Comparing cell viability and ethanol fermentation of the thermotolerant yeast *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* on steam-exploded biomass treated with laccase

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Abstract

In this study, the thermotolerant yeast *Kluyveromyces marxianus* CECT 10875 was compared to *Saccharomyces cerevisae* Ethanol Red for lignocellulosic ethanol production. For it, whole slurry from steam-exploded wheat straw was used as raw material, and two process configurations, simultaneous saccharification and fermentation (SSF) and presaccharification and simultaneous saccharification and fermentation (PSSF), were evaluated. Compared to *S. cerevisiae*, which was able to produce ethanol in both process configurations, *K. marxianus* was practically inhibited, and neither growth nor ethanol production occurred during the processes. However, the prior laccase treatment of the whole slurry, which removed specifically the lignin phenols content from the overall inhibitory compounds present in slurry, triggered the fermentation by *k. marxianus*, attaining ethanol concentrations and yields comparable to those obtained by *S. cerevisiae*.

Keywords: cell viability; ethanol fermentation; *Kluyveromyces marxianus* CECT 10875; laccase detoxification; *Saccharomyces cerevisiae* Ethanol Red

1. Introduction

Fossil fuels exhaustions besides the negative environmental effects have increased the interest in biorefinery concept as a source of chemicals, materials and biofuels (Kamm et al., 2010). Among these renewable products, the development of biofuels is being specially promoted in the entire world by ambitious energy policies (IEA, 2011). In this context, the progress towards the production of lignocellulosic ethanol is fundamental. As opposed to cornstarch or sugar-based biomass, lignocellulosic materials, including agriculture residues, forest products or energy crops, are abundant, low-cost, and not used for food (Han-Hägerdal et al., 2006). During its biochemical conversion to ethanol, the polysaccharides contained in lignocellulose, including cellulose and hemicelluloses are hydrolyzed by cellulolytic enzymes to their monomer sugars and converted by fermenting microorganisms to ethanol.

Due to the recalcitrant nature of the lignocellulose, a pretreatment step is required for increasing fermentable sugars in the hydrolysis step. Among the different available pretreatments, steam explosion is one of the more widely used. This pretreatment disrupts fiber structure, resulting in the partial hydrolysis of hemicelluloses, leaving cellulose, the rest of hemicelluloses and insoluble chemically modified lignin (Alvira et al., 2010). This partial removal of hemicelluloses together with lignin modification contributes to a better accessibility of the cellulolytic enzymes to cellulose (Alvira et al., 2010). However, steam explosion partially degrades the cellulose and hemicellulose derived sugars, as well as the lignin polymer, to some soluble inhibitory compounds. They are classified according to their chemical structure and include furan derivates from sugars degradation, weak acids (mainly acetic acid) and phenolic compounds from lignin. Besides affecting enzymes in the hydrolysis step, these inhibitors can also retard yeast growth and reduce sugars conversion rates and

ethanol productivity during fermentation (Palmqvist and Hahn-Hägerdal, 2000a; Klinke et al., 2004; Panagiotou and Olsson, 2007). Several methods have been assayed to reduce these toxic effects (Palmqvist and Hahn-Hägerdal, 2000b; Taherzadeh and Karimi, 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; Moreno et al., 2012).

Laccases (EC 1.10.3.1) are multi-copper oxidases produced by microorganisms and plants that oxidize a large variety of phenolic compounds and aromatic amines using molecular oxygen as electron acceptor (Thurston 1994). Their capacity to catalyze the depolymerization and polymerization of lignin make them suitable candidates as white biocatalysts on the development of the lignocellulose biorefinery concept. In this sense, laccase technology can be used either in those processes where lignin removal is the main objective (ethanol production and manufacture of cellulose-based chemicals and materials, including paper), or in those having lignin polymerization as the main goal (functionalization of fibers, production of adhesives, and modification of lignins and other aromatic compounds) (Cañas and Camarero, 2010). The detoxification mechanism by laccases is also based on the oxidative polymerization of phenolic compounds generated during steam-explosion pretreatment (Jurado et al., 2012).

Enzymatic hydrolysis and fermentation of the hydrolyzed biomass can be performed in different process configurations, being simultaneous saccharification and fermentation (SSF) strategy an interesting option for lignocellulosic ethanol production (Olofsson et al., 2008). During this process, the glucose released by the action of hydrolytic enzymes is converted simultaneously to ethanol by fermenting microorganisms, minimizing inhibition by cellobiose and glucose accumulation and allowing higher substrate loadings (Olofsson et al., 2008). Neverthless, SSF is usually

conducted at temperatures inferior to optimun of the cellulolytic enzymes. Whereas saccharification has an optimun temperature around 50 ºC, most fermenting yeasts has an optimun temperature ranging from 30 to 37 ºC (Alfani et al., 2000). Therefore, the use of thermotolerant microorganisms provides a number of potential advantages in terms of cost reduction and yield, such as cooling cost reduction and better sacharification and ethanol yields (Abdel-Banat et al., 2010). In this context, *Kluyveromyces marxianus* CECT 10875, a strain adapted and selected in our laboratory (Ballesteros et al., 1991), is gaining great significance due to its capability of growing and fermenting at temperature above 40 ºC, close to optimun temperature of enzymatic hydrolysis, which can lead to an integration of both saccharification and fermentation processes (Tomás-Pejó et al., 2009). However, high temperatures could elevate the yeast susceptibility to inhibitory compounds (Tomás-Pejó et al., 2009; Abdel-Banat et al., 2010), restricting the use of thermotolerant yeasts on pretreated materials with high inhibitors content, such as whole slurry obtained after steam-explosion.

In this context, the present work studies the fermentation performance of the thermotolerant yeast *K. marxianus* CECT 10875 on whole slurry from steam-exploded wheat straw detoxified by *Pycnoporus cinnabarinus* laccase. For this purpose, two different process configurations, SSF (simultaneous saccharification and fermentation) and PSSF (presaccharification and simultaneous saccharification and fermentation), were used; and cell viability and both glucose consumption and ethanol production rates were evaluated and compared with *Saccharomyces cerevesiae* Ethanol Red, a typical strain used in fuel alcohol industry.

2. Methods

2.1. 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 220 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). For analytical purpose, 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. The remaining slurry was used as substrate for the different assays.

Chemical composition of both raw and pretreated material (WIS fraction) was determined using the Laboratory Analytical Procedures (LAP) for biomass analysis provided by the National Renewable Energies Laboratory (NREL, 2007). Sugars and degradation compounds contained in the prehydrolysate were also measured. Most of 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 to determine monomeric sugars concentration.

2.2. 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 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 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. 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.

2.3. Microorganism and growth conditions

Two hexose-fermenting yeasts were compared: *K. marxianus* CECT 10875, a thermotolerant strain selected in our laboratory (Ballesteros et al., 1991); and *S. cerevisiae* (Fermentis Ethanol Red, France), an industrial strain used in the fuel alcohol industry. Active culture for inoculation was 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 42 ºC, for *K. marxianus*, or 35 ºC, for *S. cerevisiae*, the precultures were 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. Simultaneous saccharification and fermentation

The slurry obtained was submitted to an SSF process in 250-ml flasks under no sterile conditions. SSF experiments were carried out on a rotary shaker (150 rpm) in 50

mM sodium citrate (pH 5.5) supplemented with the growth medium without glucose described above. Enzyme loading of 15 FPU/g substrate of NS50013 and 15 IU/g substrate of NS50010 and 1 g/L (dry weight) of inoculum were also added. The experiments were run at 42 ºC, for *K. marxianus*, and 35 ºC, for *S. cerevisiae*, for 72 h.

2.5. Presaccharification and simultaneous saccharification and fermentation

The presaccharification and subsequent SSF experiments were also performed in 250-ml flasks. The presaccharification stage was carried out in 50 mM sodium citrate (pH 5.5) for 8 h at 50 ºC and enzyme loading of 15 FPU/g substrate of NS50013 and 15 IU/g substrate of NS50010. After that, the temperature was reduced to 42 ºC or 35 ºC, depending of the yeast used, and nutrients and inoculum were added, which turned the process into SSF. The experiments were run for another 72 h.

2.6. Laccase detoxification

Laccase treatments (L) were different in function of process configurations. For SSF experiments (LSSF), the laccase treatments were carried out for 8 h, 10 U/g substrate of *P. cinnabarinus* enzyme, at 50 ºC, according to its optimal temperature (Ibarra et al., 2006), and 150 rpm prior to SSF. According to Moreno et al., (2012), the treatments were run on dilute slurry with 50 mM sodium citrate (pH 5.5) to final concentration, based on % (w/v) total solid content present in the pretreated slurry, of 6%. After that, the detoxified samples were subsequently submitted to SSF process as explained above. For PSSF experiments (LPSSF), performed as previously described, the presaccharification stage of dilute slurry was supplemented with 10 U/g substrate of *P. cinnabarinus* enzyme.

With the purpose to evaluate the effect of laccase on inhibitory compounds, samples from both laccase treatments (laccase treatment alone or simultaneous laccase treatment and presaccharification) were withdrawn after 8 h of treatment, centrifuged

and the supernatants analyzed for identification and quantification of inhibitory compounds. In the same way, samples from both laccase treatments submitted to SSF were periodically withdrawn, centrifuged and the supernatants analyzed for cell viability, glucose consumption and ethanol concentration.

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

2.7. 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), 4-hydroxybenzaldehyde, vanillin, syringaldehyde, *p*-coumaric acid and ferulic acid were analyzed by HPLC (Agilent, Waldbronn, Germany), using a Coregel 87H3 column (Transgenomic, San Jose, 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 and acetic acid were analyzed by HPLC (Waters, Mildford, MA) using a 2414 refractive index detector (Waters, Mildford, MA) and a Bio-Rad Aminex

HPX-87H (Bio-Rad Labs) column maintained at 65 ºC with a mobile phase (5 mmol/L H_2SO_4) at a flow rate of 0.6 mL/min.

The total phenols 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). 20 µL of sample and the serial standard solution were diluted with 88 µL of water on a 96-well microplate. After the addition of 12 µL Folin-Ciocalteu reagent, plate was incubated for 5 min at room temperature in dark conditions. The reaction was stopped with 80 µL of 7.5% sodium carbonate solution. Before reading, plate was incubated 2 h at room temperature in the dark. The absorbance was measured at 765 nm with a spectrophotometric microplate reader (Anthos Zenyth 200rt).

Cell viability was measured by cell counting using agar plates (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 $MgSO_4 \cdot 7H_2O$, 20 g/L agar). Agar plates were incubated at 42 ºC or 35 ºC, depending of the yeast used, for 24 h.

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

3. Results and Discussion

3.1. Steam explosion pretreatment

Slurry with a total solids content of 21.56% (w/v) resulted after steam explosion pretreatment of the wheat straw. WIS content of the slurry was 16.07% (w/v). Table 1 summarizes WIS and prehydrolysate composition. As shown, pretreatment at 220 ºC and 2.5 min, with a severity factor R_0 of 3.93, enhanced the cellulose proportion of WIS (66.6%) in relation to untreated raw material (40.5%). It is owing to the extensive

solubilization of hemicellulosic sugars, as reflected the high xylose content (12 g/L) in the prehydrolysate. A significant proportion of degradation products were also observed in the prehydrolysate composition, such as acetic acid, formic acid, furfural, 5-HMF and phenols. Acetic acid is released due to the solubilization of acetyl groups contained in the hemicellulose structure. Formic acid comes from furfural and 5-HMF degradation, which derive from xylose and glucose degradation, respectively (Palmqvist and Hahn-Hägerdal, 2000a). Among total phenols, different phenol monomers characteristic of herbaceous biomass were also detected (Fig. 1A), according with previous studies (Tomás-Pejó et al., 2009; Kolb et al., 2012). Among them, aldehydes derived from lignin degradation such as 4-hydroxy-benzaldehyde, vanillin and syringaldehyde, released from the three monolignol units (*p*-hydroxyphenyl-guaiacyl-siringyl) contained in the lignin of wheat straw (Buranov and Mazza, 2008); and acids such as ferulic and *p*-coumaric acids, both derived from cinnamic acids, characteristic of herbaceous biomass acting as linkages between lignin and hemicellulose (Buranov and Mazza, 2008).

3.2. Effect of laccase treatment on the different inhibitory compounds

Whole slurry obtained after pretreatment should be used as substrate for ethanol production. However, the use of the whole slurry implies the presence of high inhibitors concentrations in the media, which affect the downstream ethanol production steps. Usually, the whole slurry resulting from steam explosion is filtered and whased. However, from an economical and environmental point of view, the filtration and washing steps should be avoided because they increase both operational costs and wastewater (García-Aparicio et al., 2006). Hence, the necessity to overcoming the inhibitory effects with strategies differents to filtration and washing.

In this study, the whole slurry was submitted to laccase treatment before saccharification and fermentation experiments. Laccase treatment alone prior to SSF (LSSF) removed around 67% of the total phenols content of slurry (Fig. 2). On the contrary, the concentrations of weak acids and furan derivates were not affected (Table 2), in accordance with previous studies (Larsson et al., 1999; Martín et al., 2002; Chandel et al., 2007; Moreno et al., 2012). Unlike other detoxification methods (mainly physical and chemical methods), which show the capacity to decrease the overall inhibitor concentration in a great or lesser extent, this substrate-specific reaction is only characteristic of laccase enzymes (Palmqvist and Hahn-Hägerdal, 2000b; Taherzadeh and Karimi, 2011). This fact offers some advantages over chemical or physical methods, such as mild reaction conditions, fewer toxic sub-products and low energy requirements (Parawira and Tekere, 2011).

When the whole slurry was submitted to simultaneous laccase treatment and presaccharification step prior to SSF (LPSSF), laccase removed about 73% of the total phenols content slurry (Fig. 2), a slightly higher removal than that observed with laccase treatment alone (67%). This phenomenon could be attributed to presaccharification, which facilitates the access of laccase enzyme to phenols.

As observed in both laccase treatments, the removal of the total phenols content after 8 h of reaction was not complete, in line with previous studies (Jönsson et al., 1998; Larsson et al., 1999; Martín et al., 2002; Chandel et al., 2007; Jurado et al., 2009; Moreno et al., 2012). Some of the lignin phenol monomers identified herein were more resistant to laccase action, such as 4-hydroxy-benzaldehyde and vanillin (Fig. 1B). This is in function of the reactivity of the different lignin phenol monomers towards laccase. In this sense, Kolb et al., (2012) described in liquid hot water pretreated wheat straw supernatants three different laccase reaction groups of phenol monomers, some of them

also identified in this study. Among them, phenol monomers immediately removed (syringaldehyde, ferulic acid and *p*-coumaric acid), phenol monomers removed after reaction of 24h (vanillin), and phenol monomers no affected by laccase action (4-hydroxy-benzaldehyde). They report that an additional methoxy substituent at the benzenic ring (the difference between vanillin and syrindaldehyde) increase the reactivity of the phenolic compounds towards laccase. Moreover, *para*-substituted phenols with ethylene groups, such as ferulic acid and *p*-coumaric acid, show a greater sensitivity towards laccase.

3.3. Effect of laccase treatment on cell viability and ethanol fermentation

S. cerevisiae, the yeast more widely used in industrial fermentation processes, has an optimal fermentation temperature lower than the optimal saccharification temperature, which hinder a complete integration at optimum conditions of saccharification and fermentation processes. Thus, the use of thermotolerant microorganisms such as *K. marxianus* appears as an attractive alternative in SSF processes (Tomás-Pejó et al., 2009; Abdel-Banat et al., 2010). In this context, control and detoxified slurries, resulting from laccase treatment alone or simultaneous laccase treatment and presaccharification, were submitted to SSF for 72 h using *S. cerevisiae* Ethanol Red or *K. marxianus* CECT 10875 at 35 ºC and 42 ºC, respectively. Time courses for cell viability, glucose consumption and ethanol production were studied (Fig. 3 and 4).

3.3.1. LSSF

In a first set of experiments, the performance of both yeasts was followed on slurry submitted to laccase treatment alone. As can be seen for control sample, the lag phase of *S. cerevisiae* was extended up to 12 h (Fig. 3A and 4A). This delay or lag

phase is due to the adaptation of the yeast to fermentation conditions, which depends of different factors, such as the inhibitory compounds type, their concentrations, probably inhibition by synergistic effects between them, and the fermenting microorganism type used (Palmqvist and Hahn-Hägerdal, 2000a; Klinke et al., 2004). Furfural and 5-HMF have a direct inhibition effect on either the glycolytic or fermentative enzymes of the yeast, generally affecting more growth than ethanol production. Weak acids, such as acetic acid and formic acid, disrupt the intracellular pH by its accumulation in the yeast cells, reducing equally biomass formation and ethanol yields. Nevertheless, pH 5.5 used for SSF experiments herein reduce drastically the toxicity of these acids. As furan derivates, phenols also affect growth and ethanol production rate, but not ethanol yield. However, the mechanism is different, since phenols act directly on biological membranes, causing loss of integrity and thereby affecting their ability to serve as selective barriers and enzyme matrices (Palmqvist and Hahn-Hägerdal, 2000a; Klinke et al., 2004).

The assimilation of these inhibitors by yeast, mainly the conversion of furfural, 5-HMF and aromatic aldehydes (vanillin, syringaldehyde, 4-hydroxy-benzaldehyde) to their less inhibitors alcohol forms determines to a great extent the lag phase time (Palmqvist and Hahn-Hägerdal, 2000a; Klinke et al., 2004). As observed in this study, *S. cerevisiae*, after overcoming the lag phase, showed a gradual increment in cell viability, reaching the highest number of colony units at 48 of SSF (Fig. 3A). However, a great increment in ethanol production was observed between 12 and 24 h (Fig. 4A), attaining a maximum ethanol concentration of 11.3 g/L (Table 3). Regarding *K. marxianus*, in spite of its ability to assimilate the majority of inhibitory compounds described above (Oliva et al., 2003), it was practically inhibited. A complete cell viability yeast loss was observed after 12 h of SSF (Fig. 3B); and neither glucose

consumption nor ethanol fermentation occurred during the process (Fig. 4B). Compared to *S. cerevisiae*, this higher inhibitory effect observed when using the thermotolerant yeast *K. marxianus* could also be due to the effect caused by high temperatures on cell membrane, increasing the inhibitory effects of the degradation compounds (Tomás-Pejó et al., 2009; Abdel-Banat et al., 2010).

The specifically phenols removal by laccase treatment improved the fermentation performance of *S. cerevisiae*. Compared to control sample, laccase treatment shorted the yeast lag phase from 12 h to 6 h (Fig. 3A and 4A). On the one hand, cell viability was significantly improved, reaching the highest number of colony units at 24 h of SSF (Fig. 3A). On the other hand, faster glucose consumption and ethanol production rates were also observed (Fig. 4A), increasing the ethanol productivity value from 0.39 g/L h, for control sample, to 0.44 g/L h for laccase sample. Nevertheless, laccase treatment did not improve the ethanol production, obtaining a similar ethanol yield (0.35 g/g) compared to control sample (Table 3). These results support the inhibitory effects reported for phenols, affecting growth and ethanol production rate, but not ethanol yield (Palmqvist and Hahn-Hägerdal, 2000a; Klinke et al., 2004).

Similar enhancements on the *S. cerevisiae* performance have also been attributed to different laccase enzymes in several detoxification studies. Jönsson et al., (1998) reported higher glucose consumption rate, ethanol productivity value and ethanol yield when prehydrolysate from acid steam-exploded willow was submitted to *Trametes versicolor* laccase. Larsson et al., (1999), described similar results together with a higher yeast growth on prehydrolysate from acid steam-exploded spruce treated with *T. versicolor* laccase. In contrast, Jurado et al., (2009) described a higher influence on ethanol conversion than yeast growth when enzymatic hydrolysed from both acid and

non acid steam-exploded wheat straw were submitted to laccases from *Trametes villosa* or *Coriolopsis rigida*.

In the case of *K. marxianus*, laccase treatment triggered the fermentation of the medium. A great increment in cell viability was observed between 12 and 24 h of SSF (Fig. 3B); followed by a remarkable drop after 30 h. In addition, the yeast also consumed almost all the glucose between 12 and 24 h (Fig. 4B), attaining an ethanol concentration (10.7 g/L) and an ethanol yield (0.35 g/g) similar to those obtained with *S. cerevisiae* on either control or laccase samples (Table 3). Nevertheless, the ethanol production rate was slower compared to *S. cerevisiae*, showing an ethanol productivity value (0.36 g/L h) lower to that obtained with *S. cerevisiae* on laccase sample (0.44 g/L h) (Table 3).

3.3.2. LPSSF

In a second set of experiments, the performance of both yeasts was followed on slurry submitted to simultaneous laccase treatment and presaccharification. Enzymatic prehydrolysis prior to the addition of yeast offer some advantages, such as to make the media more fluid and easer to handle, facilitating the mixing during fermentation. Prehydrolysis also enables hydrolytic enzymes to act at their optima temperatures, enhancing the enzymatic hydrolysis and consequently increasing the ethanol production rate (Manzanares et al., 2011).

Compared to SSF with no presacharification, the enzymatic prehydrolysis altered slightly the performance of *S. cerevisiae*. In contrast to SSF with no prehydrolysis, where a gradual increment of cell viability was observed during the process (Fig. 3A), cell viability showed a great increment between 12 and 24 h when presaccharification was performed (Fig. 3C). Ethanol production was also strongly increased between 12 and 24 h (Fig. 4C), attaining a similar ethanol concentration (10.8

g/L) and a slightly higher ethanol productivity value (0.41 g/L h) with presaccharification stage (Table 3). Regarding *K. marxianus*, it was also totally inhibited with prehydrolysis. A complete cell viability yeast loss was observed after 6 h of SSF (Fig. 3D); and neither glucose consumption nor ethanol fermentation resulted during the process (Fig. 4D).

As previously described for LSSF, the supplementation of presaccharification with laccase enzyme also enhanced the fermentation performance of *S. cerevisiae*. Compared to control sample, laccase supplementation shorted the yeast lag phase from 12 h to 6 h (Fig. 3C and 4C). Cell viability was significantly improved, reaching the highest number of colony units at 12 h of SSF (Fig. 3C); and faster glucose consumption and ethanol production rates were also observed (Fig. 4C), increasing slightly the ethanol productivity value from 0.41 g/L h, for control sample, to 0.43 g/L h for laccase sample. Nevertheless, the laccase supplementation did not improve either the ethanol production, obtaining a similar ethanol yield (0.34 g/g) compared to control sample (Table 3). With regard to *K. marxianus*, as previously described for LSSF, laccase supplementation also triggered the fermentation of the medium. A gradual increment in cell viability was showed between 12 and 30 h of SSF, observing after that a remarkable drop (Fig. 3D). In the same way, the yeast also consumed almost all the glucose between 24 and 30 h (Fig. 4D), attaining an ethanol concentration (11.1 g/L) and an ethanol yield (0.36 g/g) similar to those obtained with *S. cerevisiae* on either control or laccase samples (Table 3). However, the ethanol production rate was very slow compared to *S. cerevisiae*, showing an ethanol productivity value especially lower (0.20 g/L h) to that obtained with *S. cerevisiae* on laccase sample (0.43 g/L h) (Table 3).

In spite of the presaccharification improves the laccase action, observing a slightly higher removal of phenols than laccase treatment alone, *S. cerevisiae* showed a

similar fermentation performance in both LSSF and LPSSF configurations. *K. marxianus* also showed a similar ethanol production in both process configurations (Table 3). However, the presaccharification slowed down the process, observing a gradual increment of cell viability and slower glucose consumption and ethanol production rates (Fig. 2D and 3D), attaining a drop ethanol productivity value from 0.36 g/L h, with no presaccharifacation, to 0.20 g/L h with presaccharification (Table 3).

On the light of the results presented herein, and with the aim to proceed towards the use of *K. marxianus* and laccase enzymes for lignocellulosic ethanol production, other aspects, such as different substrate loadings and pretreatment conditions need to be studied greatly.

4. Conclusion

Higher integration of saccharification and fermentation processes is a key aspect to make feasible the lignocellulosic ethanol production. In this context, thermotolerant microorganisms, with the capacity to ferment ethanol at temperatures that are optimal for saccharification, could contribute in a greater extent. However, as showed herein, the presence of inhibitory compounds in the media limit its use. *K. marxianus* CECT 10875, the thermotolerant yeast used in this study, was unable to growth and produce ethanol on slurry from steam-exploded wheat straw. However, prior laccase treatment triggered its fermentability, obtaining ethanol concentrations and yields comparable to those obtained by *S. cerevisiae*.

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Table captions

Table 1. Composition of steam-exploded wheat straw

Table 2. Inhibitory compounds composition of samples resulting from control and laccase treated slurries

Table 3. Summary of LSSF and LPSSF assays of dilute slurry using *K. marxianus* and *S. cerevisiae*

.

Figure captions

Figure 1. Comparison of HPLC profiles of samples resulting from laccase treatment alone. Control (A) and laccase (B) samples. 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 2. Total phenol content determined by Folin-Ciocalteau method of samples resulting from laccase treatment alone or simultaneous laccase treatment and presaccharification. NP, no presaccharification; P, presaccharification. Control (gray bars) and laccase (white bars) samples.

Figure 3. Viable cells during LSSF (left) and LPSSF (right) processes of slurry samples. Symbols used: control (¦) and laccase (?) samples. *S. cerevisiae* (A, C) and *k. marxianus* (B, D).

Figure 4. Time course for ethanol (filled symbols and continuous lines) and glucose (open symbols and discontinuous lines) during LSSF (left) and LPSSF (right) processes of slurry samples: control (¦ , ?) and laccase (?, ?) samples. *S. cerevisiae* (A, C) and *k. marxianus* (B, D).

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	63.0	Glucose	0.98	3.49	Furfural	3.5
Hemicellulose	2.70	Xylose	3.94	8.07	5-HMF	1.1
Lignin	35.3	Arabinose	0.27	0.20	Acetic acid	11.9
		Galactose	0.31	0.19	Formic acid	9.3
		Mannose	0.09	0.38	Phenols	9.8

Table 2. Inhibitory compounds composition of samples resulting from control and laccase treated slurries

	Sample	Inhibitors (g/L)			
		Furfural	5-HMF	Acetic acid	Formic Acid
NP	C	0.579	0.152	2.769	2.098
	L	0.566	0.150	2.845	nq
P	C	0.593	0.148	3.032	2.011
	L	0.557	0.144	3.064	nq

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

Table 3. Summary of LSSF and LPSSF assays of dilute slurry using *K. marxianus* CECT 10875 and *S. cerevisiae* Ethanol Red

		Sample	EtOH (g/L)	$Y_{E/G}$ (g/g)	$Y_{E/ET}$ (%)	Q_E (g/L h)
S. cerevisiae	NP	C	11.3	0.37	71.9	0.39
		L	10.7	0.35	68.4	0.44
	P	C	10.8	0.35	68.5	0.41
		L	10.4	0.34	66.1	0.43
K. marxianus	NP	C	1.7	0.06	10.8	0.07
		L	10.7	0.35	69.2	0.36
	P	C	1.7	0.05	10.7	0.07
		L	11.1	0.36	70.9	0.20

C, control samples; L, laccase treatment; P, presaccharification; NP, no presaccharification; $EtOH_M$, maximum ethanol concentration; $Y_{E/G}$, ethanol yield based on total glucose content present in the pretreated wheat straw; $Y_{E/ET}$, theorical ethanol yield assuming ethanol yields on glucose by *K. marxianus* and by *S. cerevisiae* 0.51 g/g; Q_E, volumetric ethanol productivity at 24 h and 72 h.

Figure 1

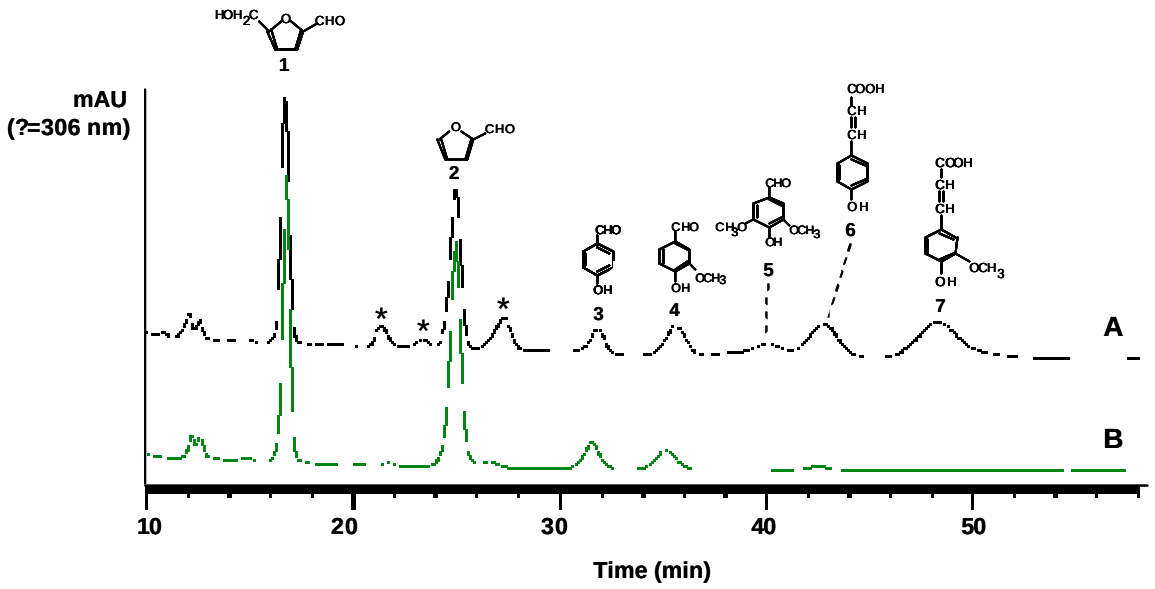


Figure 2

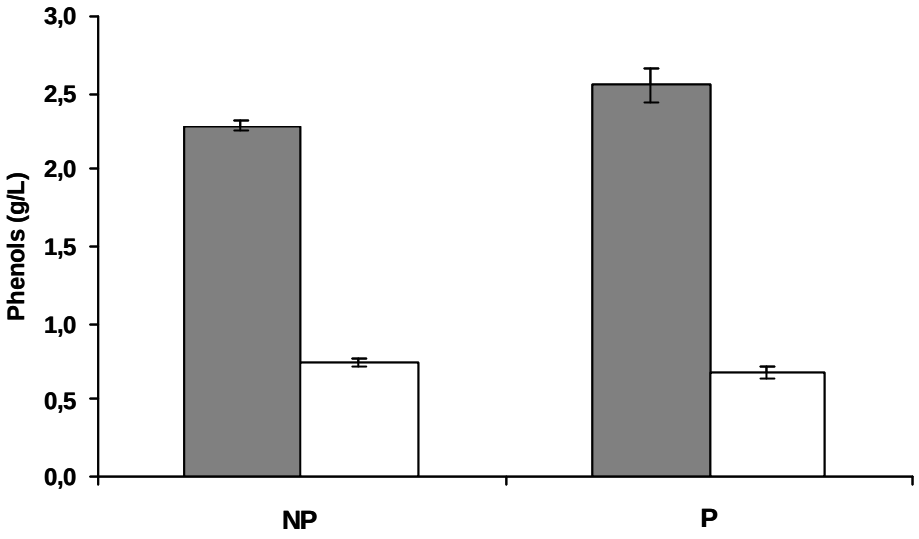


Figure 3

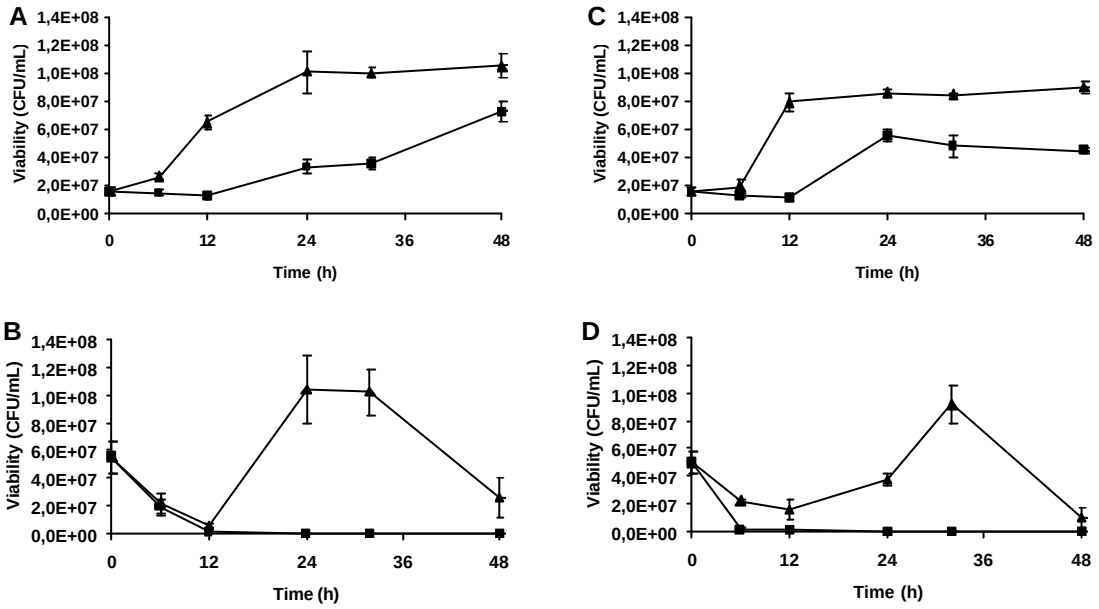
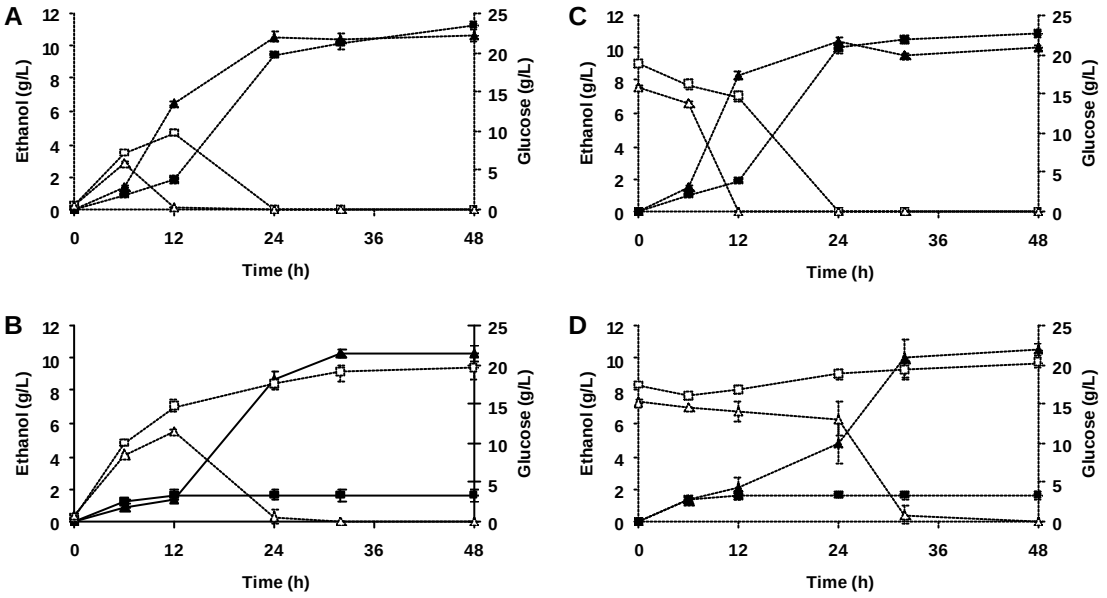

A
B
C
D
Viability (CFU/mL)
1,4E+08
1,2E+08
1,0E+08
8,0E+07
6,0E+07
4,0E+07
2,0E+07
0,0E+00
0
12
24
36
48
Time (h)

Figure 4


A
B
C
D
Ethanol (g/L)
Glucose (g/L)
Time (h)
0
12
24
36
48
0
2
4
6
8
10
12
0
5
10
15
20
25

1