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

Antonio D. Moreno[1], David Ibarra[2*], Ignacio Ballesteros[2], Alberto Gonzalez[2], Mercedes Ballesteros[2]

[1]*IMDEA Energía, Biotechnological Processes for Energy Production Unit, Móstoles, Madrid 28933, Spain.*
[2]*CIEMAT, Renewable Energy Division, Biofuels Unit, Avda. Complutense 40, Madrid 28040, Spain.*

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

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7 **Mercedes Ballesteros[2]**

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10 [1]*IMDEA Energía, Biotechnological Processes for Energy Production Unit, Móstoles,*

11 *Madrid 28933, Spain.*

12 [2]*CIEMAT, Renewable Energy Division, Biofuels Unit, Avda. Complutense 40, Madrid*

13 *28040, Spain.*

14

15
16 *Corresponding author: CIEMAT, Renewable Energy Division, Biofuels Unit, Avda.

17 Complutense 40, Madrid 28040, Spain. E-mail address: david.ibarra@ciemat.es. Tel.

18 +34 91 346 6388 Ext. 6388; fax: +34 91 346 0939.

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1 **Abstract**

2 In this study, the thermotolerant yeast *Kluyveromyces marxianus* CECT 10875 was

3 compared to *Saccharomyces cerevisae* Ethanol Red for lignocellulosic ethanol

4 production. For it, whole slurry from steam-exploded wheat straw was used as raw

5 material, and two process configurations, simultaneous saccharification and

6 fermentation (SSF) and presaccharification and simultaneous saccharification and

7 fermentation (PSSF), were evaluated. Compared to *S. cerevisiae*, which was able to

8 produce ethanol in both process configurations, *K. marxianus* was practically inhibited,

9 and neither growth nor ethanol production occurred during the processes. However, the

10 prior laccase treatment of the whole slurry, which removed specifically the lignin

11 phenols content from the overall inhibitory compounds present in slurry, triggered the

12 fermentation by *k. marxianus*, attaining ethanol concentrations and yields comparable to

13 those obtained by *S. cerevisiae*.

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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

1 ethanol productivity during fermentation (Palmqvist and Hahn-Hägerdal, 2000a; Klinke

2 et al., 2004; Panagiotou and Olsson, 2007). Several methods have been assayed to

3 reduce these toxic effects (Palmqvist and Hahn-Hägerdal, 2000b; Taherzadeh and

4 Karimi, 2011), including the use of enzymes such as laccases (Jönsson et al., 1998;

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

6 Moreno et al., 2012).

7   Laccases (EC 1.10.3.1) are multi-copper oxidases produced by microorganisms

8 and plants that oxidize a large variety of phenolic compounds and aromatic amines

9 using molecular oxygen as electron acceptor (Thurston 1994). Their capacity to catalyze

10 the depolymerization and polymerization of lignin make them suitable candidates as

11 white biocatalysts on the development of the lignocellulose biorefinery concept. In this

12 sense, laccase technology can be used either in those processes where lignin removal is

13 the main objective (ethanol production and manufacture of cellulose-based chemicals

14 and materials, including paper), or in those having lignin polymerization as the main

15 goal (functionalization of fibers, production of adhesives, and modification of lignins

16 and other aromatic compounds) (Cañas and Camarero, 2010). The detoxification

17 mechanism by laccases is also based on the oxidative polymerization of phenolic

18 compounds generated during steam-explosion pretreatment (Jurado et al., 2012).

19   Enzymatic hydrolysis and fermentation of the hydrolyzed biomass can be

20 performed in different process configurations, being simultaneous saccharification and

21 fermentation (SSF) strategy an interesting option for lignocellulosic ethanol production

22 (Olofsson et al., 2008). During this process, the glucose released by the action of

23 hydrolytic enzymes is converted simultaneously to ethanol by fermenting

24 microorganisms, minimizing inhibition by cellobiose and glucose accumulation and

25 allowing higher substrate loadings (Olofsson et al., 2008). Neverthless, SSF is usually

1 conducted at temperatures inferior to optimun of the cellulolytic enzymes. Whereas

2 saccharification has an optimun temperature around 50 ℃, most fermenting yeasts has

3 an optimun temperature ranging from 30 to 37 ℃ (Alfani et al., 2000). Therefore, the

4 use of thermotolerant microorganisms provides a number of potential advantages in

5 terms of cost reduction and yield, such as cooling cost reduction and better

6 sacharification and ethanol yields (Abdel-Banat et al., 2010). In this context,

7 *Kluyveromyces marxianus* CECT 10875, a strain adapted and selected in our laboratory

8 (Ballesteros et al., 1991), is gaining great significance due to its capability of growing

9 and fermenting at temperature above 40 ℃, close to optimun temperature of enzymatic

10 hydrolysis, which can lead to an integration of both saccharification and fermentation

11 processes (Tomás-Pejó et al., 2009). However, high temperatures could elevate the

12 yeast susceptibility to inhibitory compounds (Tomás-Pejó et al., 2009; Abdel-Banat et

13 al., 2010), restricting the use of thermotolerant yeasts on pretreated materials with high

14 inhibitors content, such as whole slurry obtained after steam-explosion.

15   In this context, the present work studies the fermentation performance of the

16 thermotolerant yeast *K. marxianus* CECT 10875 on whole slurry from steam-exploded

17 wheat straw detoxified by *Pycnoporus cinnabarinus* laccase. For this purpose, two

18 different process configurations, SSF (simultaneous saccharification and fermentation)

19 and PSSF (presaccharification and simultaneous saccharification and fermentation),

20 were used; and cell viability and both glucose consumption and ethanol production rates

21 were evaluated and compared with *Saccharomyces cerevesiae* Ethanol Red, a typical

22 strain used in fuel alcohol industry.

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24 **2. Methods**

25 *2.1. Raw material and steam explosion pretreatment*

1   Wheat straw, supplied by Ecocarburantes de Castilla y León (Salamanca, Spain),

2   was used as raw material. It presented the following composition (% dry weight):

3   cellulose, 40.5; hemicellulose, 26.1 (xylan, 22.7; arabinan 2.1; and galactan, 1.3); lignin,

4   18.1; ashes, 5.1; and extractives, 14.6.

5   Prior to steam explosion, wheat straw was milled, using a laboratory hammer

6   mill, in order to obtain a chip size between 2 and 10 mm. Then, the raw material was

7   exploded in a 10 L reactor at 220 ºC, 2.5 min. These conditions were selected according

8   to previous optimization studies based on optimal sugars recovery and enzymatic

9   hydrolysis yield (Ballesteros et al., 2006). For analytical purpose, one portion of

10   recovered whole slurry was vacuum filtered with the aim of obtaining a liquid fraction

11   or prehydrolysate and a solid fraction, which was thoroughly washed with distilled

12   water to obtain the water insoluble (WIS) fraction. The remaining slurry was used as

13   substrate for the different assays.

14   Chemical composition of both raw and pretreated material (WIS fraction) was

15   determined using the Laboratory Analytical Procedures (LAP) for biomass analysis

16   provided by the National Renewable Energies Laboratory (NREL, 2007). Sugars and

17   degradation compounds contained in the prehydrolysate were also measured. Most of

18   sugars present in the prehydrolysate were in oligomeric form, because of that a mild

19   acid hydrolysis (4% (v/v) H_2SO_4, 120 ºC and 30 min) was needed to determine

20   monomeric sugars concentration.

21   *2.2. Enzymes*

22   *Pycnoporus cinnabarinus* laccase was produced by Beldem (Belgium). Activity

23   was measured by oxidation of 5 mM 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulphonic

24   acid) (ABTS) to its cation radical ($e_{436} = 29\ 300\ M^{-1}cm^{-1}$) in 0.1 M sodium acetate (pH

25   5) at 24 ºC.

1 A mixture NS50013 and NS50010, both produced by Novozymes (Denmark), was employed to evaluate the effect of laccase enzymes on the cellulose enzymatic hydrolysis step. NS50013 is a cellulase preparation 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).

10 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

13 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 distilled water and then dilute to obtain an inoculum level of 1 g/L (dry weight).

2.4. Simultaneous saccharification and fermentation

23 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

1   mM sodium citrate (pH 5.5) supplemented with the growth medium without glucose

2   described above. Enzyme loading of 15 FPU/g substrate of NS50013 and 15 IU/g

3   substrate of NS50010 and 1 g/L (dry weight) of inoculum were also added. The

4   experiments were run at 42 ºC, for *K. marxianus*, and 35 ºC, for *S. cerevisiae*, for 72 h.

5   *2.5. Presaccharification and simultaneous saccharification and fermentation*

6   The presaccharification and subsequent SSF experiments were also performed in

7   250-ml flasks. The presaccharification stage was carried out in 50 mM sodium citrate

8   (pH 5.5) for 8 h at 50 ºC and enzyme loading of 15 FPU/g substrate of NS50013 and 15

9   IU/g substrate of NS50010. After that, the temperature was reduced to 42 ºC or 35 ºC,

10   depending of the yeast used, and nutrients and inoculum were added, which turned the

11   process into SSF. The experiments were run for another 72 h.

12   *2.6. Laccase detoxification*

13   Laccase treatments (L) were different in function of process configurations. For

14   SSF experiments (LSSF), the laccase treatments were carried out for 8 h, 10 U/g

15   substrate of *P. cinnabarinus* enzyme, at 50 ºC, according to its optimal temperature

16   (Ibarra et al., 2006), and 150 rpm prior to SSF. According to Moreno et al., (2012), the

17   treatments were run on dilute slurry with 50 mM sodium citrate (pH 5.5) to final

18   concentration, based on % (w/v) total solid content present in the pretreated slurry, of

19   6%. After that, the detoxified samples were subsequently submitted to SSF process as

20   explained above. For PSSF experiments (LPSSF), performed as previously described,

21   the presaccharification stage of dilute slurry was supplemented with 10 U/g substrate of

22   *P. cinnabarinus* enzyme.

23   With the purpose to evaluate the effect of laccase on inhibitory compounds,

24   samples from both laccase treatments (laccase treatment alone or simultaneous laccase

25   treatment and presaccharification) were withdrawn after 8 h of treatment, centrifuged

1 and the supernatants analyzed for identification and quantification of inhibitory

2 compounds. In the same way, samples from both laccase treatments submitted to SSF

3 were periodically withdrawn, centrifuged and the supernatants analyzed for cell

4 viability, glucose consumption and ethanol concentration.

5 In both laccase treatments, control assays were performed under the same

6 conditions without addition of laccase. All the experiments were carried out by

7 triplicate.

8 *2.7. Analytical methods*

9 Ethanol was analyzed by gas chromatography, using a 7890A GC System

10 (Agilent Tecnology) equipped with an Agilent 7683B series injector, a flame ionization

11 detector and a column of Carbowax 20 M at 85 ºC. Injector and detector temperature

12 was maintained at 175 ºC.

13 Sugar concentration was quantified by high-performance liquid chromatography

14 (HPLC) in a Waters chromatograph equipped with a refractive index detector (Waters,

15 Mildford, MA). A CarboSep CHO-682 carbohydrate analysis column (Transgenomic,

16 San Jose, CA) operated at 80 ºC with ultrapure water as a mobile-phase (0.5 mL/min)

17 was employed for the separation.

18 Furfural, 5-hydroxymethylfurfural (5-HMF), 4-hydroxybenzaldehyde, vanillin,

19 syringaldehyde, *p*-coumaric acid and ferulic acid were analyzed by HPLC (Agilent,

20 Waldbronn, Germany), using a Coregel 87H3 column (Transgenomic, San Jose, CA) at

21 65 ºC equipped with a 1050 photodiode-array detector (Agilent, Waldbronn, Germany).

22 As mobile phase, 89% 5 mM H_2SO_4 and 11% acetonitrile at a flow rate of 0.7 mL/min

23 were used.

24 Formic acid and acetic acid were analyzed by HPLC (Waters, Mildford, MA)

25 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_o 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

1   solubilization of hemicellulosic sugars, as reflected the high xylose content (12 g/L) in

2   the prehydrolysate. A significant proportion of degradation products were also observed

3   in the prehydrolysate composition, such as acetic acid, formic acid, furfural, 5-HMF and

4   phenols. Acetic acid is released due to the solubilization of acetyl groups contained in

5   the hemicellulose structure. Formic acid comes from furfural and 5-HMF degradation,

6   which derive from xylose and glucose degradation, respectively (Palmqvist and Hahn-

7   Hägerdal, 2000a). Among total phenols, different phenol monomers characteristic of

8   herbaceous biomass were also detected (Fig. 1A), according with previous studies

9   (Tomás-Pejó et al., 2009; Kolb et al., 2012). Among them, aldehydes derived from

10   lignin degradation such as 4-hydroxy-benzaldehyde, vanillin and syringaldehyde,

11   released from the three monolignol units (*p*-hydroxyphenyl-guaiacyl-siringyl) contained

12   in the lignin of wheat straw (Buranov and Mazza, 2008); and acids such as ferulic and

13   *p*-coumaric acids, both derived from cinnamic acids, characteristic of herbaceous

14   biomass acting as linkages between lignin and hemicellulose (Buranov and Mazza,

15   2008).

16   *3.2. Effect of laccase treatment on the different inhibitory compounds*

17   Whole slurry obtained after pretreatment should be used as substrate for ethanol

18   production. However, the use of the whole slurry implies the presence of high inhibitors

19   concentrations in the media, which affect the downstream ethanol production steps.

20   Usually, the whole slurry resulting from steam explosion is filtered and whased.

21   However, from an economical and environmental point of view, the filtration and

22   washing steps should be avoided because they increase both operational costs and

23   wastewater (García-Aparicio et al., 2006). Hence, the necessity to overcoming the

24   inhibitory effects with strategies differents to filtration and washing.

1   In this study, the whole slurry was submitted to laccase treatment before

2   saccharification and fermentation experiments. Laccase treatment alone prior to SSF

3   (LSSF) removed around 67% of the total phenols content of slurry (Fig. 2). On the

4   contrary, the concentrations of weak acids and furan derivates were not affected (Table

5   2), in accordance with previous studies (Larsson et al., 1999; Martín et al., 2002;

6   Chandel et al., 2007; Moreno et al., 2012). Unlike other detoxification methods (mainly

7   physical and chemical methods), which show the capacity to decrease the overall

8   inhibitor concentration in a great or lesser extent, this substrate-specific reaction is only

9   characteristic of laccase enzymes (Palmqvist and Hahn-Hägerdal, 2000b; Taherzadeh

10   and Karimi, 2011). This fact offers some advantages over chemical or physical

11   methods, such as mild reaction conditions, fewer toxic sub-products and low energy

12   requirements (Parawira and Tekere, 2011).

13   When the whole slurry was submitted to simultaneous laccase treatment and

14   presaccharification step prior to SSF (LPSSF), laccase removed about 73% of the total

15   phenols content slurry (Fig. 2), a slightly higher removal than that observed with laccase

16   treatment alone (67%). This phenomenon could be attributed to presaccharification,

17   which facilitates the access of laccase enzyme to phenols.

18   As observed in both laccase treatments, the removal of the total phenols content

19   after 8 h of reaction was not complete, in line with previous studies (Jönsson et al.,

20   1998; Larsson et al., 1999; Martín et al., 2002; Chandel et al., 2007; Jurado et al., 2009;

21   Moreno et al., 2012). Some of the lignin phenol monomers identified herein were more

22   resistant to laccase action, such as 4-hydroxy-benzaldehyde and vanillin (Fig. 1B). This

23   is in function of the reactivity of the different lignin phenol monomers towards laccase.

24   In this sense, Kolb et al., (2012) described in liquid hot water pretreated wheat straw

25   supernatants three different laccase reaction groups of phenol monomers, some of them

1   also identified in this study. Among them, phenol monomers immediately removed

2   (syringaldehyde, ferulic acid and *p*-coumaric acid), phenol monomers removed after

3   reaction of 24h (vanillin), and phenol monomers no affected by laccase action (4-

4   hydroxy-benzaldehyde). They report that an additional methoxy substituent at the

5   benzenic ring (the difference between vanillin and syrindaldehyde) increase the

6   reactivity of the phenolic compounds towards laccase. Moreover, *para*-substituted

7   phenols with ethylene groups, such as ferulic acid and *p*-coumaric acid, show a greater

8   sensitivity towards laccase.

9   *3.3. Effect of laccase treatment on cell viability and ethanol fermentation*

10   *S. cerevisiae*, the yeast more widely used in industrial fermentation processes,

11   has an optimal fermentation temperature lower than the optimal saccharification

12   temperature, which hinder a complete integration at optimum conditions of

13   saccharification and fermentation processes. Thus, the use of thermotolerant

14   microorganisms such as *K. marxianus* appears as an attractive alternative in SSF

15   processes (Tomás-Pejó et al., 2009; Abdel-Banat et al., 2010). In this context, control

16   and detoxified slurries, resulting from laccase treatment alone or simultaneous laccase

17   treatment and presaccharification, were submitted to SSF for 72 h using *S. cerevisiae*

18   Ethanol Red or *K. marxianus* CECT 10875 at 35 ºC and 42 ºC, respectively. Time

19   courses for cell viability, glucose consumption and ethanol production were studied

20   (Fig. 3 and 4).

21   *3.3.1. LSSF*

22   In a first set of experiments, the performance of both yeasts was followed on

23   slurry submitted to laccase treatment alone. As can be seen for control sample, the lag

24   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

1   consumption nor ethanol fermentation occurred during the process (Fig. 4B). Compared

2   to *S. cerevisiae*, this higher inhibitory effect observed when using the thermotolerant

3   yeast *K. marxianus* could also be due to the effect caused by high temperatures on cell

4   membrane, increasing the inhibitory effects of the degradation compounds (Tomás-Pejó

5   et al., 2009; Abdel-Banat et al., 2010).

6   The specifically phenols removal by laccase treatment improved the

7   fermentation performance of *S. cerevisiae*. Compared to control sample, laccase

8   treatment shorted the yeast lag phase from 12 h to 6 h (Fig. 3A and 4A). On the one

9   hand, cell viability was significantly improved, reaching the highest number of colony

10   units at 24 h of SSF (Fig. 3A). On the other hand, faster glucose consumption and

11   ethanol production rates were also observed (Fig. 4A), increasing the ethanol

12   productivity value from 0.39 g/L h, for control sample, to 0.44 g/L h for laccase sample.

13   Nevertheless, laccase treatment did not improve the ethanol production, obtaining a

14   similar ethanol yield (0.35 g/g) compared to control sample (Table 3). These results

15   support the inhibitory effects reported for phenols, affecting growth and ethanol

16   production rate, but not ethanol yield (Palmqvist and Hahn-Hägerdal, 2000a; Klinke et

17   al., 2004).

18   Similar enhancements on the *S. cerevisiae* performance have also been attributed

19   to different laccase enzymes in several detoxification studies. Jönsson et al., (1998)

20   reported higher glucose consumption rate, ethanol productivity value and ethanol yield

21   when prehydrolysate from acid steam-exploded willow was submitted to *Trametes*

22   *versicolor* laccase. Larsson et al., (1999), described similar results together with a

23   higher yeast growth on prehydrolysate from acid steam-exploded spruce treated with *T.*

24   *versicolor* laccase. In contrast, Jurado et al., (2009) described a higher influence on

25   ethanol conversion than yeast growth when enzymatic hydrolysed from both acid and

1   non acid steam-exploded wheat straw were submitted to laccases from *Trametes villosa*

2   or *Coriolopsis rigida*.

3   In the case of *K. marxianus*, laccase treatment triggered the fermentation of the

4   medium. A great increment in cell viability was observed between 12 and 24 h of SSF

5   (Fig. 3B); followed by a remarkable drop after 30 h. In addition, the yeast also

6   consumed almost all the glucose between 12 and 24 h (Fig. 4B), attaining an ethanol

7   concentration (10.7 g/L) and an ethanol yield (0.35 g/g) similar to those obtained with *S.*

8   *cerevisiae* on either control or laccase samples (Table 3). Nevertheless, the ethanol

9   production rate was slower compared to *S. cerevisiae*, showing an ethanol productivity

10 value (0.36 g/L h) lower to that obtained with *S. cerevisiae* on laccase sample (0.44 g/L

11 h) (Table 3).

12 *3.3.2. LPSSF*

13   In a second set of experiments, the performance of both yeasts was followed on

14 slurry submitted to simultaneous laccase treatment and presaccharification. Enzymatic

15 prehydrolysis prior to the addition of yeast offer some advantages, such as to make the

16 media more fluid and easer to handle, facilitating the mixing during fermentation.

17 Prehydrolysis also enables hydrolytic enzymes to act at their optima temperatures,

18 enhancing the enzymatic hydrolysis and consequently increasing the ethanol production

19 rate (Manzanares et al., 2011).

20   Compared to SSF with no presacharification, the enzymatic prehydrolysis

21 altered slightly the performance of *S. cerevisiae*. In contrast to SSF with no

22 prehydrolysis, where a gradual increment of cell viability was observed during the

23 process (Fig. 3A), cell viability showed a great increment between 12 and 24 h when

24 presaccharification was performed (Fig. 3C). Ethanol production was also strongly

25 increased between 12 and 24 h (Fig. 4C), attaining a similar ethanol concentration (10.8

1   g/L) and a slightly higher ethanol productivity value (0.41 g/L h) with

2   presaccharification stage (Table 3). Regarding *K. marxianus*, it was also totally

3   inhibited with prehydrolysis. A complete cell viability yeast loss was observed after 6 h

4   of SSF (Fig. 3D); and neither glucose consumption nor ethanol fermentation resulted

5   during the process (Fig. 4D).

6   As previously described for LSSF, the supplementation of presaccharification

7   with laccase enzyme also enhanced the fermentation performance of *S. cerevisiae*.

8   Compared to control sample, laccase supplementation shorted the yeast lag phase from

9   12 h to 6 h (Fig. 3C and 4C). Cell viability was significantly improved, reaching the

10   highest number of colony units at 12 h of SSF (Fig. 3C); and faster glucose

11   consumption and ethanol production rates were also observed (Fig. 4C), increasing

12   slightly the ethanol productivity value from 0.41 g/L h, for control sample, to 0.43 g/L h

13   for laccase sample. Nevertheless, the laccase supplementation did not improve either the

14   ethanol production, obtaining a similar ethanol yield (0.34 g/g) compared to control

15   sample (Table 3). With regard to *K. marxianus*, as previously described for LSSF,

16   laccase supplementation also triggered the fermentation of the medium. A gradual

17   increment in cell viability was showed between 12 and 30 h of SSF, observing after that

18   a remarkable drop (Fig. 3D). In the same way, the yeast also consumed almost all the

19   glucose between 24 and 30 h (Fig. 4D), attaining an ethanol concentration (11.1 g/L)

20   and an ethanol yield (0.36 g/g) similar to those obtained with *S. cerevisiae* on either

21   control or laccase samples (Table 3). However, the ethanol production rate was very

22   slow compared to *S. cerevisiae*, showing an ethanol productivity value especially lower

23   (0.20 g/L h) to that obtained with *S. cerevisiae* on laccase sample (0.43 g/L h) (Table 3).

24   In spite of the presaccharification improves the laccase action, observing a

25   slightly higher removal of phenols than laccase treatment alone, *S. cerevisiae* showed a

1 similar fermentation performance in both LSSF and LPSSF configurations. *K.*

2 *marxianus* also showed a similar ethanol production in both process configurations

3 (Table 3). However, the presaccharification slowed down the process, observing a

4 gradual increment of cell viability and slower glucose consumption and ethanol

5 production rates (Fig. 2D and 3D), attaining a drop ethanol productivity value from 0.36

6 g/L h, with no presaccharifacation, to 0.20 g/L h with presaccharification (Table 3).

7   On the light of the results presented herein, and with the aim to proceed towards

8 the use of *K. marxianus* and laccase enzymes for lignocellulosic ethanol production,

9 other aspects, such as different substrate loadings and pretreatment conditions need to

10 be studied greatly.

11

12 4. Conclusion

13   Higher integration of saccharification and fermentation processes is a key aspect

14 to make feasible the lignocellulosic ethanol production. In this context, thermotolerant

15 microorganisms, with the capacity to ferment ethanol at temperatures that are optimal

16 for saccharification, could contribute in a greater extent. However, as showed herein,

17 the presence of inhibitory compounds in the media limit its use. *K. marxianus* CECT

18 10875, the thermotolerant yeast used in this study, was unable to growth and produce

19 ethanol on slurry from steam-exploded wheat straw. However, prior laccase treatment

20 triggered its fermentability, obtaining ethanol concentrations and yields comparable to

21 those obtained by *S. cerevisiae*.

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1 **Table captions**

2 **Table 1**. Composition of steam-exploded wheat straw

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4 **Table 2**. Inhibitory compounds composition of samples resulting from control and laccase

5 treated slurries

6

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

8 *cerevisiae*

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1   **Figure captions**

2   **Figure 1**. Comparison of HPLC profiles of sample s resulting from laccase treatment alone.

3   Control (A) and laccase (B) samples. Identified peaks: 1, 5-HMF; 2, furfural; 3, 4-

4   hydroxybenzaldehyde; 4, vanillin; 5, syringaldehyde; 6, *p*-coumaric acid; and 7, ferulic acid.

5   *Phenols no identified.

6

7   **Figure 2**. Total phenol content determined by Folin-Ciocalteau method of samples resulting

8   from laccase treatment alone or simultaneous laccase treatment and presaccharification. NP, no

9   presaccharification; P, presaccharification. Control (gray bars) and laccase (white bars) samples.

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11   **Figure 3**. Viable cells during LSSF (left) and LPSSF (right) processes of slurry samples.

12   Symbols used: control (¦) and laccase (?) samples. *S. cerevisiae* (A, C) and *k. marxianus* (B,

13   D).

14

15   **Figure 4**. Time course for ethanol (filled symbols and continuous lines) and glucose (open

16   symbols and discontinuous lines) during LSSF (left) and LPSSF (right) processes of slurry

17   samples: control (¦ , ?) and laccase (?, ?) samples. *S. cerevisiae* (A, C) and *k. marxianus* (B,

18   D).

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2 **Table 1**. Composition of steam-exploded wheat straw

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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

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

3 treated slurries

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	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.

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2 **Table 3**. Summary of LSSF and LPSSF assays of dilute slurry using *K. marxianus* CECT 10875

3 and *S. cerevisiae* Ethanol Red

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		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.
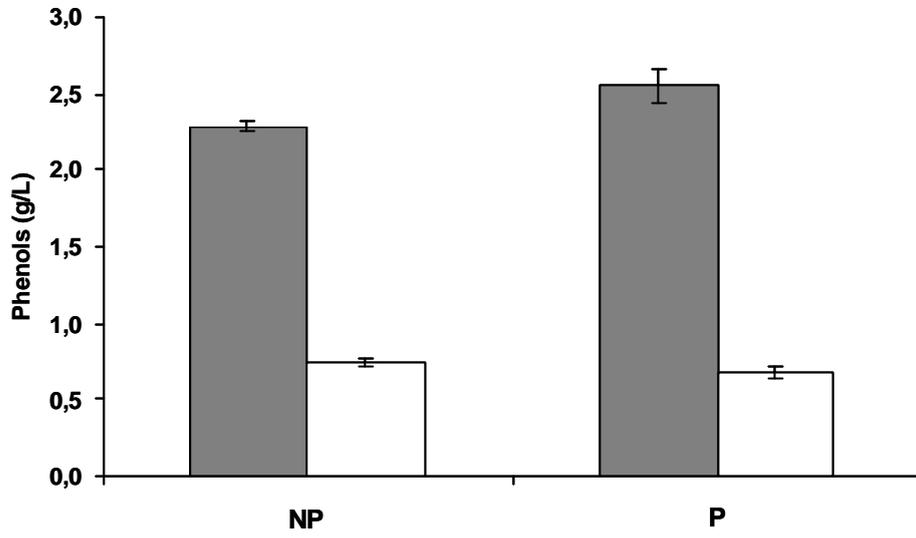
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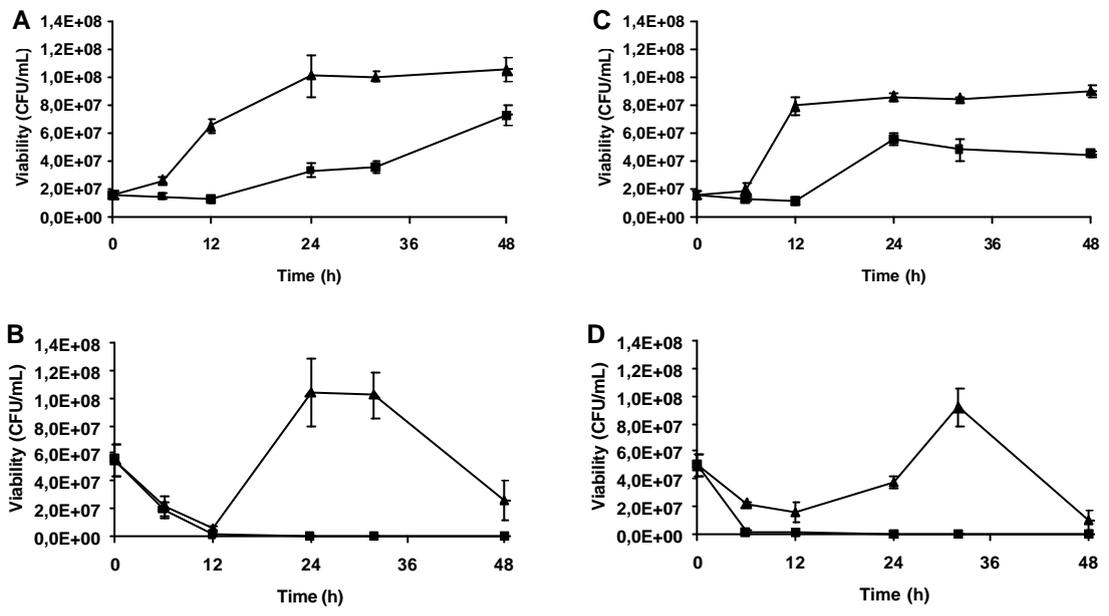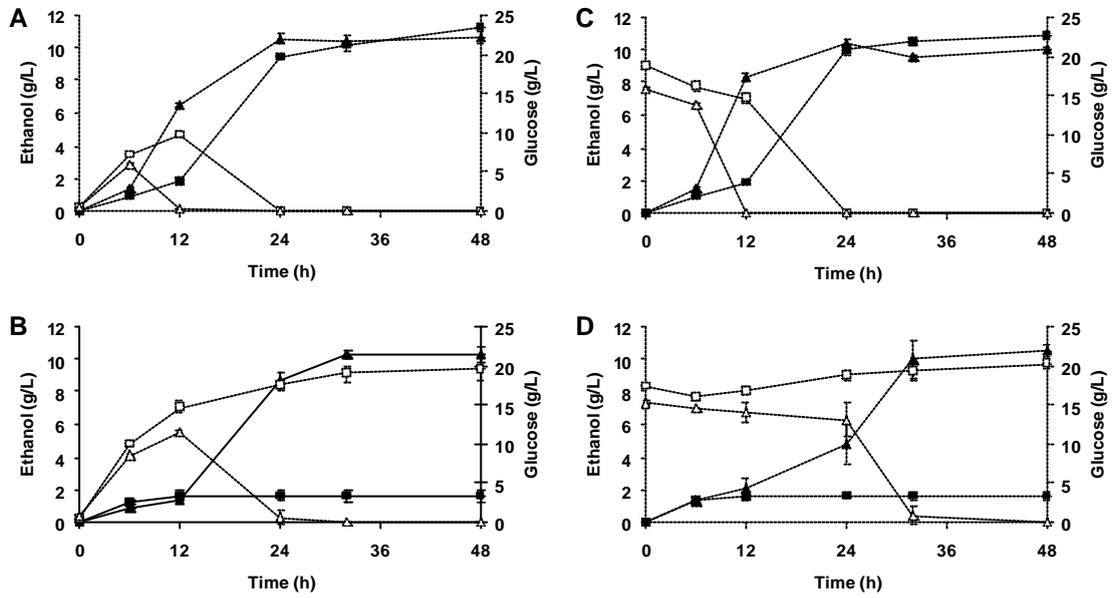15
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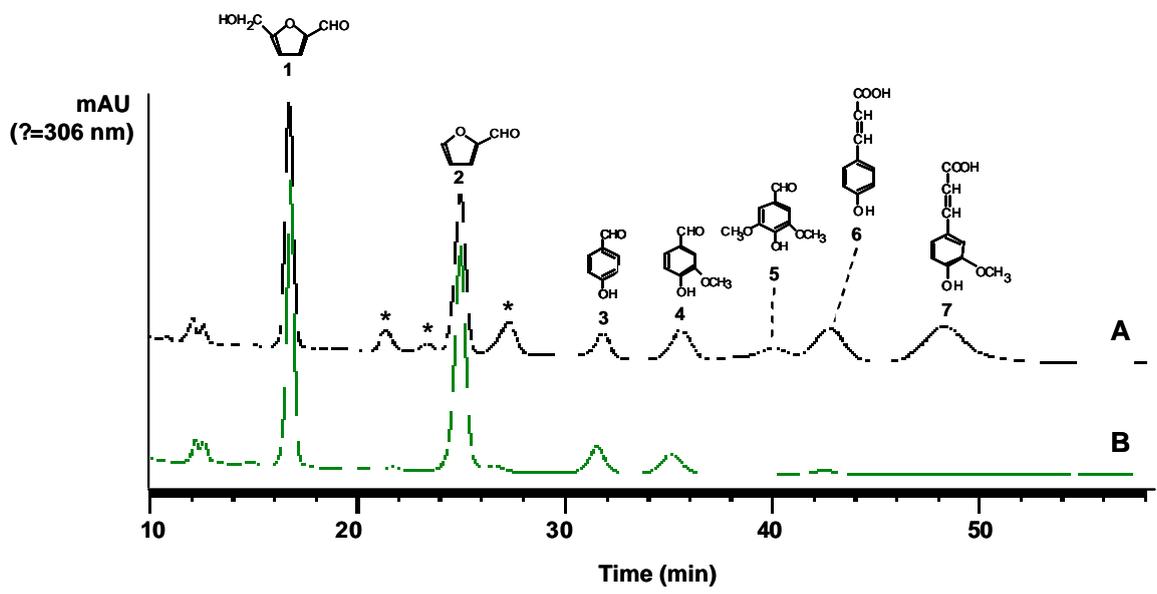
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Figure 2

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