization studies based on optimal sugars recovery and enzymatic hydrolysis yield (Ballesteros et al., 2006). After pretreatment the whole slurry obtained was vacuum-filtered and separated into a solid fraction and a liquid fraction or prehydrolysate. The solid fraction was thoroughly washed with tap water obtaining 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

Saccharomyces cerevisiae (Fermentis Ethanol red, France) was used for fermentation in PSSF assays. It is an industrial strain used in the fuel alcohol industry, which ferment glucose but not xylose. Active cultures for inoculation were obtained in 100-mL 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 35 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. Presaccharification and simultaneous saccharification and fermentation

WIS was used as a substrate for PSSF assays. All experiments were performed in 100-ml Erlenmeyer flasks agitated on a rotatory shaker at 180 rpm. In a first set of experiments, the presaccharification stage was carried out at 17%, 20% and 25% (w/w) of solids content in 50 mM sodium citrate (pH 5.5) for 48 h at 50 ºC and an enzyme loading of 15 FPU/g substrate of NS50013 (60 FPU/mL and 122 IU/mL of cellulase and xylanase activities, respectively) and 15 IU/g substrate of NS50010 (810 IU/mL of β-glucosidase activity). After that, the temperature was reduced to 35 ºC and 1 g/L (dry weight) of yeast was added without nutrients, which turned the process into SSF. The experiments were run for another 72 h. In a second set of experiments, the PSSF assays were run as previously described but with a presaccharification stage at 17%, 20% and 25% (w/w) of solids content for 24 h and an enzyme loading of 15 FPU/g substrate of CTec2 (100 FPU/mL and 3950 IU /mL of cellulase and β-glucosidase activities, respectively) and 57 IU/g substrate of HTec2 (1300 IU/mL).

2.5. Laccase supplementation

Presaccharification broths were supplemented with laccase in two different ways. For presaccharification experiments of 48 h performed with the standard mixture NS50013 and NS50010 enzymes, laccase supplementation, using 10U/g substrate of *P. cinnabarinus* laccase, was carried out at 36 h of presaccharification. After 12 h of laccase supplementation, the samples were subsequently submitted to SSF process as explained above. For presaccharification experiments of 24 h performed with the mixture CTec2 and HTec2 enzymes, laccase supplementation, using 10U/g substrate of *P. cinnabarinus* laccase, was carried out at 21 h of presaccharification. After 3 h of laccase supplementation, the samples were subsequently submitted to SSF process as previously described. In both set of experiments, control assays consisted of presaccharification without laccase supplementation. All the experiments were carried out by triplicate.

Prior to SSF process, laccase treated samples from presaccharification broths were taken, centrifuged and the supernatants analyzed with the purpose to evaluate the effect of laccase on inhibitory compounds. For it, an identification and quantification of inhibitory compounds was performed. Moreover, glucose and xylose concentrations were also determined. In the same way, samples from laccase treatments submitted to SSF were periodically withdrawn, centrifuged and the supernatants analyzed for glucose consumption and ethanol concentration.

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), benzaldehyde, 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 analyzed 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.

The total phenolic content was analyzed in the supernatants after presaccharification and laccase supplementation according to a slightly modified version of Folin-Ciocalteau method (Folin and Ciocalteau, 1927; Zhang et al., 2006). Load 20 µL of each sample solution and the serial standard solution on a 96-well microplate. Add 12 µL Folin-Ciocalteu reagent and 88 µL, mix well and wait 5 min. Add µ80 L of 7.5% sodium carbonate solution and mix well. Cover the plate and leave it in the dark at room temperature for 2 h. Measure absorbance at 765 nm with a spectrophotometric microplate reader (Anthos Zenyth 200rt).

3. Results and discussion

3.1Pretreatment

After steam explosion pretreatment, slurry obtained presented a total solid content of 34%, with a WIS content of 17.4%. Table 1 summarizes WIS a prehydrolysate composition. As shown, pretreatment at selected conditions increased the cellulose proportion of WIS (51.1%) in relation to untreated raw material (40.5%). It is due to the solubilization of the hemicellulose fraction, as reflected the high xylose content (38.9 g/L) in the prehydrolysate. Furthermore, the prehydrolysate also showed a significant proportion of sugars degradation products. Among them, acetic acid, formic acid, furfural and 5-HMF were the most abundant. Acetic acid is produced because of the solubilization on acetyl groups present in the hemicellulose. Formic acid derives from furfural and 5-HMF degradation, which results from xylose and glucose degradation, respectively (Palmqvist and Hahn-Hägerdal, 2000a). In addition, some phenolic compounds were also detected in the prehydrolysate. On the one hand ferulic acid and *p*-coumaric acid, both derived from cinnamic acids. They are

characteristic of herbaceous plants forming cross-linkages between lignin and hemicellulose (Sun et al., 2002). On the other hand 4-hydroxybenzaldehyde, vanillin and syringaldehyde, all of them derived from lignin degradation (García-Aparicio et al., 2006).

3.2 Presaccharification

The WIS fraction resulting from filtration and washing of steam-exploded wheat straw was submitted to presaccharification prior to SSF. High substrate loadings, 17, 20 and 25% (w/w), and two different hydrolytic enzymes mixtures were used. The production of higher ethanol concentrations is closely related with higher substrate loadings in the SSF broths. However, the high viscosity of most lignocellulosic materials at high solids content implies mass transfer limitations and mixing difficulties (Rosgaard et al., 2007). In this context, presaccharification makes the media more fluid and easer to handle, facilitating the mixing during fermentation, and consequently making the process possible at higher substrate loadings. Furthermore, prehydrolysis enables to hydrolytic enzymes to act at their optima temperatures (Öhgren et al., 2007).

Presaccharification of WIS with the standard NS50013 / NS50010 enzymes mixture resulted in higher glucose concentrations as the substrate loading increases (Table 2), obtaining the highest glucose concentration (100.5 g/L) at 25% (w/w). In all the loadings assayed, the major degree of glucan conversion was achieved within the first 24 h of the hydrolysis (Table 2), in accordance with previous studies (Kristensen et al., 2009; Manzanares et al., 2011). After that, only at 25% (w/w) of substrate loading a slight glucose release was observed after 48 h of hydrolysis (Table 2). The glucan conversion decreased with increasing substrate loadings (constant enzyme to substrate ratio), obtaining the lower conversion value (53.7%) at 25% (w/w) (Table 2). These results are in line with other reported on different steam-exploded lignocellulose (García-Aparicio et al., 2011; Lu et al., 2010; Manzanares et

al., 2011). The mechanism behind the decreasing conversion has been attributed to different factors, including end-product inhibition or inhibition by degradation products, changes in substrate reactivity during enzymatic hydrolysis, mass transfer limitations, and un-productive adsorption of enzymes (Rosgaard et al., 2007; Kristensen et al., 2009).

Taking into account the results described above for the standard enzymes mixture, where the major degree of glucan conversion was achieved within the first 24 h of the hydrolysis, the presaccharification step using the CTec2 / HTec2 enzymes mixture was not prolonged more than 24 h. Compared to the standard NS50013 / NS50010 enzymes mixture, the use of both CTec2 and HTec2 enzymes increased the glucose concentration of broths, particularly at 20% and 25% (w/w) (Table 2). In both cases, glucan conversion increased, from 59.8% with the standard mixture to 66.4% with CTec2 and HTec2 enzymes at 20% (w/w); and from 49.9% with the standard mixture to 55.2% with CTec2 and HTec2 enzymes at 25% (w/w). These improvements could be explained by the high xylanase activity contained in the CTec2 / HTec2 enzymes mixture, provided mainly by HTec2 enzyme. Addition of xylanases is being largely studied to increase enzymatic hydrolysis yields (García-Aparicio et al., 2011; Alvira et al., 2011). They can improve the accessibility of cellulose to cellulases due to xylan removal (Berlin et al., 2007). In this sense, presaccharification of WIS at 20% (w/w) with the CTec2 / HTec2 enzymes mixture showed higher xylose concentration (11.2 g/L) after 24 of hydrolysis than that obtained (8.7 g/L) with the standard mixture. In addition, the xylan removal can lead to an indirect delignification (Kumar and Wyman 2008), which can also promote the accessibility of cellulose to cellulases.

As observed for the standard enzymes mixture, the glucan conversion also decreased as the substrate loading increased when both CTec2 and HTec2 enzymes were used for presaccharification of WIS (Table 2).

3.3 Laccase supplementation

Inhibitor compounds, including weak acids, furan derivates and phenolic compounds, were determined and quantified after 48 h and 24 h of presaccharification with the standard and CTec2 / HTec2 enzymes mixtures, respectively (Table 3 and Fig. 1). In spite of the filtration and washing steps performed on steam-exploded wheat straw prior to presaccharification, successful operations in removing large quantities of inhibitory compounds (García-Aparicio et al., 2006), enzymatic hydrolysis of WIS fraction released again inhibitors to the media (Table 3 and Fig. 1). In addition to mix the WIS fraction into the presaccharification buffer, releasing inhibitory compounds trapped within solid fraction (Gurram et al., 2011), the action of hydrolytic enzymes can also cause the inhibitors release. In this sense, certain activities, such as xylanase, acetyl esterase and phenolic acid esterase, are especially involved in the release of acetic acid and phenolic acids compounds (Thomsen et al., 2009). The inhibitors concentrations were higher as the substrate loading increases (Table 3 and Fig. 1).

Presaccharification broths were supplemented with *P. cinnabarinus* laccase at 36 h and 21 h of the enzymatic prehydrolysis performed with the standard and CTec2 / HTec2 enzymes mixtures, respectively. After finishing laccase suplemented presaccharification step, inhibitor compounds were determined and quantified (Table 3 and Fig. 1). Compared to presaccharification without laccase supplementation, the results showed that *P. cinnabarinus* laccase reduced strongly the total phenolic content of the enzymatic hydrolysates in all the loadings assayed (Fig. 1), in agreement with previous studies (Martín et al., 2002; Jurado et al., 2009; Moreno et al., 2012). At higher substrate loading the efficiency of laccase for reducing the total phenols diminished, observing a total phenols removal from 79% at 17% (w/w) to 65% at 25% (w/w) for presaccharification with the standard enzymes mixture; and

from 60% at 17% (w/w) to 53% at at 25% (w/w) for presaccharification with the CTec2 / HTec2 enzymes mixture. It could be due to the high viscosity of the medium as the substrate loading increases, which difficult the blending of laccase with the material. On the other hand, the laccase supplementation did not alter the concentration of weak acids and furan derivates (Table 3). This substrate-specific reaction of *P. cinnabarinus* laccase towards phenols has already been reported by several authors (Larsson et al., 1999; Martín et al., 2002; Chandel et al., 2007; Moreno et al., 2012).

Laccases catalyze the oxidation of phenols generating unstable phenoxy radicals that lead to polymerization into less toxic aromatic compounds (Jönsson et al., 1998; Jurado et al., 2009). Fig. 2 shows the performance of laccase on the different individual phenolic monomers identified in the presaccharification broths obtained with the standard enzymes mixture. Whilst syringaldehyde, *p*-coumaric acid and ferulic acid decreased completely, together with other phenols no identified, vanillin was only slightly affected by laccase and 4-hydroxybenzaldehyde remained constant. Similar laccase performance was observed in the presaccharification broths obtained with the CTec2 / HTec2 enzymes mixture (chromatograms not showed). Kolb et al., (2012) described the same effects with *Trametes versicolor* laccase on the phenolic monomers contained in the prehydrolysate of liquid hot water pretreated wheat straw. They observed a complete removal of syringaldehyde, *p*-coumaric acid and ferulic acid by laccase within one hour. The removal of vanillin was slower, being completely removed within one day. Finally, no significant effect of laccase on 4-hydroxybenzaldehyde was described within a 1 week reaction time. These reactivity differences of the different phenols towards laccase depend largely on their structural characteristics (Kolb et al., 2012; Moreno et al., 2012). Laccase activity towards phenols is enhanced by the presence of electron-donating substituents at the benzene ring that decrease

their electrochemical potential. In this sense, an additional methoxy-group (the structural difference between vanillin and syringaldehyde) increase the affinity of the phenolic compounds towards laccase. Moreover, the presence of ethylene groups in *para*-substituted phenols, such as p-coumaric acid and ferulic acid, increase the activity of laccases.

3.4 SSF

Presaccharification broths at different high substrate loadings, with or without laccase supplementation, were submitted to SSF using *S. cerevisiae* as fermenting organism and without nutrients addition. The process was performed during 72 h, taking samples at different times for glucose consumption and ethanol production analysis.

Fig. 3 shows time courses for glucose consumption and ethanol production during SSF of presaccharification broths obtained with the standard enzymes mixture and no laccase supplementation. Despite no nutrients addition to the media, a complete fermentation without glucose accumulation was obtained within 72 h of SSF in all the loadings assayed, attaining the highest ethanol concentration (48.2 g/L) at 25% (w/w) (Table 4). Jørgensen et al., (2007) also showed successfully fermentation of pretreated wheat straw at high substrate loadings in SSF with no nutrients addition. As seen in the figure, *S. cerevisiae* showed slower glucose consumption and ethanol production rates as the substrate loading increases, especially at 25% (w/w) with a marked drop in the ethanol volumetric productivity value (Table 4). García-Aparicio et al., (2011) and Linde et al., (2007) reported similar performances on steam-pretreated barley straw with *Kluyveromyces marxianus* and *S. cerevisiae*, respectively. This phenomenon is due to the extension of the yeast lag phase, period that the microorganism uses to adapt to the fermentation conditions. Its duration is related with different factors, among them the inhibitory compounds type and their concentrations play a decisive role (Palmqvist and Hahn-Hägerdal, 2000a; Klinke et al., 2004). Moreover, the ethanol yields decreased, from

0.32 g/g at 17% (w/w) to 0.25 g/g at 25% (w/w) (Table 4), according with previous studies (García-Aparicio et al., 2011; Linde et al., 2007). These negative effects observed could be explained by the increments of degradation compounds as substrate loadings increase (Table 3 and Fig. 1), which could affect negatively the yeast fermentation performance by cumulative concentration of them. Nevertheless, probably inhibition by synergistic effects between different degradation compounds could also occur (Palmqvist and Hahn-Hägerdal, 2000a; Klinke et al., 2004).

Laccase supplementation enhanced the yeast fermentation performance in all the loadings assayed. As seen in Fig. 3, *S. cerevisiae* shorted its lag phase, observing faster glucose consumption and ethanol production rates, and higher volumetric productivity values (Table 4). Moreover, laccase supplementation also enhanced slightly the ethanol yields (Table 4). The action of *P. cinnabarinus* laccase, removing phenols specifically, demonstrates the inhibitory effects of the phenolic compounds on the yeast during ethanol production from steam-exploded wheat-straw at high substrate loadings. Phenols produce yeast cells damage and direct interferences with biological membranes, which affect their ability to serve as selective barriers and enzyme matrices (Palmqvist and Hahn-Hägerdal, 2000a; Klinke et al., 2004). Despite the laccase supplementation, both the ethanol production yields and volumetric productivity values decreased as the substrate loading increased (Table 4). Thus the presence of weak acids and furan derivates, no affected by the action of laccase, also shows negative effects on the yeast performance. Weak acids produce a disruption of intracellular pH by its accumulation in the yeast cells, while furan derivates inhibit directly both glycolytic and non-glycolytic enzymes of the yeast (Palmqvist and Hahn-Hägerdal, 2000a; Klinke et al., 2004). In addition, both high initial sugars and high final ethanol concentrations, rising as substrate

loadings increase (Table 3 and 4), also affect the performance of *S. cerevisiae* (Jørgensen et al., 2007).

Complete fermentation without glucose accumulation was also obtained at 17% and 20% (w/w) within 72 h of SSF using presaccharification broths obtained with the CTec2 / HTec2 enzymes mixture and no laccase supplementation (Fig. 4). In both substrate loadings, higher ethanol concentrations were obtained, 42.5 g/L with CTec2 and HTec2 enzymes compared to 37.1 g/L with the standard mixture at 17% (w/w); and 51.1 g/L with CTec2 and HTec2 enzymes compared to 40.0 g/L with the standard mixture at 20% (w/w) (Table 4). On the other hand, the fermentation was not completed at 25% (w/w) within 72 h of SSF studied (Fig. 4), being necessary 24 h more to finish the fermentation (58.2 g/L). Longer lag phases and lower volumetric productivity values were also observed as the substrate loading increased (Table 4 and Fig. 4). These negative effects were specially pronounced at 25% (w/w), observing a lag phase of 48 h (Fig. 4c) with a barley volumetric productivity value (0.09 g/L/h) and a very low ethanol yield (0.14 g/g). As previously described for presaccharification broths obtained with the standard enzymes mixture, laccase supplementation enhanced the yeast fermentation performance, observing faster glucose consumption and ethanol production rates (Fig. 4), and both higher volumetric productivity values and ethanol yields (Table 4). These enhancements were very marked at 25% (w/w), increasing strongly the ethanol volumetric productivity value, from 0.09 g/L/h with no laccase supplementation to 0.73 g/L/h; the ethanol yield, from 0.14 g/g with no laccase supplementation to 0.29 g/g; and obtaining the highest ethanol concentration (58.6 g/L).

4. Conclusion

High ethanol concentrations in a short fermentation time are an economically relevant factor in industrial ethanol production. As indicated by the results, phenols release during PSSF of steam-exploded wheat straw at very high substrate loadings affected negatively the performance of *S. cerevisiae*, extending its lag phase and lowering the ethanol yields. The laccase integration into the process, supplementing the presaccharification step, removed the major part of phenols. It led to improve the fermentation performance of *S. cerevisiae*, shortening its lag phase and enhancing the ethanol yields. Moreover, the addition of other hydrolytic enzyme activities to conventional cellulase and β-glucosidase, such as xylanase, resulted in higher glucose release and, therefore, higher final ethanol concentrations.

Acknowledgements

This work has been supported by the RESTOENE project (Consejería de Educación-Comunidad de Madrid, Spain). P.A. acknowledges …… 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

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

Alvira P, Negro MJ, Ballesteros M. Effect of endoxylanase and a-L-arabinofuranosidase supplementation on the enzymatic hydrolysis of steam exploded wheat straw. Bioresour Technol 2011; 102:4552- 4558.

Bailey MJ, Biely P, Poutanen K. Interlaboratory testing of methods for assay xylanase activity. J Biotechnol 1991; 23:257–270.

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

Ballesteros I, Negro MJ, Oliva JM, Cabañas A, Manzanares P, Ballesteros M. Ethanol production from steam-explosion pretreated wheat straw. Appl Biochem Biotechnol 2006**;** 129:496–508.

Berlin A, Maximenko V, Gilkes N, Sadler J. Optimization of enzymes complexes for lignocellulose hydrolysis. Biotechnol Bioeng 2007; 97:287–96.

Chandel AK, Kapoor RK, Singh A, Kuhad RC. Detoxification of sugarcane bagasse hydrolysate improves ethanol production by *Candida shehatae* NCIM 3501. Bioresour Technol 2007; 98: 1947-1950.

Folin O, Ciocalteu V. On tyrosine and tryptophane determinations in proteins. J Biol Chem 1927; 73: 627–650.

García-Aparicio MP, Ballesteros I, González A, Oliva JM, Ballesteros M, Negro MJ. Effects of inhibitors released during steam-explosion pretreatment of barley straw on enzymatic hydrolysis. Appl Biochem Biotechnol 2006; 192–132:278–288.

García-Aparicio MP, Oliva JM, Manzanares P, Ballesteros M, Ballesteros I, González I, Negro MJ. Second-generation ethanol production from steam exploded barley straw by *Kluyveromyces marxianus* CECT 10875. Fuel 2011; 90: 1624–1630.

Ghose TK. Measurement of cellulase activity. Pure Appl Chem 1987; 59:257–68.

Gurram RN, Datta S, Lin YJ, Snyder SW, Menkhaus TJ. Removal of enzymatic and fermentation inhibitory compounds from biomass slurries for enhanced biorefinery efficiencies. Bioresour Technol 2011; 102:7850–7859.

Hahn-Hägerdal B, Galbe M, Gorwa-Grauslund MF, Linden G, Zacchi G. Bio-ethanol – the fuel of tomorrow from the residues of today. Trends Biotechnol 2006; 24:549–556.

Jönsson JL, Palmqvist E, Nilvebrant N-O, Hahn-Hägerdal B. Detoxification of wood hydrolysates with laccase and peroxidase from the white-rot fungus *Trametes versicolor*. Appl Microbiol Biotechnol 1998; 49:691-697.

Jørgensen H, Vibe-Pedersen J, Larsen J, Felby C. Liquefaction of lignocellulose at high-solids concentrations. Biotechnol Bioeng 2007; 96:862- 870.

Jurado M, Prieto A, Martínez-Alcalá A, Martínez AT, Martínez MJ. Laccase detoxification of steam-exploded wheat straw for second generation bioethanol. Bioresour Technol 2009; 100: 6378-6384.

Jurado M, Saparrat M, Martínez AT, Martínez MJ**.** Application of white-rot fungi in transformation, detoxification, or revalorization of agriculture wastes: role of laccase in the processes. Comprehen Biotechnol 2011; 6:595- 603.

Kalyani D, Dhiman SS, Kim H, Jeya M, Kim I-W, Lee J-K Charazterization of a novel laccase from the isolated *Coltricia perennis* and its application to detoxification of biomass. Process Biochem 2012; 47:671- 678.

Kim S, Dale BE. Global potential bioethanol production from wasted crops and crop residues. Biomass Bioenerg 2003; 26:361- 375.

Klinke HB, Thomsen AB, Ahring BK. Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pretreatment of biomass. Appl Microbiol Biotechnol 2004; 66:10-26.

Kolb M, Sieber V, Amann M, Faulstich M, Schieder D. Removal of monomer delignification products by laccase from *Trametes versicolor*. Bioresour Technol 2012; 104:298- 304.

Kristensen JB, Felby C, Jorgensen H. Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose. Biotechnol Biofuels 2011; 2:11.

Kumar R, Wyman CE. Effect of enzyme supplementation at moderate cellulase loadings on initial glucose and xylose release from corn stover solids pretreated by leading technologies. Biotechnol Bioeng 2008; 102:457- 467.

Larsson S, Reimann A, Nilvebrant N-O, Jönsson JL. Comparison of different methods for the detoxification of lignocellulose hydrolyzates of spruce. Appl Microbiol Biotechnol 1999; 77-79: 91-103.

Linde M, Galbe M, Zacchi G. Simultaneous saccharification and fermentation of steam-pretreated barley straw at low enzyme loadings and low yeas concentration. Enzyme Microb Technol 2007; 40:1100- 1107.

Lu Y, Wang Y, Xu G, Chu J, Zhuang Y, Zhang S. Influence of high solid concentration on enzymatic hydrolysis and fermentation of steam-exploded corn stover biomass. Appl Biochem Biotechnol2010; 160:360-369.

Martín C, Galbe M, Wahlbom CF, Hahn-Hägerdal B, Jönsson JL. Ethanol production from enzymatic hydrolysates of sugarcane bagasse using recombinant xyloseutilising *Saccharomyces cerevisae*. Enzyme Microbiol Technol 2002; 31:274-282.

Manzanares P, Negro MJ, Oliva JM, Saéz F, Ballesteros I, Cara C, Castro E, Ruiz E. Different process configurations for bioethanol production from pretreated olive pruning biomass. J Chem Technol Biotechnol 2011; 86:881-887.

Mohagheghi A, Tucker M, Grohmann K, Wyman C. High solids simultaneous saccharification and fermentation of pretreated wheat straw to ethanol. Appl Biochem Biotechnol 1992; 33:67- 81.

Moreno AD, Ibarra D, Fernández JL, Ballesteros M. Different laccase detoxification strategies for ethanol production from lignocellulosic biomass by the thermotolerant yeast Kluyveromyces marxianus CECT 10875. Bioresour Technol 2012; 106:101- 109.

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

International Energy Agency. Technology Roadmap-Biofuels for Transport, OECD/IEA, Paris; 2011. http://www.iea.org/papers/2011/Biofuels_Roadmap.pdf

Olofsson K, Bertilsson M, Liden G. A short review on SSF-an interesting option for ethanol production from lignocellulosic feedstock. Biotechnol Biofuels 2008; 1:1–14.

Öhgren K, Vehmaanperä J, Siika-Aho M, Galbe M, Viikari L, Zacchi G. High temperature enzymatic prehydrolysis prior to simultaneous saccharification and fermentation of steam pretreated corn stover for ethanol production. Enzyme Microb Technol 2007; 40:607-613.

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

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

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

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

Rosgaard L, Andric P, Dam-Johansen K, Pedersen S, Meyer AS. Effects of substrate loading on enzymatic hydrolysis and viscosity of pretreated barley straw. Appl Biochem Biotechnol 2007; 143:27- 40.

Sun RC, Sun XF, Wang SQ, Zhu W, Wang XY. Ester and ether linkages between hydroxycinnamic acids and lignins from wheat, rice, rye, and barley straws, maize stems, and fast-growing poplar wood. Ind Crops Prod 2002; 15:179- 188.

Thomsen MT, Thygesen A, Thomsen AB. Identification and characterization of fermentation inhibitors formed during hydrothermal treatment and following SSF of wheat straw. Appl Microbiol Biotechnol 2009; 83: 1867- 1871.

Zhang Q, Zhang **J**, Shen J, Silva A, Dennis DA, Barrow CJ. A simple 96-well microplate method for estimation of total polyphenol content in seaweeds. J Appl Phycol 2006; 18:445–450.

Table captions

Table 1. Composition of steam-exploded wheat straw.

Table 2. Summary of presaccharification assays.

Table 3. Inhibitory compounds composition of hydrolysates after laccase supplementation.

Table 4. Summary of SSF assays.

Figure captions

Figure 1. Total phenol content determined by Folin-Ciocalteau method of hydrolysates resulting from presaccharification with the standard NS50013 / NS50010 enzymes mixture (a) and with the CTec2 / HTec2 enzymes mixture (b). Without (black bars) and with laccase supplementation (gray bars).

Figure 2. Comparison of HPLC profiles of hydrolysates resulting from presaccharification with the standard NS50013 / NS50010 enzymes mixture at 20% (w/w). Without (top) and with laccase supplementation (botton). Identified peaks: 1, 5-HMF; 2, furfural; 3, 4-hydroxybenzaldehyde; 4, vanillin; 5, syringaldehyde; 6, *p*-coumaric acid; and 7, ferulic acid. * Phenols no identified.

Figure 3. Time course for ethanol production (filled symbols and continuous lines) and glucose consumption (open symbols and discontinuous lines) during SSF of hydrolysates resulting from presaccharification with the standard NS50013 / NS50010 enzymes mixture. Symbols used: Without (¦ , ?); and with laccase supplementation (?, ?). Substrate loadings at 17% (a), 20% (b) and 25% (w/w) (c).

Figure 4. Time course for ethanol production (filled symbols and continuous lines) and glucose consumption (open symbols and discontinuous lines) during SSF of hydrolysates resulting from presaccharification with the CTec2 / HTec2 enzymes mixture. Symbols used: Without (¦ , ?); and with laccase supplementation (?, ?). Substrate loadings at 17% (a), 20% (b) and 25% (w/w) (c).

Table 1.

WIS		Prehydrolysate			
Component	% Dry weight	Monosaccharides	g/L	Inhibitors	g/L
Cellulose	51.1	Glucose	7.13	Furfural	2.45
Hemicellulose	7.8	Xylose	38.9	5-HMF	0.68
Lignin	30.5	Arabinose	1.72	Acetic acid	8.22
		Galactose	2.07	Formic acid	6.59
		Mannose	0.70	Benzaldehyde	0.007
				Vanillin	0.065
				Syringaldehyde	0.018
				p-Coumaric acid	0.022
				Ferulic acid	0.038

Table 2.

		NS50013 / NS50010		CTec2 / HTec2	
% WIS (w/w)	Sample	Presaccharification (Glu g/L)		Presaccharification (Glu g/L)*	
		24 h	48 h	24 h	48 h
17	C	76.2 (67.8)	77.2 (68.7)	78.4 (69.7)	nq
	L	77.2 (68.7)	76.7 (68.2)	78.6 (69.9)	nq
20	C	84.1 (59.8)	84.3 (60.0)	93.4 (66.4)	nq
	L	85.5 (60.9)	84.7 (60.3)	94.6 (67.3)	nq
25	C	93.5 (49.9)	100.5 (53.7)	103.4 (55.2)	nq
	L	95.4 (50.9)	100.1 (53.4)	101.9 (54.4)	nq

C, without laccase supplementation; L, with laccase supplementation; Glucan conversion (%) is calculated as the hydrolyzed glucan (glucose content in the media) by the glucan content in the WIS; * Glucose contribution of the CTec2 / HTec2 enzymes mixture does not consider; nq, not quantified.

Table 3.

% WIS (w/w)	Sample	Inhibitors (g/L)			
		Furfural	5-HMF	Acetic acid	Formic Acid
NS50013 / NS50010					
17	C	0.209	0.069	1.913	0.841
	L	0.218	0.067	1.740	nq
20	C	0.275	0.092	2.225	1.005
	L	0.261	0.083	2.140	nq
25	C	0.290	0.100	2.494	1.078
	L	0.279	0.089	2.407	nq
CTec2 / HTec2					
17	C	0.229	0.076	1.731	0.371
	L	0.238	0.074	1.850	nq
20	C	0.286	0.104	2.098	0.515
	L	0.274	0.097	1.945	nq
25	C	0.295	0.106	2.330	0.575
	L	0.274	0.097	2.309	nq

C, without laccase supplementation; L, with laccase supplementation; nq, not quantified.

Table 4.

		NS50013 / NS50010			CTec2 / HTec2		
% WIS (w/w)	Sample	Fermentation			Fermentation		
		$EtOH_M$ (g/L)	$Y_{E/G}$ (g/g)	Q_E (g/L h)	$EtOH_M$ (g/L)	$Y_{E/G}$ (g/g)	Q_E (g/L h)
17	C	37.1 (64.7)	0.32	0.87	42.5 (68.2)	0.34	0.72
	L	37.5 (65.5)	0.33	1.17	44.4 (71.3)	0.36	1.44
20	C	40.0 (55.8)	0.28	0.89	51.1 (66.0)	0.33	0.55
	L	41.0 (57.2)	0.29	1.32	51.7 (66.8)	0.34	1.39
25	C	48.2 (50.4)	0.25	0.45	30.0 (29.1)	0.14	0.09
	L	49.0 (51.3)	0.26	0.90	58.6 (56.9)	0.29	0.73

C, without laccase supplementation; L, with laccase supplementation; $EtOH_M$, maximum ethanol concentration after 72 h of SSF; $Y_{E/G}$, ethanol yield based on total glucan content in the WIS fraction; (%), theoretical ethanol yield assuming ethanol yields on glucose by *S. cerevisiae* of 0.51 g/g; Q_E, volumetric ethanol productivity calculated at 24 h of SSF.

Figure. 1

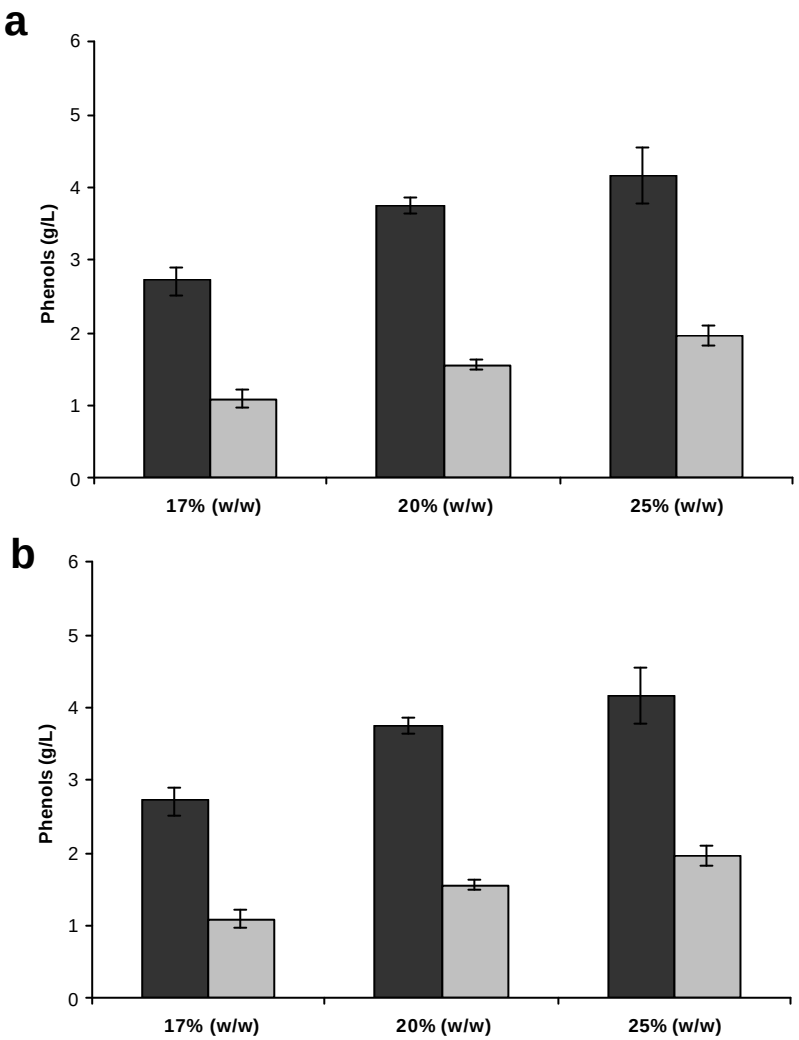
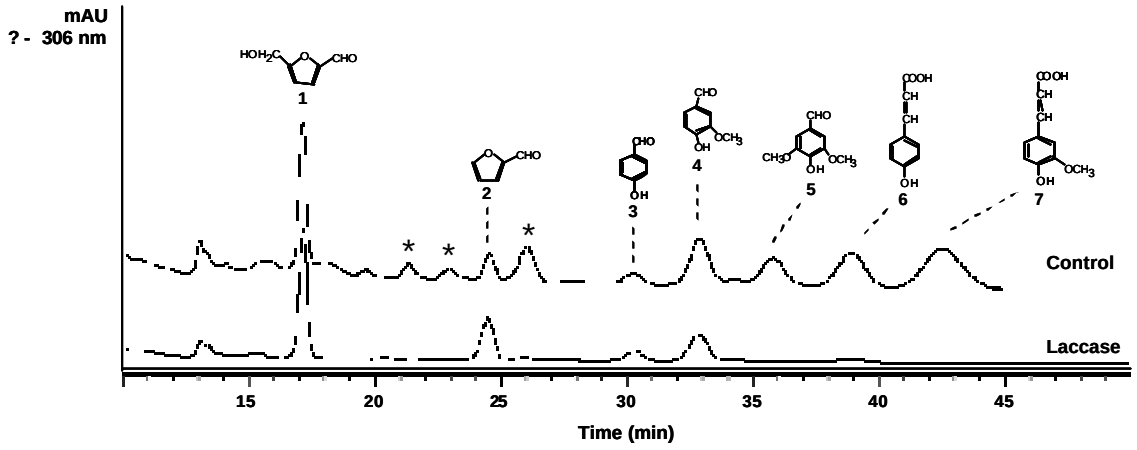

a
Phenols (g/L)
0
1
2
3
4
5
6
17% (w/w)
20% (w/w)
25% (w/w)
b
Phenols (g/L)
0
1
2
3
4
5
6
17% (w/w)
20% (w/w)
25% (w/w)

Figure. 2


mAU
? - 306 nm
1
2
3
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7
Control
Laccase
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25
30
35
40
45
Time (min)

Figure. 3

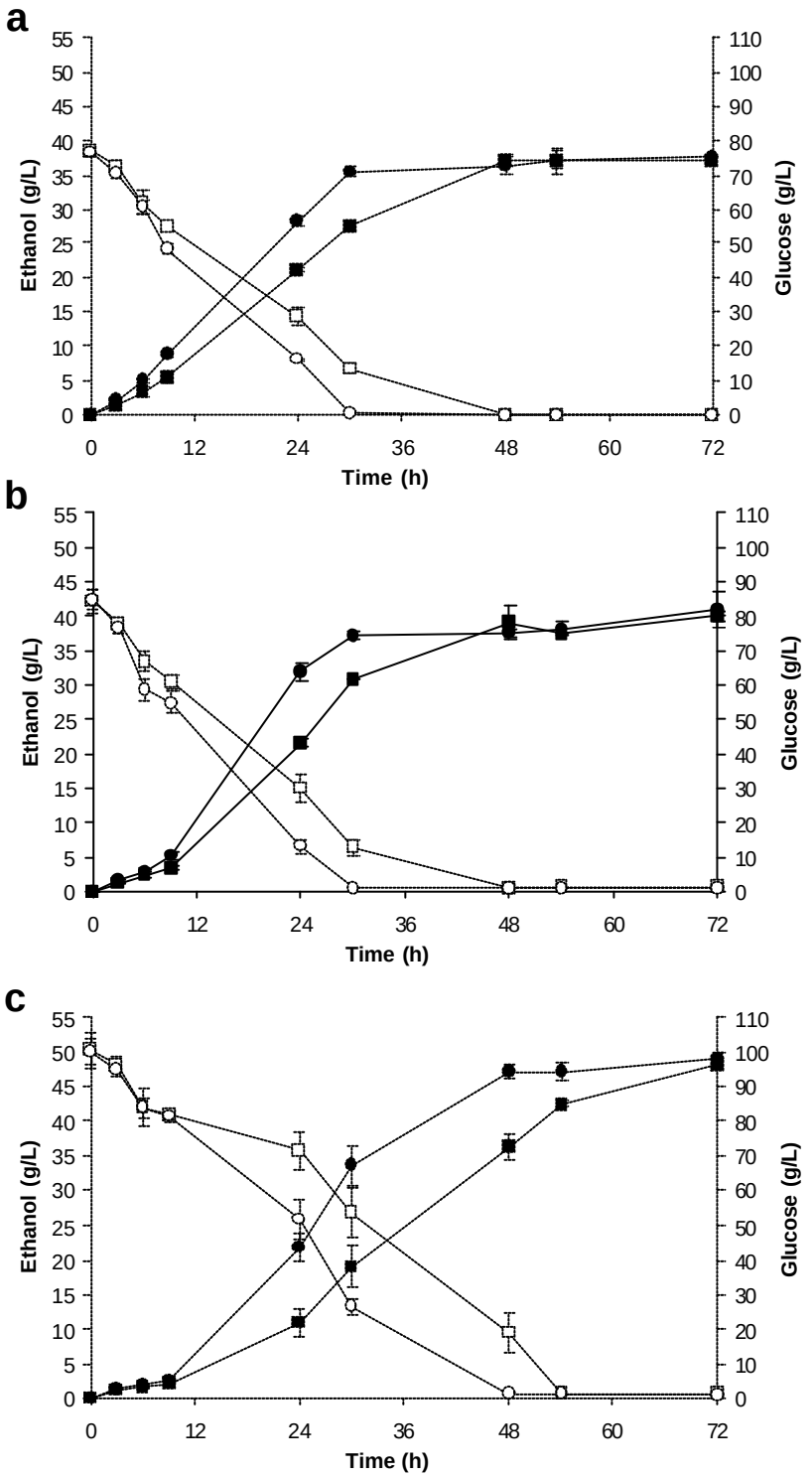
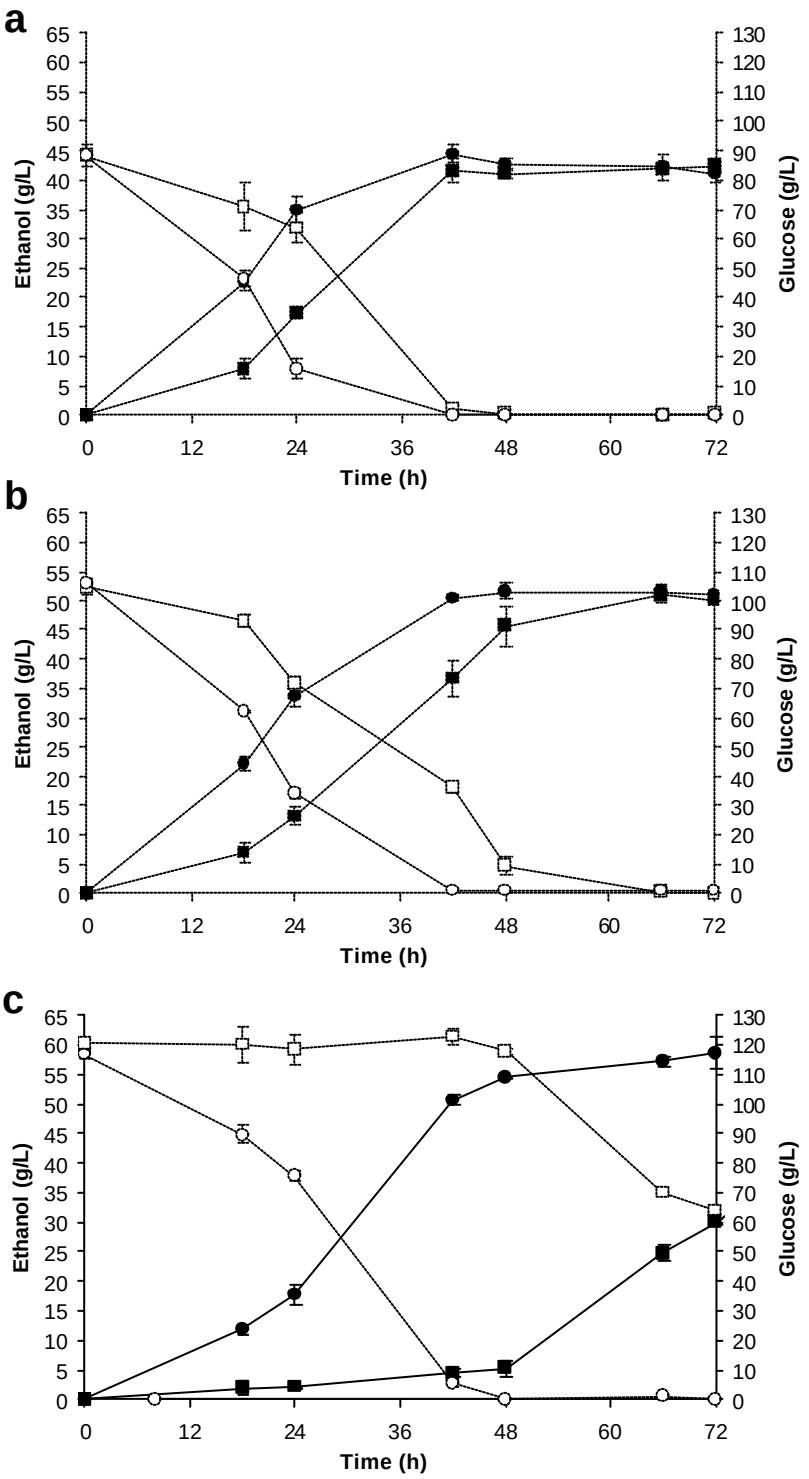

a
b
c
Ethanol (g/L)
Glucose (g/L)
Time (h)
0
5
10
15
20
25
30
35
40
45
50
55
0
10
20
30
40
50
60
70
80
90
100
110
0
12
24
36
48
60
72

Figure. 4


a
b
c
Ethanol (g/L)
Glucose (g/L)
Time (h)
0
12
24
36
48
60
72
0
5
10
15
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25
30
35
40
45
50
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60
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0
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100
110
120
130