Ethanol from laccase-detoxified lignocellulose by the thermotolerant yeast *Kluyvermoyces marxianus* – Effects of steam pretreatment conditions, process configurations and substrate loadings

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This is the peer reviewed version of the following article:
Moreno, A.D.; Ibarra, D.; Ballesteros, I.; Fernández, J.L.; Ballesteros, M. Ethanol from laccase-detoxified lignocellulose by the thermotolerant yeast *Kluyveromyces marxianus* – Effects of steam pretreatment conditions, process configurations and substrate loadings. Biochemical Engineering Journal 2013, 79:94-103.
which has been published in final form at:
https://doi.org/10.1016/j.bej.2013.07.006

Abstract

In our previous work, the ability of laccase enzymes to improve the fermentation performance of the thermotolerant yeast *Kluyveromyces marxianus* CECT 10875 on steam-exploded wheat straw slurry was demonstrated. As a continuation of this study, the present research evaluates different aspects, including pretreatment conditions, process configurations and substrate loadings, with the aim to proceed towards the use of *K. marxianus* and laccases for second generation ethanol production. For it, two wheat straw slurries resulting from different steam explosion pretreatment conditions (200 ºC, 2.5 min and 220 ºC, 2.5 min) were employed at various substrate loadings [5-14% (w/v)] under two process configurations: SSF (simultaneous saccharification and fermentation) and PSSF (presaccharification and simultaneous saccharification and fermentation).The better performance of *K. marxianus* was observed on the slurry produced at softer conditions. Its lower inhibitors content allowed to increase the total solids loading up to 10% (w/v) in both process configurations, reaching higher ethanol concentrations (12 g/L). Moreover, laccase detoxification improved these results, particularly in SSF processes, increasing the substrate loading up to 12% (w/v) and, consequently, obtaining the highest ethanol concentration (16.7 g/L).

Keywords: Lignocellulosic ethanol; *Kluyveromyces marxianus*; Laccase detoxification; Steam explosion; Process configurations; Substrate loadings

1. Introduction

Biofuels made from biomass offer both a reduction of greenhouse gas (GHG) emissions as well as a partially replacement of liquid fossil fuels for transportation. In recent years, some policies have been adopted for the introduction of these alternatives into the current fuel distribution systems. For instance, the European Union directive 2009/28/EC establishes a share of 10% of biofuels in the transport sector by 2020 under several binding sustainability standards [1]. To reach this target, the development towards a cost-effective lignocellulosic ethanol industry is fundamental.

Lignocellulosic ethanol performs better than conventional sugar or starch based biofuels in terms of energy balance, GHG emissions and land-use requirements. Moreover, the lignocellulosic materials are abundant, cheap and do not compete with food [2]. In this context, wheat straw is a readily available candidate for ethanol production in Europe [3].

Ethanol can be made from lignocellulosic biomass through the enzymatic hydrolysis and the subsequent fermentation by microorganisms of the carbohydrates contained in the plant cell walls. Unfortunately, due to the recalcitrant nature of lignocellulose, a pretreatment step is required to improve the saccharification and to increase the fermentable sugars yields [4]. Steam explosion, a process that combines high pressures and temperatures, is a very suitable pretreatment technology that enhances the accessibility of enzymes to cellulose by an extensive alteration of the lignocellulosic structure [4]. However, this pretreatment leads to a partial sugars and lignin degradation, forming some soluble inhibitory compounds that can affect the downstream hydrolysis and fermentation steps [5–7].

According to their chemical structure, the formed inhibitors are classified into weak acids, furan derivatives and phenols and their concentration depend on the severity of the pretreatment and the raw material used [6]. Several procedures have been assayed for the removal of these compounds in order to prevent their inhibitory action. After steam explosion, the liquid fraction is usually separated from the solid fraction, which in turn is thoroughly washed to obtain the water insoluble solids (WIS) fraction, used as substrate. From an economical and environmental point of view, however, it would be desirable the use of the whole slurry obtained after steam explosion as there is no need for extra equipment (filtration and washing system), the amount of wastewater generated and

freshwater required is reduced and the concentration of fermentable sugars is increased [8]. For these reasons, other methods, including biological, physical and chemical treatments, have been tested for the detoxification of pretreated materials [5,9,10]. Among biological methods, a wide variety of laccases have been successfully applied on different steam-exploded materials, showing good detoxification abilities [11–16]. Laccases are multicopper-containing oxidases with phenoloxidase activity, which catalyze the oxidation of phenols generating unstable phenoxy radicals that lead to polymerization into less toxic aromatic compounds [12,13].

In terms of process configuration, simultaneous saccharification and fermentation (SSF) appears as an attractive option for lignocellulosic ethanol production [17]. During this process, the glucose released by the action of hydrolytic enzymes is converted directly to ethanol by the fermenting microorganism, minimizing the end-product inhibition of enzymes caused by cellobiose and glucose accumulation [17]. Nevertheless, the main drawback of SSF is that it is usually conducted at temperatures below the optimal for the hydrolytic enzymes. Whereas saccharification has an optimum temperature around 50 ºC, most fermenting yeasts have an optimum temperature ranging from 30 to 37 ºC [18]. In this case, an enzymatic presaccharification prior to simultaneous saccharification and fermentation (PSSF) has been proposed to enable hydrolytic enzymes to act at their optimal temperature, enhancing the saccharification and, consequently, the ethanol yields [19]. Furthermore, this stage promotes the liquefaction of the broth, making it more fluid and easier to handle and facilitating the mixing during the fermentation [20]. Another interesting approach to overcome this disadvantage of SSF processes is the use of thermotolerant strains that can ferment sugars at temperatures close to the optimum of the enzymatic hydrolysis [21]. In this sense, *Kluyveromyces marxianus* CECT 10875, a yeast adapted and selected by Ballesteros et al. [22], is gaining great significance due to its ability of growing and fermenting at 42 ºC. Furthermore, the use of thermotolerant strains during SSF can lead to other advantages such as the reduction of cooling costs and contamination risks, the increase of saccharification yields or the continuous ethanol removal [21].

The feasibility of *K. marxianus* for ethanol production has been successfully reported on various steam-exploded materials, using WIS fraction as substrate [23–25]. However, some of these studies have also showed the restriction of the yeast when using

the whole slurry due to the presence of inhibitory compounds [25]. Recently, this restriction has been overcome thanks to the use of laccases, observing growth and ethanol production on steam-exploded wheat straw slurry [16]. In order to proceed towards the use of *K. marxianus* and laccases for ethanol production, an optimization study about pretreatment conditions, process configurations and substrate loadings was carried out. Thus, the whole slurries obtained by steam explosion of wheat straw at two severity conditions were subjected for SSF and PSSF processes at increasing substrate loadings. To evaluate these aspects, inhibitors content, cell viability and both glucose consumption and ethanol production were investigated.

2. Materials and 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 pretreated in a 10 L reactor at two conditions: 220 ºC, 2.5 min and 200 ºC, 2.5 min. For analytical purpose, one portion of recovered slurry was vacuum filtered with the aim of obtaining a liquid fraction or prehydrolysate and a solid fraction. To obtain the WIS fraction, the solid fraction was thoroughly washed with distilled water until the filtrate was clean. The remaining slurry was used as substrate for the different assays.

Chemical composition of both raw and pretreated material (WIS) 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 [26]. Dry weight (DW) of slurry and WIS were determined by drying the samples at 105 ºC for 24 h (LAP-001).

Liquid fraction was also analyzed in terms of sugars and degradation compounds. In the case of sugars quantification, a mild acid hydrolysis [4% (v/v) H_2SO_4, 120 ºC for 30 min] was required in order to convert the oligomers into monomers.

147 *2.2. Enzymes*

148 *Pycnoporus cinnabarinus* laccase (60 IU/mL of laccase activity; 7-8 mg/mL of
149 protein content), from Beldem (Belgium), was used for detoxification. Activity was
150 measured by oxidation of 5 mM 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
151 (ABTS) to its cation radical (ε_{436} = 29 300 $M^{-1}cm^{-1}$) in 0.1 M sodium acetate buffer (pH 5)
152 at 24°C.

153 For saccharification, a mixture of NS50013 and NS50010, both produced by
154 Novozymes (Denmark), was employed. NS50013 (60 FPU/mL of cellulase activity; 140
155 mg/mL of protein content) is a cellulase preparation produced by *Trichoderma* spp. that
156 presents low β-glucosidase activity; therefore supplementation with NS50010 (810 IU/mL
157 of β-glucosidase activity; 188 mg/mL of protein content), produced by *Aspergillus niger*
158 and that mainly presents β-glucosidase activity, is typically applied in the biochemical
159 transformation processes of lignocellulosic biomass into ethanol. The overall cellulase
160 activity was determined using filter paper (Whatman No. 1 filter paper strips) and β-
161 glucosidase activity was measured using cellobiose as substrate. Both enzymatic activities
162 were followed by the release of reducing sugars [27], defining one unit of enzyme activity
163 as the amount of enzyme that transforms 1 μmol of substrate per minute.

164 In addition to the activity, total protein content from all enzymatic preparations was
165 analyzed by BCA protein assay kit (Pierce Ref. 23225), using bovine serum albumin as
166 standard.

167 *2.3. Microorganism and growth conditions*

168 The fermentative yeast used in this study was *K. marxianus* CECT 10875, a
169 thermotolerant strain selected by Ballesteros et al. [22]. Active cultures for inoculation were
170 obtained in 100-mL flasks with 50 mL of growth medium containing 30 g/L glucose, 5 g/L
171 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
172 rotary shaker at 150 rpm and 42 ºC, the preculture was centrifuged at 9000 rpm for 10 min.
173 Supernatant was discarded and cells were washed once with distilled water and then diluted
174 to obtain the desired inoculum size.

175 *2.4. Laccase detoxification*

176 The slurries obtained after pretreatment at 220 ºC, 2.5 min and 200 ºC, 2.5 min were
177 subjected to different laccase detoxification assays. Before adding laccase, 2.5 g DW of the
178 corresponding slurries were diluted with 50 mM sodium citrate buffer (pH 5) in 100-mL
179 flasks to reach a final concentration of total solids of 5, 6, 7, 10, 12 or 14% (w/v).
180 In a first set of experiments, the diluted slurries were treated with laccase only (L).
181 Hence, an enzyme loading of 10 IU/g DW substrate of laccase was added and samples were
182 incubated for 8 h at 50 ºC and 150 rpm in a rotary shaker, according to its optimal
183 parameters [28].
184 On the other hand, the diluted slurries were supplemented with laccase (10 IU/g
185 DW substrate) together with hydrolytic enzymes (15 FPU/g DW substrate of NS50013 and
186 15 IU/g DW substrate of NS50010) for a simultaneous detoxification and
187 presaccharification (LP) process. In the same way than the previous detoxification step,
188 samples were incubated for 8 h at 50 ºC and 150 rpm.
189 Both treatments (L and LP) were carried out under non-sterile conditions without O_2
190 bubbling and control assays (untreated samples) were performed with the same procedure
191 without the addition of laccase. Before start SSF processes, representative L and LP
192 samples were withdrawn and centrifuged and the collected supernatants were analyzed for
193 the identification and quantification of inhibitory compounds.

194 *2.5. Simultaneous saccharification and fermentation*

195 Untreated and laccase treated samples resulting from L and LP detoxification assays
196 were subjected to SSF processes. From the first set of detoxification experiments (L), the
197 diluted slurries were supplemented with 15 FPU/g DW substrate of NS50013, 15 IU/g DW
198 substrate of NS50010 and the nutrients from the described growth medium (without
199 glucose) and afterwards, they were inoculated with 1 g/L DW of *K. marxianus*. On the
200 contrary, samples obtained from LP assays were only supplemented with nutrients and
201 inoculated with 1 g/L DW of *K. marxianus*.
202 For SSF processes, the temperature was reduced until 42 ºC and the pH adjusted to
203 5.5. Under these conditions, flasks were incubated in a rotary shaker (150 rpm) for a
204 further 72 h and representative samples from untreated (SSF and PSSF) and lacase treated
205 samples (LSSF and LPSSF) were withdrawn and analyzed for cell viability and glucose and
206 ethanol concentrations.

2.6. Analytical methods

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

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

Furfural, 5-hydroxymethylfurfural (5-HMF), vanillin, syringaldehyde, *p*-coumaric acid and ferulic acid were analyzed and quantified 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 also 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 mM H_2SO_4) at a flow rate of 0.6 mL/min.

Cell viability was determined as colony forming units (CFU/mL) by cell counting using agar plates containing the following media: 30 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 $MgSO_4 \cdot 7H_2O$. Agar plates were incubated at 42 ºC for 24 h.

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

3. Results and discussion

3.1. Pretreated wheat straw composition

Steam explosion pretreatments were performed at two severity conditions obtaining slurries with different compositions (Table 1). Compared to cellulose content of the

237 untreated wheat straw (40.5%), both pretreatments increased the cellulose proportion of
238 WIS fractions (53.5% and 63.0% at 200 ºC and 220 ºC, respectively), due to an extensive
239 hemicellulose solubilization and degradation. This hemicellulose removal was more
240 pronounced at 220 ºC, as reflected the lower proportion of remaining hemicellulose (2.7%)
241 in the WIS fraction. Moreover, a significant sugar degradation was also seen at 220 ºC,
242 obtaining a lower sugar concentration in the liquid fraction. In addition, slightly higher
243 phenolic content from lignin degradation was also observed.

244 The effect of temperature and time on the fractionation of raw material and the
245 formation of degradation products during pretreatment has been described as severity factor
246 Log(R_o). This parameter increases when increasing the temperature [29]. In this context, a
247 temperature increment from 200 ºC to 220 ºC resulted in an increase of Log(R_o) from 3.34
248 to 3.93, that consequently produced a higher sugar and lignin degradation (Table 1).

249 Among degradation products, acetic acid, formic acid, furfural and 5-HMF were the
250 most predominant in both liquid fractions (Table 1). Acetic acid is formed by the hydrolysis
251 of acetyl groups contained in the hemicellulose structure. Formic acid derives from furfural
252 and 5-HMF degradation, which in turn results from pentoses (mainly xylose) and hexoses
253 degradation, respectively [6,30]. Both liquid fractions also showed low amounts of some
254 phenols such as ferulic and *p*-coumaric acids, both derived from *p*-hydroxycinnamic acids.
255 These compounds are characteristic of herbaceous plants, acting as linkages between lignin
256 and hemicellulose by ether and esters bonds, respectively [31]. In addition, vanillin, derived
257 from guaiacyl propane lignin units, and syringaldehyde, released from syringyl propane
258 lignin units, were also found [8].

259 *3.2. Effect of laccase on degradation products*

260 Slurries obtained after pretreatment at 220 ºC and 200 ºC were diluted [from 5 to
261 14% (w/v)] and subjected to different laccase detoxification experiments (L or LP).
262 Compared to other detoxification methods, the use of laccases involves fewer inhibitory
263 sub-products, little waste generation and mild reaction conditions. Moreover, laccases offer
264 the possibility to be used directly, without the need to perform any additional separate step
265 that increases the process costs [9,10].

266 *3.2.1. Laccase treatment (L)*

Table 2 shows the degradation products content measured in untreated and laccase treated samples from the slurry obtained at 220 ºC at lower substrate loadings [5%, 6%, and 7% (w/v)]. Untreated samples showed higher inhibitors concentrations as the substrate loadings increased. However, laccase treated samples exhibited a remarkable reduction in the measured phenols (between 86-92% in all treated diluted samples), in the same range that the removal reported by Moreno et al. [16]. In contrast to the phenols reduction, weak acids and furan derivatives were not altered by laccase, being higher as the substrate loadings increased. Similar effects were observed using slurry produced at 200 ºC and lower substrate loadings (data not shown).

The absence of laccase action observed on weak acids and furan derivatives have been already reported in previous studies [11–16]. Regarding phenols removal, it followed a similar pattern to that observed by Moreno et al. [16]. Syringaldehyde, *p*-coumaric acid and ferulic acid were completely removed by laccase. In contrast, vanillin was less susceptible to laccase action.

Compared to untreated samples from the slurry pretreated at 220 ºC and lower substrate loadings, the untreated samples from slurry at 200 ºC and higher solids content [10%, 12%, and 14% (w/v)] showed higher inhibitors concentrations (Table 3). Furthermore, the efficiency of laccase treatment for reducing the phenolic content diminished, observing a phenols removal of about 44%. As recently explained by Alvira et al. [32], this effect could be attributed to the high viscosity of the medium when a higher solids content is used, which difficults the blending of laccase with the material.

3.2.2. Enzymatic presaccharification with laccase treatment (LP)

In the second set of experiments, the diluted slurries were subjected to an enzymatic presaccharification with laccase supplementation. Comparing the untreated samples of both set of experiments (L or LP), an increment of the inhibitors content was observed in the samples under enzymatic presaccharification (Tables 2 and 3). According to Thomsen et al. [33], presaccharification increased the degradation products content, especially acetic acid, *p*-coumaric acid and ferulic acid (Tables 2 and 3). Acetic acid is released by the hydrolysis of acetyl groups in hemicellulose, which involves a synergistic action of both hemicellulase and acetyl esterase activities [33]. In this sense, NS50013 preparation, produced by

297 *Trichoderma* spp. strains, presents some additional xylanase and acetyl esterase activitities
298 together with its main cellulase activity [34]. In addition, the complementary action of
299 xylanase and phenolic acid esterase activities could explain the release of *p*-coumaric acid
300 and ferulic acid. The latter activity, mainly feruloyl esterase, is naturally produced by
301 *Aspergillus niger*, the source strain for glucosidase NS50010 preparation [35].
302 In spite of the phenols increment produced by the enzymatic prehydrolysis, the
303 phenols removal efficiency of laccase was similar or even better than laccase treatment
304 without enzymatic presaccharification. With slurry obtained at 220 ºC and lower substrate
305 loadings [5%, 6%, and 7% (w/v)], the phenols reduction by laccase resulted in the same
306 degree with or without presaccharification (between 86-92%) (Table 2). Similar results
307 were obtained with the pretreated slurry at 200 ºC and lower substrate concentrations (data
308 not shown). In contrast, at higher solids content [10%, 12%, and 14% (w/v)]
309 presaccharification improved the accessibilty of laccase to phenolic compounds, increasing
310 the phenols reduction from 44% to 95% compared to laccase treatment alone (L) (Table 3).

311 *3.3. Effect of laccase on yeast growth and ethanol production*

312 Untreated and laccase treated samples resulting from both set of laccase
313 detoxification experiments (L or LP) were subsequently subjected to SSF process using the
314 thermotolerant yeast *K. marxianus* with a total solids content ranging from 5 to 14% (w/v).
315 The substrate concentration is an important aspect for ethanol production. By increasing
316 substrate loading during saccharification, a higher sugar content can be obtained, which in
317 turn may yield higher ethanol concentration after fermentation. This approach could reduce
318 operational cost for hydrolysis and fermentation processes and minimize the energy
319 consumption during the subsequent distillation and evaporation stages [36].

320 *3.3.1. Untreated SSF configuration*

321 With the untreated sample at 5% (w/v) from the slurry obtained at 220 ºC, cell
322 viability in the form of CFU/mL decreased within the first 32 h of SSF (Fig. 1a), which
323 delayed the glucose consumption and the ethanol production. As a consenquence, glucose
324 and other sugars (not shown) released by the action of hydrolytic enzymes were
325 accumulated in the broth (Fig. 2a). This delay correspond to the adaptation of the yeast to
326 the fermentation broths, which depends on different factors such as the inhibitory

327 compounds type, their concentrations, the synergistic effects between them and the
328 fermenting microorganism used [5,6]. Regarding *K. marxianus* CECT 10875, Oliva et al.
329 [30,37] have attributed several negative effects to different inhibitors. Furfural has shown a
330 strong inhibition of growth and ethanol production in *K. marxianus*. Moreover, furfural in
331 the presence of vanillin or acetic acid has a stronger negative effect on its yeast
332 assimilation, resulting in a longer exposure of the yeast to furfural. By contrast, *K.*
333 *marxianus* has shown a major resistance towards 5-HMF. Weak acids, such as formic acid
334 or acetic acid, have also produced inhibitory effects, mainly on *K. marxianus* growth.
335 However, the pH conditions (pH 5.5) used in this study during fermentation reduces
336 considerably the toxicity of these acids. Vanillin and syringaldehyde have also produced
337 similar toxic effects than furfural on *K. marxianus*. In addition, they could not be
338 metabolised by *K. marxianus* in the presence of furfural. Finally, inhibitory effects of *p*-
339 coumaric acid and ferulic acid have been described on *Saccharomyces cerevisiae* [38], but
340 not on *K. marxianus*.
341 The assimilation of these toxics by *K. marxianus*, chiefly the conversion of furfural,
342 5-HMF and aromatic aldehydes (vanillin and syringaldehyde) to their less inhibitory
343 alcohol forms [30], determines to a great extent the adaptation time of the yeast [5,6]. After
344 overcoming this period, *K. marxianus* showed a gradual increment in viability, reaching the
345 highest number of colony forming units between 56 and 72 h of SSF (Fig. 1a). However,
346 the glucose concentration was completely reduced between 32 and 48 h (Fig. 2a), obtaining
347 a maximum ethanol concentration of 9.6 g/L (Table 4). This ethanol concentration
348 corresponds to an ethanol yield of 83% of the theoretical based on the glucose content
349 present in the pretreated wheat straw. Some wild-type and mutant yeast strains have also
350 been reported to produce ethanol at temperatures above 40 ºC with efficiencies between
351 75% and 90% [39]. Nevertheless, the majority of these yeasts were assayed on synthetic
352 media without the presence of inhibitors, in contrast to the present work in which a real
353 medium such as the whole slurry is used.
354 A complete growth inhibition was observed with the untreated sample at 6% (w/v)
355 from the slurry obtained at 220 ºC, phenomenon that can be attributed to the increment of
356 inhibitors content. As shown in Fig. 1b, a remarkable reduction of the number of CFU/mL
357 was observed within 12 h of fermentation, followed by a total cell viability yeast loss.

Consequently, neither glucose consumption nor ethanol production took place and a glucose accumulation was seen along 72 h of SSF (Fig. 2b).

These negative effects have also been described with other pretreated materials and fermenting microorganisms with a major tolerance to toxics. Stenberg et al. [40] showed the total inhibition of *S. cerevisiae* with steam-pretreated softwood slurry at 10% (w/v) of substrate loading. However, the yeast grew using WIS fraction at the same concentration. Studies from barley straw also showed *S. cerevisiae* growth with WIS fraction at 7.5% (w/v), but a high yeast inhibition during SSF with the slurry at the same solids content was observed [41]. Regarding wheat straw, good results have been published using the slurry (10% (w/v) of consistency) produced at 210 ºC for 2.5 min and the industrial strain *S. cerevisiae* Ethanol Red as fermenting microorganism [42]. However, in the mentioned study the inhibitors concentration was lower compared to the content observed herein for the pretreated slurry at 220 ºC, owing to the milder pretreatment conditions. Taking into account these results, the less toxic slurry obtained at 200 ºC was used. In this case, *K. marxianus* grew, consumed all the glucose and produced ethanol using the untreated samples from 5 to 10% (w/v) of substrate loading (data not shown). At 10% (w/v), a marked reduction of cell viability together with a sugar accumulation was observed during the first 24 h of SSF (Figs. 1c and 2c). After that, a pronounced increment of the number of CFU/mL occurred, reaching the highest value at 48 h (Fig. 1c); however, the maximum ethanol concentration (12.3 g/L) was attained at the end of the process (Fig. 2c and Table 4). When the substrate loading was fixed over 10% (w/v), *K. marxianus* was completely inhibited due to the higher inhibitors content. As shown in Figs. 1d and 2d, no cell growth occurred during the process and consequently, ethanol was not produced and glucose was accumulated.

3.3.2. LSSF configuration

Compared to untreated samples, the specifically phenols removal by laccase enhanced the fermentation performance of *K. marxianus*, in accordance with Moreno et al. [16]. At 5% (w/v) concentration of the slurry produced at 220 ºC, laccase treatment shortened the adaptation time from 32 h for untreated samples, to 6 h. Furthermore, cell viability was significantly improved, reaching the maximum CFU/mL at 32 h of SSF (Fig. 1a), after which, a remarkable decrease took place. In addition to the viability

improvement, faster glucose consumption and ethanol production rates were also observed (Fig. 2a), as shown the barely glucose accumulation in the early stages of SSF and the maximun ethanol concentration (10.2 g/L) reached at 24 h. It resulted in an ethanol productivity increment from 0.09 g/L h for untreated samples to 0.31 g/L h for laccase treated samples (Table 4). Moreover, laccase also enhanced slightly the ethanol yield from 0.37 g/g to 0.40 g/g (Table 4). At 6% (w/v) of substrate loading, where a total growth inhibition was observed with untreated samples, laccase treatment triggered the yeast growth and the ethanol production. After a drop in the cell viability within the first 12 h, *K. marxianus* showed a pronounced increment, with a maximum CFU/mL between 24 and 32 h (Fig. 1b). Then, cells ceased to grow. In this case, the maximum ethanol concentration was 10.7 g/L, reached at 32 h (Fig. 2b and Table 4). Over 6% (w/v) the yeast was totally inhibited (data not shown), in spite of the phenols reduction produced by laccase (Table 2).

The laccase treatment also showed similar improvements on diluted samples from the slurry obtained at 200 ºC. At 10% (w/v) laccase reduced the adaptation time from 24 h for untreated samples, to 12 h. The highest number of colony forming units was obtained at 24 h of SSF (Fig. 1c), and the glucose accumulation was only observed during the first 12 h (Fig. 2c). Then, the yeast consumed almost all the accumulated glucose between 12 and 24 h, producing a maximum ethanol concentration of 13.8 g/L. This adaptation time reduction by laccase was reflected in a slight increment of the ethanol productivity value from 0.17 g/L h for untreated samples, to 0.19 g/L h (Table 4). Moreover, laccase treatment also enhanced the ethanol yield from 0.29 g/g to 0.33 g/g (Table 4). In the case of diluted slurries at 12% (w/v), where no cell growth was observed for untreated samples, laccase enabled the growth of *K. marxianus*. As seen in Fig. 1d, a cell viability drop was observed during the first 24 h of SSF, followed by a notable increment that reached the highest number of CFU/mL at 48 h. Glucose was accumulated within 24 h, afterwards it started to be consumed (Fig. 2d), obtaining a maximum ethanol concentration of 16.7 g/L (Table 4), a production much higher than those values reported in the other samples (Table 4) or in our previous work (11 g/L) [16]. Finally, over 12% (w/v) of substrate loadings, laccase treatment did not lead to yeast growth nor ethanol production (data not shown), in spite of the phenols removal observed (Table 3).

Similar positive effects of laccases have been also described on other microorganisms and material types. Jurado et al. [13] observed higher yeast concentration, sugar consumption and ethanol yield after laccase detoxification of steam-exploded wheat straw and a subsequent separate hydrolysis and fermentation (SHF) with *S. cerevisiae*. Jönsson et al. [12] and Larsson et al. [14] reported the same performance for *S. cerevisiae* on laccase detoxified liquid fraction from steam-acid exploded wood. In the same way, Martín et al. [15] described similar enhancements for a xylose-fermenting *S. cerevisiae* on laccase detoxified enzymatic hydrolysates from sugarcane bagasse; and Chandel et al. [11] for *Candida shehatae* on laccase detoxified acid hydrolysates from sugarcane bagasse.

3.3.3. Untreated PSSF configuration

Compared to untreated samples without enzymatic presaccharification, the increment of the degradation products content by enzymatic prehydrolysis (P) affected negatively the yeast fermentation performance during the subsequent SSF process. In this context, the diluted samples at consistencies of 5% and 6% (w/v) from the slurry obtained at 220 ºC showed a total yeast inhibition under PSSF configuration. Cell growth was not observed after 72 h of SSF (Figs. 3a and b), and neither glucose consumption nor ethanol production took place (Figs. 4a and b). This is in accordance with Tomás-Pejó et al. [25], who described a total inhibition of *K. marxianus* on wheat straw slurry (220 ºC, 2.5 min) at 5% (w/v) under a PSSF configuration. In contrast, in the same study a high ethanol concentration (30.2 g/L) was achieved when the WIS fraction was used with a substrate consistency of 14% (w/v).

Negative effects were also observed with untreated samples from slurry produced at 200 ºC. In this case, the presaccharification of the diluted sample at 10% (w/v) of substrate loading prolonged the adaptation time from 24 h during SSF with no prehydrolysis, to 48 h with presaccharification. A remarkable cell viability drop was showed within 12 h, followed by a long period in which cell growth was not observed. After that, a sudden increase in cell viability could be seen between 48 and 72 h (Fig. 3c). With regard to glucose, it was accumulated during the first 48 h, afterward a total consumption occurred (Fig. 4c). Nevertheless, no differences were found in the ethanol concentrations (12 g/L) and yields (0.29 g/g) during SSF processes with and without presaccharification (Table 4). In the same way that it had previously observed during SSF with no prehydrolysis, PSSF

samples at 12 % (w/v) of substrate loading showed a complete inhibition of the yeast, and neither growth nor ethanol production took place (Figs. 3d and 4d).

The better performance of SSF versus PSSF observed in this work has also been described for *K. marxianus* using the WIS fraction from steam-exploded barley straw [24]. In addition to the effects observed by the higher inhibitors content, other factors described during the presaccharification such as end-product inhibition and thermal deactivation of enzymes could explain the better eficiency of SSF [41].

3.3.4. LPSSF configuration

In addition to the negative effects of the phenols on the yeast fermentation performance, it is known that phenolic compounds also reduce both rate and yields of cellulose hydrolysis [43,44]. Vanillin and syringaldehyde inhibite cellulase enzymes, especially β-glucosidase, whereas ferulic acid and p-coumaric acid deactivate them [43,44]. However, the phenols removal by laccase described herein did not improve the enzymatic hydrolysis. In contrast, the laccase supplementation of enzymatic presaccharification affected negatively the sugar recovery in all diluted samples (Fig. 4). The same phenomenon was described by Moreno et al. [16] who reported fewer sugars released after enzymatic hydrolysis of slurry samples treated with *P. cinnabarinus* or *T. villosa* laccases. This negative performance of enzymatic presaccharification could be attributed to several causes derived from laccase action, including an inhibition or deactivation of cellulases by the products formed, an increase in the non-productive binding of hydrolytic enzymes to lignin and a strengthening of the lignin-carbohydrate complexes [45,46].

Despite the lower sugar recovery observed, enzymatic presaccharification with laccase supplementation (LP) enhanced the fermentation performance of *K. marxianus* in the subsequent SSF processes. In contrast to those samples with presaccharification but without laccase, where the yeast was completely inhibited when using the slurry pretreated at 220 ºC, laccase supplementation triggered the yeast growth and the ethanol production. With the diluted samples at 5% (w/v) the highest number of CFU/mL was reached at 32 h (Fig. 3a), with a depletion of the glucose released and an ethanol production of 9.3 g/L (Fig. 4a and Table 4). The same effects were observed at 6% (w/v) (Figs. 3b and 4b), with a maximum ethanol concentration of 11.1 g/L (Table 4). However, the yeast was inhibited over 6% (w/v) of substrate loadings (data not shown).

Compared to laccase treatment (L), presaccharification with laccase supplementation (LP) of the slurry produced at 220 ºC did not result in better ethanol concentrations and yields. By contrast, at 5% (w/v) of substrate concentration the ethanol yield decreased from 0.40 g/L with laccase treatment, to 0.36 g/L with presaccharification and laccase supplementation (Table 4).

With regard to the slurry produced at 200 ºC, laccase supplementation of presaccharification at 10% (w/v) shortened the adaptation time from 48 h with no laccase supplementation, to 12 h. A faster yeast growth was observed, obtaining the maximum CFU/mL at 24 h of SSF (Fig. 3c); and faster glucose consumption and ethanol production rates were also seen (Fig. 4c). Moreover, the ethanol yield was improved from 0.29 g/g with no laccase supplementation, to 0.33 g/g (Table 4). However, over 10% (w/v) of substrate consistency the laccase supplementation did not lead to yeast growth nor ethanol production (Figs. 3d and 4d), in spite of the great phenols reduction achieved (around 95%) (Table 3). This is contrary to the previous results described with laccase treatment (L), where at 12% (w/v) of substrate loading the yeast grew and produced ethanol, attaining the highest concentration (16.7 g/L) with a lower phenols reduction (around 44%).

4. Conclusions

The thermotolerant yeast *K. marxianus* CECT 10875 was able to ferment the whole slurry obtained by steam explosion pretreatment of wheat straw at 220 ºC for 2.5 min in a SSF process at low substrate loading (5% w/v), reaching an ethanol concentration of 9.6 g/L. However, presaccharification prior to SSF inhibited totally the yeast. A better yeast performance was observed on the slurry obtained at softer conditions (200 ºC for 2.5 min). Its lower inhibitors content allowed the use of the yeast in both SSF and PSSF processes at higher consistency (10% w/v), obtaining an ethanol concentration of 12.3 g/L.

The specific removal of phenols by laccase reduced the inhibitory effects of slurry samples. It led to enhance the yeast fermentation performance and to increase the substrate loadings of broths during SSF and PSSF processes, increasing consequently the ethanol production. In spite of the boosted laccase action by presaccharification, even at higher substrate loadings, the highest ethanol concentration (16.7 g/L) was achieved when the laccase treatment was carried out without presaccharification on the slurry produced at

lower severity conditions (200 ºC for 2.5 min) and higher consistency (12% w/v). Nevertheless, this ethanol concentration is not enough for a cost-effective bioethanol production (ethanol concentrations over 4% v/v). Therefore, further researchs with new operation modes such as fed batch processes, are necessaries in order to obtain higher ethanol concentrations. In addition, taking into account the great xylose content presents in the liquid fraction, specially in the slurry pretreated at 200 ºC, the use of strains for fermenting pentoses could also increase the ethanol production.

Acknowledgements

This work has been supported by the RESTOENE project (Consejería de Educación – Comunidad de Madrid, Spain). A.D.M. acknowledges a CPI contract (Consejería de Educación-Comunidad de Madrid, Spain).

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Table captions

Table 1. Composition of steam-exploded wheat straw at 220 ºC, 2.5 min and 200 ºC, 2.5 min

Table 2. Inhibitory compounds concentration (mg/L) of slurry samples (220 ºC, 2.5 min) treated with laccase (L) or enzymatic presaccharification with laccase supplementation (LP). The concentrations are measured before simultaneous saccharification and fermentation (SSF) assays

Table 3. Inhibitory compounds concentration (mg/L) of slurry samples (200 ºC, 2.5 min) treated with laccase (L) or enzymatic presaccharification with laccase supplementation (LP). The concentrations are measured before simultaneous saccharification and fermentation (SSF) assays

Table 4. Summary of simultaneous saccharification and fermentation assays with (PSSF) or without (SSF) prehydrolysis of slurry samples (220 ºC, 2.5 min and 200 ºC, 2.5 min) and with laccase treatment (LSSF) or enzymatic presaccharification with laccase supplementation (LPSSF)

Figure captions

Fig. 1. Viable cells during simultaneous saccharification and fermentation (SSF) assays of dilute slurry samples (220 ºC, 2.5 min, left; 200 ºC, 2.5 min, right) treated with laccase (L). Symbols used: untreated (○) and laccase treated (●) samples. Dilute slurries at 5% (a), 6% (b), 10% (c), and 12% (d) (w/v) of substrate loading. Mean values and standard deviations were calculated from the triplicates to present the results.

Fig. 2. Time course for ethanol production and glucose consumption during simultaneous saccharification and fermentation (SSF) assays of dilute slurry samples (220 ºC, 2.5 min, left; 200 ºC, 2.5 min, right) treated with laccase (L). Symbols used: glucose, untreated (□) and laccase treated (■) samples; ethanol, untreated (△) and laccase treated (▲) samples. Dilute slurries at 5% (a), 6% (b), 10% (c), and 12% (d) (w/v) of substrate loading. Mean values and standard deviations were calculated from the triplicates to present the results.

Fig. 3. Viable cells during simultaneous saccharification and fermentation (SSF) assays of dilute slurry samples (220 ºC, 2.5 min, left; 200 ºC, 2.5 min, right) subjected to an enzymatic presaccharification with laccase supplementation (LP). Symbols used: untreated (○) and laccase treated (●) samples. Dilute slurries at 5% (a), 6% (b), 10% (c), and 12% (d) (w/v) of substrate loading. Mean values and standard deviations were calculated from the triplicates to present the results.

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701 **Table 1**

702 Table 1. Composition of steam-exploded wheat straw at 220 °C, 2.5 min and 200 °C, 2.5 min.

Pretreatment conditions	**220 ºC 2.5 min**		**200 ºC 2.5 min**	
Total solids (% dry weight)	22.61		27.61	
WIS content (% dry weight)	16.62		18.07	
WIS composition (% dry weight)				
Cellulose	63.0		53.5	
Hemicellulose	2.7		11.7	
Lignin	35.3		30.4	
Prehydrolysate composition (g/L)				
Monosaccharides	**220 ºC 2.5 min**		**200 ºC 2.5 min**	
	Monomeric form	**Oligomeric form**	**Monomeric form**	**Oligomeric form**
Glucose	0.73	0.35	2.29	12.37
Xylose	4.89	3.01	2.76	29.21
Arabinose	0.18	0.09	1.30	1.08
Galactose	0.32	0.00	0.42	1.39
Mannose	0.10	0.28	nq	nq
Degradation Products				
Formic acid	6.1		6.3	
Acetic acid	9.7		6.9	
5-HMF	0.82		0.32	
Furfural	3.0		0.80	
Vanillin	0.05		0.04	
Syringaldehyde	0.03		0.01	
p-Coumaric acid	0.02		0.02	
Ferulic acid	0.03		0.03	

nq, not quantified

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Table 2

Table 2. Inhibitory compounds concentration (mg/L) of slurry samples (220 °C, 2.5 min) treated with laccase (L) or enzymatic presaccharification with laccase supplementation (LP). The concentrations are measured before simultaneous saccharification and fermentation (SSF) assays.

220ºC	**Inhibitor**	**Slurry 5% (w/v)**		**Slurry 6% (w/v)**		**Slurry 7% (w/v)**	
		C	L	C	L	C	L
NP	Formic acid	1536	nq	1684	nq	2102	nq
	Acetic acid	2360	2357	2256	2233	2699	2608
	5-HMF	120	120	140	150	165	165
	Furfural	500	430	650	635	715	675
	Vanillin	20	8	28	14	28	14
	Syringaldehyde	8	0	11	0	12	0
	p-Coumaric acid	27	0	23	0	28	0
	Ferulic acid	30	0	36	0	40	0
P	Formic acid	1228	nq	1472	nq	1872	nq
	Acetic acid	2433	2344	2913	2808	3508	3300
	5-HMF	127	120	143	144	173	177
	Furfural	443	413	522	410	737	697
	Vanillin	19	12	25	12	26	16
	Syringaldehyde	5	0	12	0	10	0
	p-Coumaric acid	29	0	39	0	42	0
	Ferulic acid	62	0	66	0	85	0

[NP] no enzymatic presaccharification; [P] enzymatic presaccharification; [C] untreated samples; [L] laccase samples; [nq] not quantified.

Table 3

Table 3. Inhibitory compounds concentration (mg/L) of slurry samples (200 °C, 2.5 min) treated with laccase (L) or enzymatic presaccharification with laccase supplementation (LP). The concentrations are measured before simultaneous saccharification and fermentation (SSF) assays.

200ºC	Inhibitor	Slurry 10% (w/v)		Slurry 12% (w/v)		Slurry 14% (w/v)	
		C	L	C	L	C	L
NP	Formic acid	1852	nq	2310	nq	3496	nq
	Acetic acid	2295	2248	2925	2880	4018	3823
	5-HMF	98	97	111	110	155	140
	Furfural	288	271	311	290	348	347
	Vanillin	20	20	22	22	25	19
	Syringaldehyde	8	5	10	7	9	2
	p-Coumaric acid	30	22	37	28	39	30
	Ferulic acid	26	0	33	0	30	0
P	Formic acid	1863	nq	2144	nq	3337	nq
	Acetic acid	3272	3172	3875	3559	5139	4726
	5-HMF	95	98	104	104	136	120
	Furfural	253	229	318	286	346	318
	Vanillin	22	10	23	17	24	9
	Syringaldehyde	8	0	9	0	9	0
	p-Coumaric acid	77	0	94	0	93	0
	Ferulic acid	110	0	122	0	123	0

[NP] no presaccharification; [P] enzymatic presaccharification; [C] untreated samples; [L] laccase samples; [nq] not quantified.

Table 4

Table 4. Summary of simultaneous saccharification and fermentation assays with (PSSF) or without (SSF) prehydrolysis of slurry samples (220 °C, 2.5 min and 200 °C, 2.5 min) and with laccase treatment (LSSF) or enzymatic presaccharification with laccase supplementation (LPSSF).

	Substrate loading (w/v)	Sample	EtOH (g/L)	$Y_{E/G}$ (g/g)	$Y_{E/ET}$ (%)	Q_E (g/L h)
220ºC	5% SSF	C	9.6	0.37	83.2	0.09[a]
		L	10.2	0.40	88.6	0.31[a]
	5% PSSF	C	1.7	0.07	14.5	0.05[a]
		L	9.3	0.36	81.0	0.28[a]
	6% SSF	C	1.7	0.06	12.3	0.05[a]
		L	10.7	0.35	78.4	0.32[a]
	6% PSSF	C	1.7	0.05	12.2	0.05[a]
		L	11.1	0.36	80.3	0.31[a]
200ºC	10% SSF	C	12.3	0.29	65.5	0.17[b]
		L	13.8	0.33	73.1	0.19[b]
	10% PSSF	C	12.0	0.29	63.6	0.17[b]
		L	13.8	0.33	73.5	0.18[b]
	12% SSF	C	1.36	0.03	6.0	0.02[b]
		L	16.7	0.33	74.2	0.23[b]
	12% PSSF	C	0.3	0.01	2.2	0[b]
		L	0.3	0.01	2.4	0[b]

[C] untreated samples; [L] laccase samples; [EtOHM] maximum ethanol concentration for 72 h of SSF; [YE/G] ethanol yield based on total glucose content present in the whole slurry (WIS fraction and liquid fraction); [YE/ET] theorical ethanol yield assuming ethanol yields on glucose by *K. marxianus* of 0.45 g/g; [QE] volumetric ethanol productivity based on time when maximum ethanol concentration is achieved: 32 h[a] and 72 h[b].

728 **Figure 1**

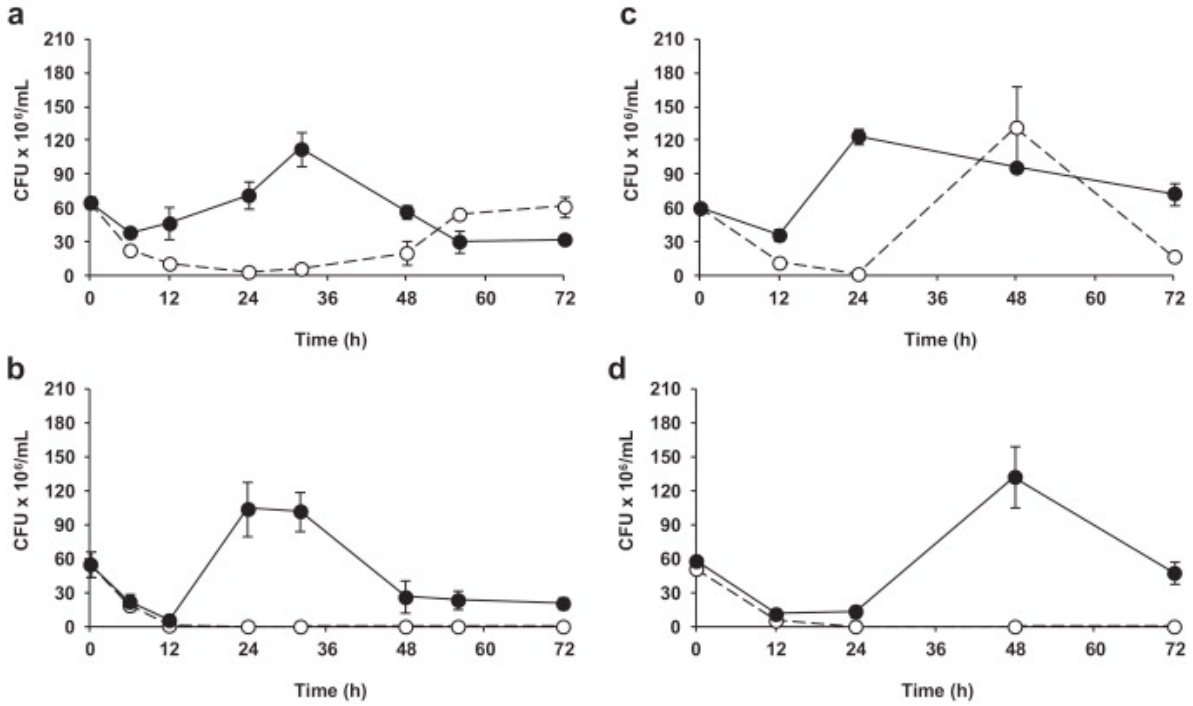

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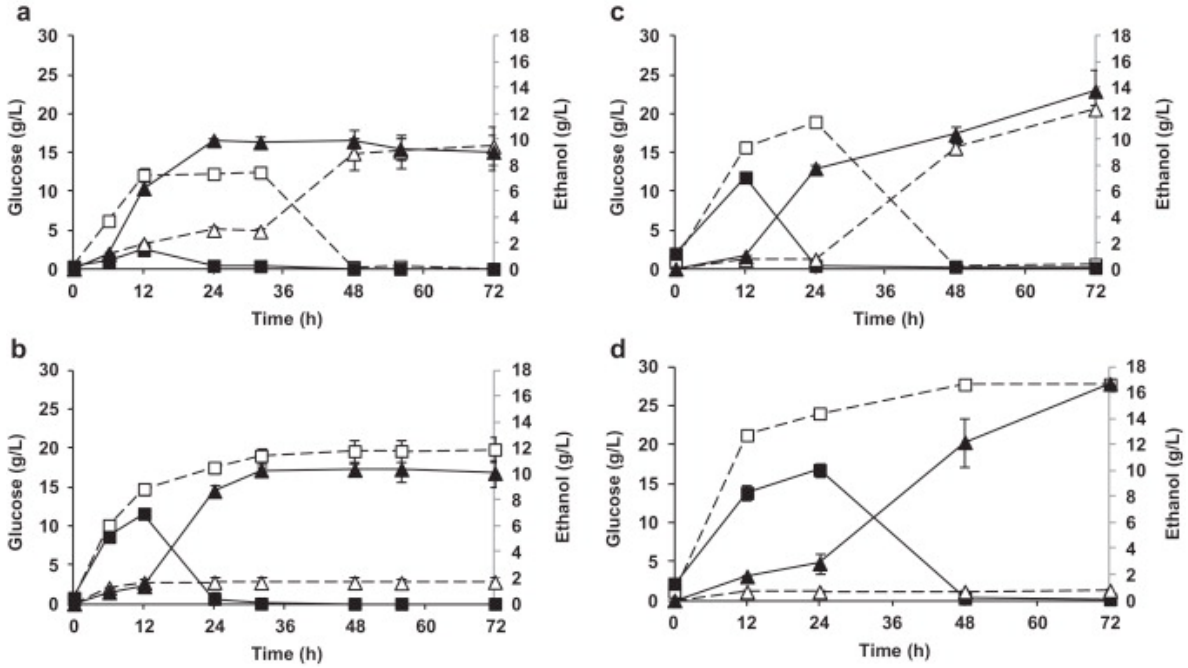
Figure 2


a
b
c
d
Glucose (g/L)
Ethanol (g/L)
Time (h)
0
12
24
36
48
60
72
0
5
10
15
20
25
30
0
2
4
6
8
10
12
14
16
18

736 **Figure 3**

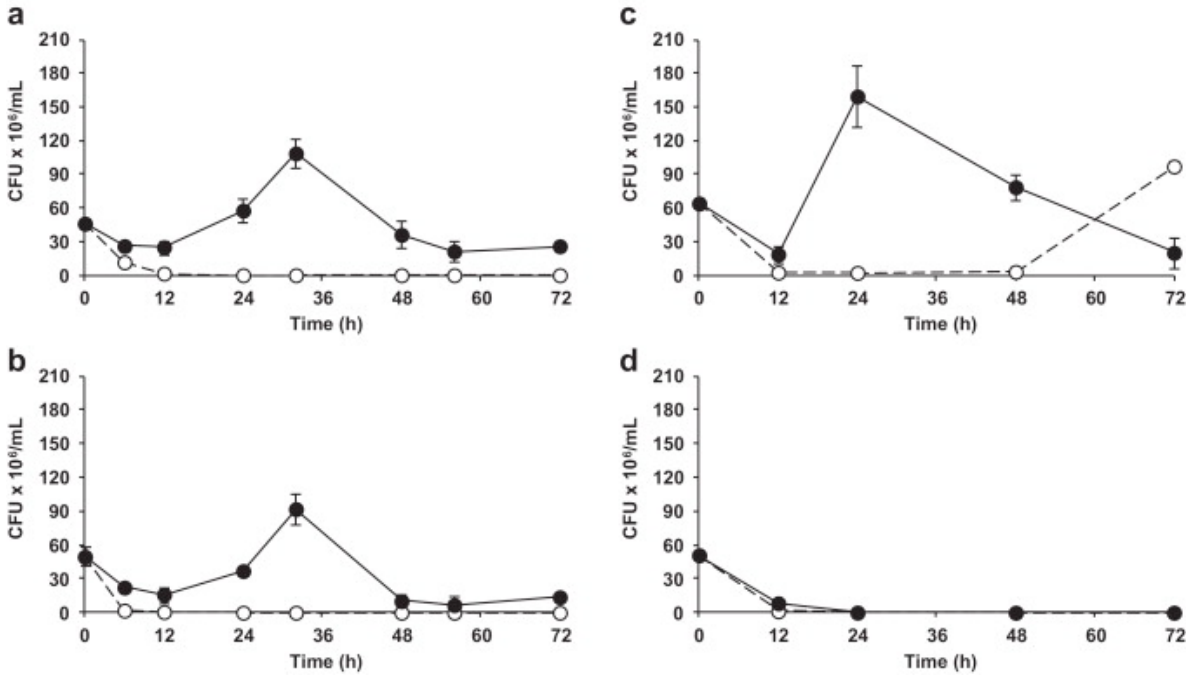


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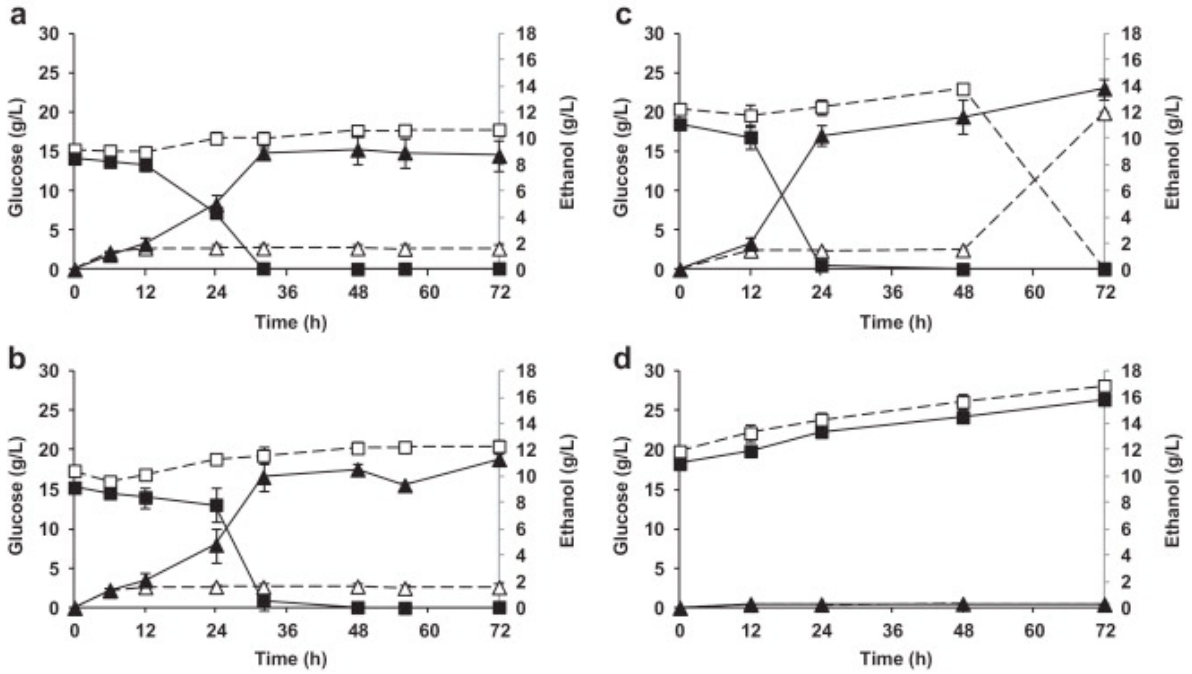
Figure 4


a
b
c
d
Glucose (g/L)
Ethanol (g/L)
Time (h)
0
12
24
36
48
60
72
0
5
10
15
20
25
30
0
2
4
6
8
10
12
14
16
18

744 **Graphical abstract**

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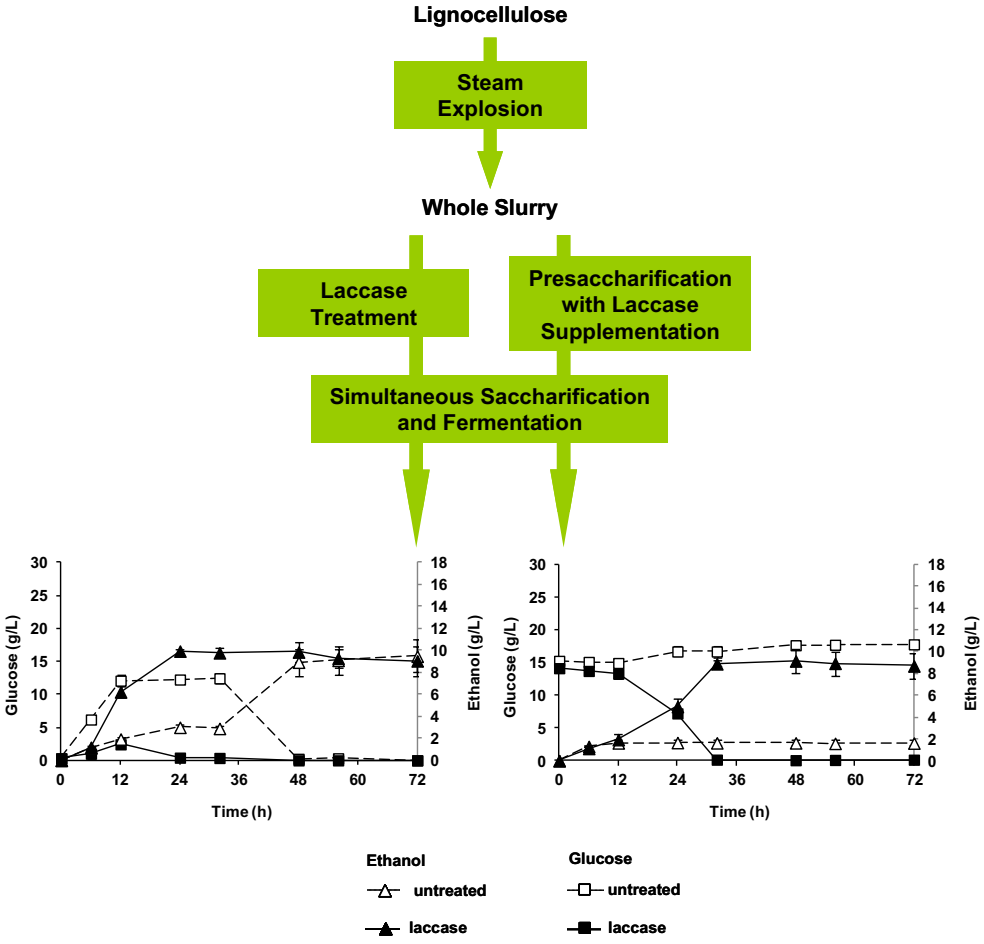

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