In situ laccase treatment enhances the fermentability of steam-exploded wheat straw in SSCF processes at high dry matter consistencies

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Abstract

This work evaluates the *in situ* detoxification of inhibitory lignocellulosic broths by laccases to facilitate their fermentation by the xylose-consuming *Saccharomyces cerevisiae* F12. Treatment of wheat straw slurries with laccases prior to SSCF processes decreased the total phenolic content by 50-80%, reducing the lag phase and increasing the cell viability. After laccase treatment, a negative impact on enzymatic hydrolysis was observed. This effect, together with the low enzymatic hydrolysis yields when increasing consistency, resulted in a decrease in final ethanol yields. Furthermore, when using high substrate loading (20% DM (w/v)), high concentration of inhibitors prevailed in broths and the absence of an extra nitrogen source led to a total cell growth inhibition within the first 24 h in non-treated samples. This inhibition of growth at 20% DM (w/v) was overcome by laccase treatment with no addition of nitrogen, allowing *S. cerevisiae* F12 to produce more than 22 g/L of ethanol.

Keywords: lignocellulose, bioethanol, simultaneous saccharification and co-fermentation, *in situ* laccase detoxification, steam explosion, *S. cerevisiae* F12.

1. Introduction

The current challenges to developing a cost-effective industrial bioethanol production include obtaining high product concentration, efficient process integration and an improvement in water economy. In this context, the use of whole slurries obtained after physico-chemical pretreatments such as steam explosion are considered suitable alternatives towards these goals. When using the whole slurry there is no need for extra equipment (filtration and washing systems), the amount of wastewater generated and freshwater required is reduced and the concentration of fermentable sugars is increased. However, the main drawback in using whole pretreated materials is the presence of inhibitory compounds released during pretreatment (weak acids, furan derivatives and phenols) that hinder sugar conversion into ethanol by fermenting microorganisms (Palmqvist and Hahn-Hägerdal, 2000; Klinke et al., 2004; Panagiotou and Olsson, 2007). Among several detoxification methods developed to overcome the effect of such inhibitory compounds, *in situ* laccase treatment represents a suitable option since it is carried out in the same vessel under mild reaction conditions, requires low energy and generates few by-products (Palmqvist and Hahn-Hägerdal, 2000). Laccases are multicopper-containing phenoloxidases which catalyze the oxidation of substituted phenols, anilines and aromatic thiols at the expense of molecular oxygen (Parawira and Tekere, 2011). This capacity allows laccases to act specifically on phenolic compounds present in pretreated materials (Martín et al., 2002; Jurado et al., 2009; Moreno et al., 2012, 2013).

Xylose content in agricultural residues such as wheat straw can constitute up to 30% of the total sugars, hence the importance of using microorganisms able to ferment to ethanol both glucose and xylose simultaneously. *Saccharomyces cerevisiae* F12 has been genetically engineered and evolved to ferment xylose (Sonderegger et al., 2004; Tomás-Pejó et al., 2010). Furthermore, this strain has been successfully employed to produce ethanol from different lignocellulosic raw materials (Panagiotou and Olsson, 2007; Tomás-Pejó et al., 2008).

Simultaneous saccharification and co-fermentation (SSCF) processes in which hydrolysis of the fibres and co-fermentation of glucose and xylose are integrated in one step are known to be a good alternative to separate processes. SSCF leads to cost reduction (one single vessel is used) and less end-product inhibition of most commonly

used enzymes. In the present work, the combination of *in situ* detoxification of steam-exploded wheat straw by laccase before SSCF processes was evaluated using the evolved xylose-recombinant *S. cerevisiae* F12 as fermenting microorganism.

2. Materials and Methods

2.1. Raw material and pretreatment

Wheat straw employed as a raw material was supplied by Ecocarburantes de Castilla y León (Salamanca, Spain) and had the following composition (% dry weight (w/v)): cellulose, 40.5; hemicelluloses, 26.1; lignin, 18.1 and others, 15.3 (Alvira et al., 2011).

The biomass was firstly milled using a laboratory hammer mill in order to obtain a chip size of between 2 and 10 mm. Two batches of milled wheat straw were pretreated without acid impregnation at 200 ºC and 210 ºC for 2.5 min by steam explosion in a 2 L reactor vessel. The pH of the recovered materials was 3.9 and 3.7, respectively, and small portions of both batches were vacuum filtered to obtain two fractions, one for analysis of water insoluble solids (WIS) and one for analysis of liquid fraction. Afterwards, the material was kept at 4 ºC until use.

2.2. Enzymes

Pycnoporus cinnabarinus laccase (Beldem, Belgium) was used for detoxification. The activity of this enzyme (60 IU/mL) was measured by oxidation of 5 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) to its cation radical ($\varepsilon 436 = 29300\ M^{-1}\ cm^{-1}$) in 0.1 M sodium acetate (pH 5) at 24 ºC.

An enzyme mixture of Cellic CTec2 and Cellic Htec2 (Novozymes, Denmark) was used for saccharification. Cellic Ctec2 is a cellulase preparation that shows high β-glucosidase activity, whilst Cellic Htec2 is a hemicellulase preparation with mainly endoxylanase activity. The overall cellulase activity was determined using filter paper (Whatman No. 1 filter paper strips) and β-glucosidase activity was measured using cellobiose as substrate on Cellic Ctec2 (100 FPU/mL and 3950 IU/mL of cellulase and β–glucosidase activities, respectively) (Ghose, 1987). Furthermore, xylanase activity

was determined using birchwood xylan on Cellic Htec2 (1300 IU/mL) (Bailey et al., 1991).

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

2.3. Microorganisms and media

Evolved *S. cerevisiae* F12 was used as fermenting microorganism (Tomás-Pejó et al. 2010). This recombinant *S. cerevisiae* strain was modified to ferment xylose by over-expressing the endogenous gene encoding xylulokinase (XK) and by introducing the genes encoding for xylose reductase (XR) and xylitol dehydrogenase (XDH) from *Pichia stipitis* (Sonderegger et al., 2004). Preinoculums were grown for 24 h at 150 rpm and 30 ºC in 250 mL Erlenmeyer flasks containing 50 mL of Delft medium as follows: 20 g/L glucose, 20 g/L xylose, 7.5 g/L $(NH_4)_2SO_4$, 3.5 g/L KH_2PO_4, 0.75 g/L $MgSO_4 \cdot 7H_2O$, 2 mL/L trace metal solution and 1 mL/L vitamin solution. Cells were harvested by centrifugation at 5,300 g for 5 min at room temperature. The supernatant was discarded and the cell pellet was washed once with sterile water and centrifuged again. The cell pellet was weighed and diluted with sterile water to obtain the desired inoculum size.

2.4. Laccase treatment

P. cinnabarinus laccase was used to detoxify the steam-exploded wheat straw before enzymatic hydrolysis or SSCF. The slurry obtained after pretreatment at 200 ºC was diluted to 10%, 13.5%, 16% and 20% dry matter (DM) (w/v) with citrate buffer 50 mM, pH 5.5, while in the case of the slurry obtained at 210 ºC only 12% and 16% DM (w/v) substrate loading were tested. Laccase was added at 10 IU/g DM substrate to the diluted slurries and incubated for 12 h at 50 ºC and 180 rpm before addition of hydrolytic enzymes and/or yeast.

2.5. Enzymatic hydrolysis of wheat straw

In order to evaluate the laccase treatment during the saccharification step, both treated and non-treated samples from the slurry obtained at 210 ºC and diluted at 16% DM (w/v) were subjected to enzymatic hydrolysis. In this case, 15 FPU/g DM substrate

of the cellulolytic cocktail Cellic CTec2 and 60 IU/g DM substrate of Cellic HTec2 were added to the broths. Samples were incubated in triplicate for 72 h under optimal saccharification conditions, 50 ºC and 180 rpm.

2.6. Simultaneous saccharification and co-fermentation processes

All untreated and laccase-treated slurries were subjected to SSCF processes in 100 mL shake flasks using rubber caps with a needle to allow CO_2 outflow. All the experiments were run in triplicate at 35 ºC and pH 5.5 for 144 h with 1 g/L (dry weight) of the evolved xylose-consuming *S. cerevisiae* F12. In all cases, 5 g/L of diammonium phosphate (DAP) were supplemented as nitrogen source unless the contrary is specified.

For saccharification, an enzymatic mixture consisting of Cellic Ctec2 and Cellic HTec2 (Novozymes, Denmark) was added at 15 FPU/g and 60 IU/g DM substrate, respectively, according to Barta et al. (2011).

2.7. Analytical methods

WIS fraction was analyzed using the National Renewable Energy Laboratory (NREL) standard methods for determination of structural carbohydrates and lignin in biomass (LAP-002, LAP-003, and LAP-019) (NREL). Dry weight of slurry and WIS were determined by drying the samples at 105 ºC for 24 h (LAP-001).

Total phenolic content was analyzed in the supernatants according to a slightly modified version of the Folin-Ciocalteau method (Alvira et al., 2013).

Extracellular metabolites, sugars, 5-hydroxymethylfurfural (5-HMF) and furfural were analyzed by high performance liquid chromatography (HPLC) using an Aminex HPX-87H column with a 30 x 4.6 mm Cation-H Biorad micro-guard column maintained at 45°C. The eluent was 5 mM H_2SO_4 at a flow rate of 0.6 ml/min. Formic acid and acetic acid were determined at the same conditions maintaining the column at 65 ºC.

Samples were taken at different times along the SSCF process and centrifuged at 18,800 g for 3 min. Supernatant was filtered with 0.20 µm nylon filters and stored at -20 ºC until analysis.

Cell viability was determined as colony forming unit (CFU/mL) by cell counting using agar plates containing the following media: 20 g/L glucose, 20 g/L agar, 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 agar plates were incubated at 30 ºC for 48 h.

3. Results and Discussion

3.1. Pretreated material

After steam explosion pretreatment, slurries with a DM content of 33.2% (w/v) (200 ºC, 2.5 min) and 26.0% (w/v) (210 ºC, 2.5 min), respectively, were collected (Table 1). Both materials showed an increase in the cellulosic fraction from 40.5% to 48.2% and 57.3%, respectively, due to an extensive hemicellulose solubilization. Supporting this, high xylose concentration (mainly in the oligomeric form) and sugar degradation products were recovered and identified in their liquid fractions (Table 2).

Acetic acid, formic acid, furfural, 5-HMF and phenolic compounds were found to be the main inhibitors. These compounds are generated from different lignocellulosic fractions. Furfural and 5-HMF derive from pentoses and hexoses decomposition, respectively, and further degradation of both these compounds leads to the production of formic acid. The hydrolysis of acetyl groups forms acetic acid and phenolic compounds are solubilized lignin units (Oliva et al., 2003; Alvira et al., 2010). Depending on their nature, these inhibitors can alter the growth of the fermenting microorganisms and inhibit cellulolytic enzymes, decreasing final yields and productivities (Ximenes et al., 2010, 2011; Almeida et al., 2007; Taherzadeh and Karimi, 2011). Weak acids reduce biomass formation by modifying the intracellular pH that promotes an imbalance in the ATP/ADP ratio; furan derivatives affect different intracellular pathways, extending the microorganism lag phase; and finally, phenolic compounds can alter biological membranes, thus decreasing growth rates and also inhibiting or deactivating hydrolytic enzymes.

3.2. Effect of laccase treatment on phenolic compounds

Total phenolic content was measured in untreated and laccase-treated diluted slurries before cellulases and yeast addition (Figure 1). Laccase-treated samples showed a reduction in the total phenolic content of between 50-80% compared to untreated

196 slurries. In terms of phenol concentration, the highest reduction was observed when
197 detoxifying slurries pretreated at 200 ºC with 20% DM (w/v).

198 With higher substrate loadings, the reduction of the phenolic compounds was
199 more remarkable (Figure 1). With pretreated slurry at 200 ºC, about 2 g/L and 4.5 g/L of
200 total phenols were removed at 10% and 20% DM (w/v), respectively. Regarding
201 pretreated slurry at 210 ºC, 3 g/L and 3.6 g/L phenols were removed with 12% and 16%
202 DM (w/v), respectively.

203 In terms of efficiency, about 60% fewer phenols were measured in all diluted
204 samples obtained at 200 ºC after lacasse treatment; whilst in the case of samples from
205 pretreated material at 210 ºC, efficiency decreased from 77% at 12% DM (w/v) to 67%
206 at 16% DM (w/v) (Figure 1). It should be noticed, however, that higher efficiency
207 values were obtained with the pretreated material at 210 ºC. This result can be explained
208 by the differences in viscosity between both materials. The higher severity pretreatment
209 conditions at 210 ºC lead to a higher breakdown of lignocellulosic fibers, which helps to
210 increase the homogeneity of the material and reduce viscosity, promoting the
211 accessibility of laccase to phenolic compounds and increasing the treatment efficiency
212 (Samaniuk et al., 2011; Alvira et al., 2013). By contrast, at high substrate loading,
213 mixing and mass transfer limitations can negatively affect laccase action towards
214 phenols (Alvira et al., 2013). Thus, augmenting consistency from 12% to 16% DM
215 (w/v) (slurry obtained at 210 ºC) resulted in a reduction of the efficiency from 77% to
216 67% (Figure 1).
217 The mechanism involved in laccase detoxification is oxidative polymerization
218 (Jönsson et al., 1998). Laccases extract one electron from phenols, generating unstable
219 phenoxy-radicals, which leads to polymerization into less toxic aromatic compounds.
220 Total depletion in phenolic content seems to be impossible due to the selective action of
221 laccase (Kolb et al., 2012; Alvira et al., 2013). A wide variety of phenolic substituted
222 compounds such as 4-hydroxybenzaldehyde, vanillin, syringaldehyde, *p*-coumaric acid,
223 ferulic acid, have been identified in steam-exploded wheat straw (Tomás-Pejó et al.,
224 2009; Alvira et al., 2010; Moreno et al., 2012; 2013). Laccases can easily convert some
225 such compounds, e.g., syringaldehyde or cinnamic acids. In contrast, other phenolic
226 compounds are oxidized with lower rates (vanillin) or remain intact

(hydroxybenzaldehyde) (Kolb et al., 2012; Moreno et al., 2012; 2013; Alvira et al., 2013).

3.3. Effects of laccase on saccharification

Sugar concentration is directly correlated with ethanol production and a decrease in saccharification yields negatively affects this fermentation product. Figure 2 shows less glucose released during the hydrolysis step after the enzymatic detoxification, which implies a reduction in the saccharification yields. However, xylose released seems not to be affected. This negative effect of laccases during the saccharification step was previously described with steam-exploded wheat straw (Jurado et al., 2009; Moreno et al., 2012). Jurado et al. (2009) showed lower sugar recovery during the saccharification step of steam-exploded wheat straw after treatment with *Trametes villosa* or *Coriolopsis rigida* laccases. The same phenomenon was described by Moreno et al. (2012) who reported fewer sugars released after the enzymatic hydrolysis of samples treated with *P. cinnabarinus* or *T. villosa* laccases. However, in both mentioned cases different cellulolytic enzymes were employed.

In addition to the lower yields, a reduction in the saccharification rates was also observed (Figure 2). Compared to non-treated slurries, where the hydrolysis ended after 48 h, in laccase-treated samples this process was longer, reaching the highest glucose concentration at 144 h. This negative performance of enzymatic hydrolysis observed in laccase-treated materials could be attributed to several causes, including an inhibition or deactivation of cellulases by products formed, an increase in the non-productive binding of hydrolytic enzymes and a strengthening of the lignin-carbohydrate complexes (Moilanen et al., 2011; Tejirian and Xu, 2011). As mentioned above, the reaction mechanism of laccases involves an oxidative polymerization of phenols via formation of radicals. The resulting oligomers are less toxic to the yeast than simple phenolic compounds, but they can nevertheless exert greater inhibition than such compounds on hydrolytic enzymes (Tejirian and Xu, 2011). Beyond the oxidative polymerization of phenols, laccases probably catalyze different reactions on lignin, changing its surface properties and consequently encouraging the non-productive binding of cellulases. Finally, carbohydrates in laccase-treated samples can be more tightly bound to lignin,

rendering the substrate more difficult to hydrolyze enzymatically (Moilanen et al., 2011).

3.4. SSCF processes of untreated and laccase-treated samples

Untreated and laccase-treated samples with a DM content ranging from 10% to 20% (w/v) were used in SSCF processes using the evolved xylose-recombinant *S. cerevisiae* F12 strain. As has been shown previously, the use of high tolerant xylose-fermenting yeasts that can ferment both glucose and xylose present in toxic materials offers the possibility to increase final ethanol concentrations by converting all potential sugars and working at high substrate consistencies (Tomás-Pejó et al., 2010).

Figure 3A shows the ethanol production obtained in this study after different SSCF processes of both slurries. An increment in the final ethanol concentration was obtained when increasing consistency, reaching the highest concentration (25.0 g/L) at 20% DM (w/v) with untreated slurry obtained at 200 ºC. Taking into account all potential glucose and xylose, however, an adverse effect in ethanol yields was observed when increasing consistency (Figure 3B). Ethanol yields were calculated as the concentration of ethanol divided by the whole amount of glucose and xylose present in the slurry (WIS and hydrolysate) at different substrate loadings. Compared to samples at 10% DM (w/v), the ethanol yield decreased from 0.27 g/g to 0.20 g/g after augmenting substrate consistency up to 20% DM (w/v). This effect could be due to a correlation between the increment in substrate consistency and the decrease in enzymatic hydrolysis yields (Kristensen et al., 2009; Wang et al., 2011). Although there are many factors that can affect hydrolytic enzymes such as end-product inhibition, unproductive binding or protein deactivation or denaturalization (Jørgensen et al., 2007), recent studies have found that a decline in the binding capacity of enzymes to cellulose can cause a reduction in the enzymatic hydrolysis yields at high substrate loadings (Kristensen et al., 2009; Wang et al., 2011). In the same way, laccase-treated samples in this study showed similar effects on ethanol production and yield as for untreated samples. However, compared with the processes where no laccases were added, lower final ethanol concentrations were obtained in all cases due to the poorer performance of hydrolytic enzymes during SSCF in the presence of laccases (Figure 2).

In addition to ethanol production, viability in the form of CFU/mL was also determined during the SSCF processes (Figure 3C). When SSCF was performed at 16% and 20% DM (w/v) with material pretreated at 200 ºC, experiments were carried out with and without addition of DAP. It is known that the growth of a microorganism and its robustness is highly influenced by medium composition. Under anaerobic conditions, nitrogen is essential for cell proliferation, and the rate of ethanol production is higher in yeast growing actively (Devantier, et al., 2005; Jørgensen, 2009). Hence, the lack of good nitrogen sources in combination with a high inhibitory concentration can hamper yeast fermentation performance. In this sense, an enzymatic detoxification step prior to inoculation could reduce this synergistic effect, allowing the growth of the fermenting microorganism in broths without extra nitrogen addition. Media supplementation nutrients have a very strong effect on the cost of the bioethanol process. Thus, lacasse treatment could be a strategy to avoid nutrient addition with the resultant cost reduction.

Using the slurry obtained at 200 ºC, the number of CFU/mL was enhanced by increasing consistency when the toxicity was low (10-16% DM (w/v)); however, in samples with the highest substrate loading, 20% DM (w/v) with or without DAP, and 16% DM (w/v) without DAP, a clear drop in viability was observed (Figure 3C). When increasing consistency, inhibitory compounds were also present in higher concentrations, which may explain the drop in viability. Complete growth inhibition was observed in untreated samples at 20% DM (w/v) without DAP (Figure 4A). However, $17x10^6$ cells/mL were counted when treating the same medium with laccase in spite of non-DAP addition.

No cell growth was observed within the first 24 h in the treated samples in the absence of extra nitrogen source (Figure 4A) in contrast to the SSCF process performed in the presence of DAP, where growth started after 12 h (Figure 4B). The lag phase before starting the growth caused both glucose and xylose accumulation in the broth by the action of enzymes. Compared to untreated samples, the reduction in total phenolic content by laccase treatment triggered the SSCF process without DAP (Figure 4A), obtaining an ethanol concentration of 22.3 g/L (Figure 3A). This value is similar to that obtained with the nitrogen-supplemented laccase-treated samples (Figure 3A).

The higher pretreatment severity conditions in the case of the slurry obtained at 210 ºC increased the inhibitory profile of the pretreated material, showing a total growth

inhibition in untreated samples at 16% DM (w/v), even with DAP as nitrogen source (Figure 5A). In this case, neither sugar consumption nor ethanol production were observed (Figures 3A and 5A), as previously described for samples (200 ºC) at 20% DM (w/v) without DAP (Figure 4A). Nevertheless, laccase treatment enabled growth (Figure 5A) and contributed to reaching a final ethanol concentration of 22.1 g/L compared to 19.6 or 19.1 g/L (Figure 3A) obtained with the untreated or laccase-treated samples at 12% DM (w/v), respectively.

As mentioned before, the substantial phenol removal in laccase-treated samples showed a reduction in the fermentation lag phase (Figures 4B and 5B) and, importantly, an increase in the maximum number of colony forming units during the SSCF processes. The reduced phenolic content in less inhibitory broths led to short lag phases and enhanced cell viability compared to non-treated samples. By contrast, lower maximum CFU/mL was observed for laccase-treated samples at 20% DM (w/v) with the slurry obtained at 200 ºC. In spite of the high deviation observed at this point, this value could be attributed to less glucose being available for the yeast due to a poor enzymatic hydrolysis after laccase treatement, as discussed above (Figures 2 and 4B).

During SSCF processes, glucose and xylose accumulated during the lag phase of the microorganism (Figures 4 and 5). Afterwards, *S. cerevisiae* F12 started to consume glucose while xylose concentration did not show a clear drop. In the presence of both sugars, a delay in xylose consumption is usually observed because they share the transporter by which both sugars are incorporated into the cells (Meinander et al., 1999). Furthermore, the stress suffered by yeast due to the high inhibitory fermentation broths produced a reduction of cell viability after glucose depletion, limiting the fermentation of xylose and explaining its higher concentration at the end of the fermentation (Figures 4 and 5).

The slights differences in the final xylose concentration between untreated and treated samples in SSCF processes (Figure 4A and 5A) show evidences of xylose coconsumption. As has been mentioned before, in a simultaneous process, higher sugar release would be expected due to the reduction of the end-product inhibition which could derive in higher amount of xylose released in treated samples (Figure 4A and 5A). This fact together with the lower amount of residual xylose clearly suggests sugars coconsumption.

This continuous xylose and glucose released from the solid fraction during the SSCF process makes the determination of sugar conversion yields difficult. Further research in order to improve xylose consumption could be done by using different process configurations (fed-batch, pre-fermentation of liquid fraction, etc.) and different addition times for the laccase enzymes.

4. Conclusions

Laccase treatment decreased the inhibitory profile of slurries by reducing total phenolic content. This strategy resulted in shorter lag phases and higher cell viability for the evolved *S. cerevisiae* F12 strain than in SSCF with non-treated samples. By increasing consistency, higher ethanol concentrations were obtained, but, lower sugar recoveries decreased the overall yields. Laccase treatment enabled the fermentation of inhibitory broths non fermentable before treatment (210 ºC, 16% DM). Fermentation was boosted at 20% DM in spite of the absence of extra nitrogen, thus suggesting that this approach could contribute an important cost reduction for an industrial process.

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Table captions

Table 1. Composition of pretreated wheat straw

Table 2. Composition of liquid fractions from steam-exploded wheat straw

Figure captions

Figure 1. Total phenolic content prior fermentation of untreated (grey bars) and laccase-treated (white bars) wheat straw slurries at different consistencies.

Figure 2. Glucose (■) and xylose (▲) concentrations released during an enzymatic hydrolysis process of untreated (discontinuous lines) and laccase-treated (continuous lines) wheat straw slurry (210 ºC) at 16% DM (w/v). Enzyme loading: 15 FPU/g DM substrate Cellic CTec and 60 IU/g DM substrate Cellic HTec2.

Figure 3. Ethanol production (**A**), ethanol yield (**B**) and maximum cell viability (**C**), in terms of CFU/mL, for untreated (grey bars) and laccase-treated (white bars) wheat straw slurries at different consistencies. (*) No DAP added.

Figure 4. Time course for SSCF process of untreated (discontinuous lines) and laccase-treated (continuous lines) wheat straw slurry (200 ºC) at 20% DM (w/v) without (**A**) or with (**B**) DAP. Glucose (■) and xylose (▲) concentrations and cell viability (●) in terms of CFU/mL.

Figure 5. Time course for SSCF process of untreated (discontinuous lines) and laccase-treated (continuous lines) wheat straw slurry (210 ºC) at 16% (**A**) or 12% DM (w/v) (**B**). Glucose (■) and xylose (▲) concentrations and cell viability (●) in terms of CFU/mL.

511 **Table 1**

512 Table 1. Composition of pretreated wheat straw.

Pretreatment conditions	**200 °C, 2.5 min**	**210 °C, 2.5 min**
Total solids (% (w/v))	33.2	26.0
WIS content (% (w/v))	21.6	21.5
Composition (% (w/w) dry weight)	WIS fraction	
Cellulose	48.2	57.3
Hemicellulose	14.3	10.4
Lignin	28.1	30.0
Others	9.4	2.3

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517 **Table 2**

518 Table 2. Composition of liquid fractions from steam-exploded wheat straw.

Monosaccharides	**200 °C, 2.5 min**		**210 °C, 2.5 min**	
	Monomeric form (g/L)	**Oligomeric form (g/L)**	**Monomeric form (g/L)**	**Oligomeric form (g/L)**
Glucose	1.7	11.6	1.0	3.7
Xylose	2.2	28.1	2.6	23.6
Galactose	0.3	2.5	1.5	0.4
Arabinose	1.1	2.0	0.8	1.32
Degradation products			**g/L**	**g/L**
Formic acid			7.0	7.8
Acetic acid			6.6	5.6
5-HMF			0.3	0.3
Furfural			0.7	0.7
Total phenols			11.6	9.4

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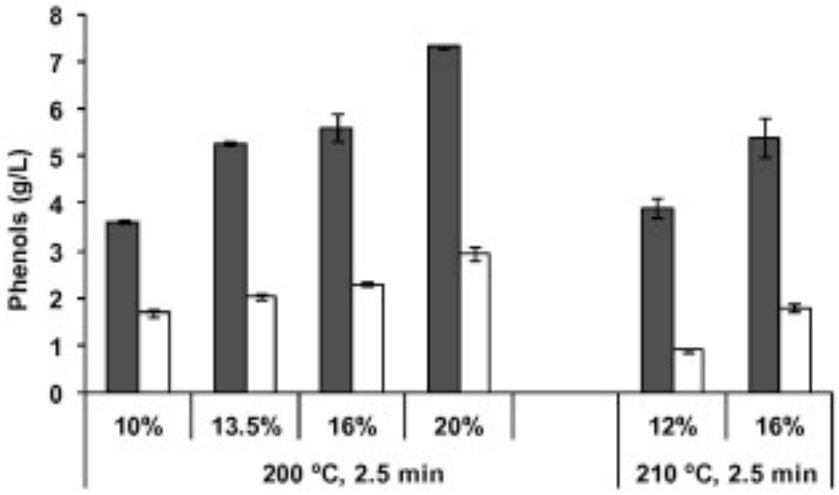
524 **Figure 1**



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528 **Figure 2**

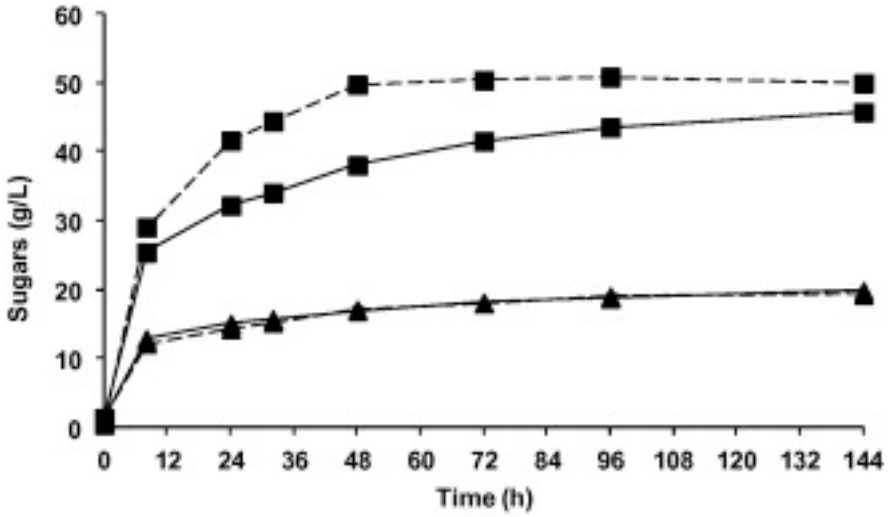

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Figure 3

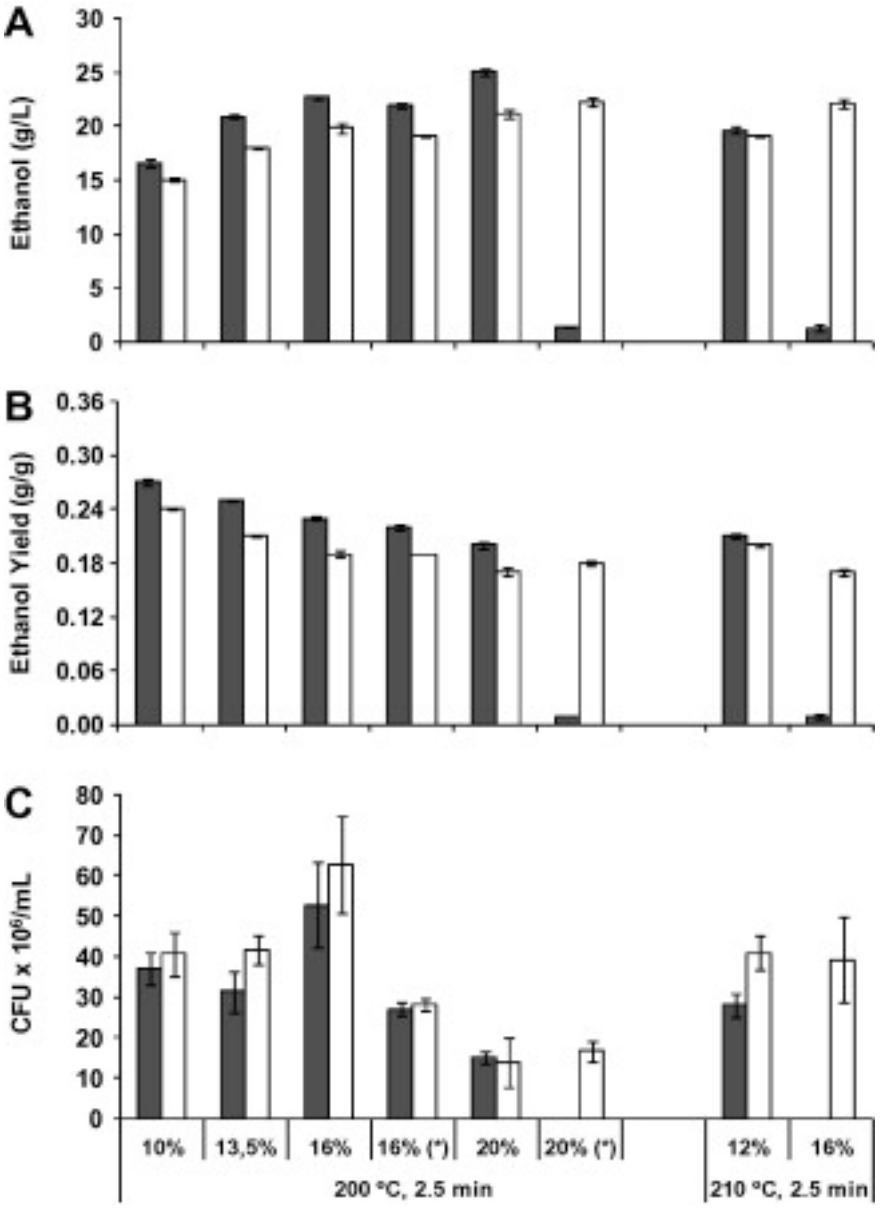

A
Ethanol (g/L)
30
25
20
15
10
5
0
B
Ethanol Yield (g/g)
0.36
0.30
0.24
0.18
0.12
0.06
0.00
C
CFU x 10⁶/mL
80
70
60
50
40
30
20
10
0
10%
13,5%
16%
16% (*)
20%
20% (*)
12%
16%
200 °C, 2.5 min
210 °C, 2.5 min

Figure 4

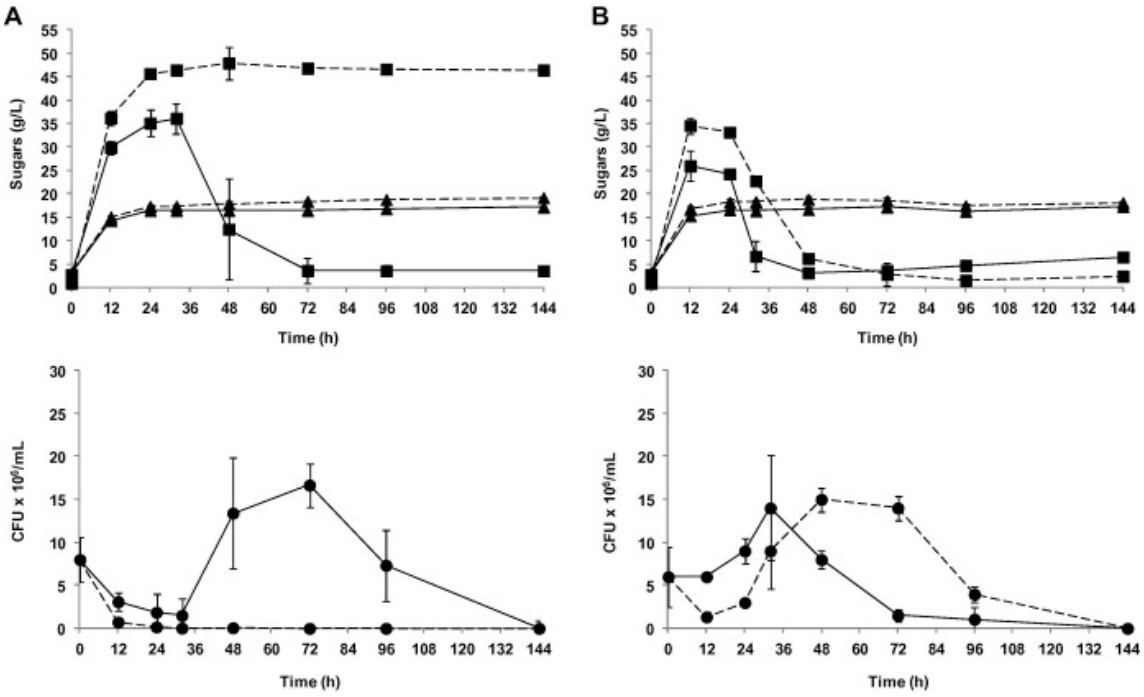
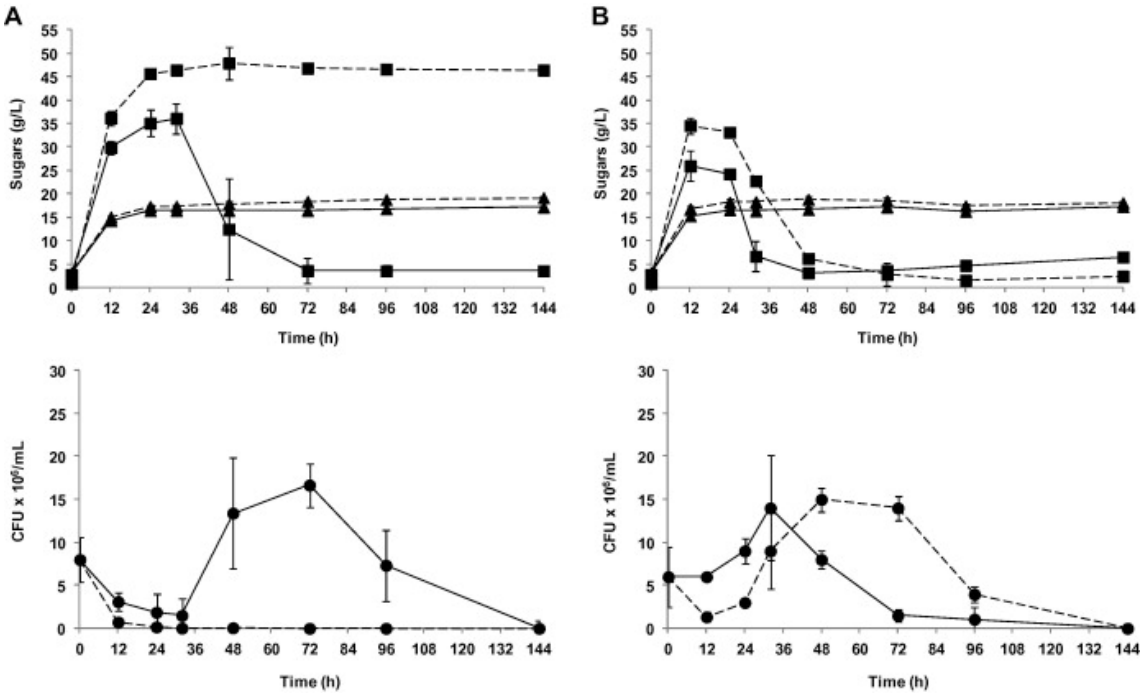

A
B
Sugars (g/L)
Time (h)
CFU x 10⁶/mL
0 12 24 36 48 60 72 84 96 108 120 132 144
0 5 10 15 20 25 30 35 40 45 50 55

Figure 5


A
B
Sugars (g/L)
Time (h)
CFU x 10⁶/mL
0 12 24 36 48 60 72 84 96 108 120 132 144