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Sugar recoveries from wheat straw following treatments with the fungus *Irpex lacteus*

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HIGHLIGHTS

- Wheat thin stillage was a suitable medium for *Irpex lacteus* pre-inoculums production.
- Mn(II) supplementation of wheat straw pretreated with the fungus gave significant improvements in glucose yield.
- The maximum glucose yield reached 68% at 21 days of incubation with Mn(II) addition.
- Xylose digestibility reached 100% under some pretreatment conditions.
- Fungal biomass on wheat straw was a positive indicator of glucose yields.

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ABSTRACT

Irpex lacteus is a white-rot fungus capable of increasing sugar recovery from wheat straw; however, in order to incorporate biopretreatment in bioethanol production, some process specifications need to be optimized. With this objective, *I. lacteus* was grown on different liquid culture media for use as inoculums. Additionally, the effect of wheat straw particle size, moisture content, organic and inorganic supplementations, and mild alkali washing during solid-state fermentation (SSF) on sugar yield were investigated. Wheat thin stillage was the best medium for producing inoculums. Supplementation of wheat straw with 0.3 mM Mn(II) during SSF resulted in glucose yields of 68% as compared to yields of 62% and 33% for cultures grown without supplementation or on untreated raw material, respectively after 21 days. Lignin loss, wheat straw digestibility, peroxidase activity, and fungal biomass were also correlated with sugar yields in the search for biopretreatment efficiency indicators.

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1. Introduction

Wheat straw, the most abundant agricultural residue in Europe and the second worldwide, presents great potential for ethanol production (Talebnia et al., 2010); however, due to the complexity of its structure, especially the lignin framework, it is a challenge to obtain high sugar release from this substrate.

Steam explosion, one of the most used physico-chemical pretreatment methods to disrupt the lignocellulosic biomass, produces undesirable compounds such as weak acids, furan derivatives, and phenolic and inorganic substances which negatively affect the fermentation step (Hahn-Hägerdal et al., 2006). Biological pretreatments could be an alternative, since some organisms, like white-rot fungi, are able to degrade lignocellulose selectively and produce fewer yeast inhibitors than steam explosion (Salvachúa et al., 2011). One disadvantage of these treatments is the long incubation time necessary to reach yields similar to those obtained with current physico-chemical pretreatments. For this reason, combinations of biopretreatment with mild physical (Yamagishi et al., 2011), alkali (Salvachúa et al., 2011; Saritha et al., 2012; Zhong et al., 2011), organosolv (Canam et al., 2011), or hot water (Wang et al., 2012) treatments have been investigated.

The basidiomycete *Irpex lacteus*, has emerged as a fungus with great biodegradation potential (Novotny et al., 2009). The fungus has an exceptional ability to degrade corn stover (Xu et al., 2010), corn stalks (Du et al., 2011; Zhong et al., 2011), and wheat straw (Pinto et al., 2012; Salvachúa et al., 2011) under SSF conditions and thus to considerably increase sugar yields from these feedstock.

The present study focused on optimizing the production of *I. lacteus* inoculums for use in SSF of wheat straw by studying fungal biomass production. In addition, SSF cultural and nutritional parameters, such as nitrogen and mineral salt supplementation,

Abbreviation: Lip, lignin peroxidase; MnP, manganese peroxidase; SSF, solid-state fermentation.

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wheat straw particle size and moisture were analyzed as well as ergosterol and enzymes secretion during SSF. The efficiency of fungal treatment complemented with mild-alkali washing was determined by sugar yield estimations at 7, 14, and 21 days.

2. Methods

2.1. Microorganism

The white-rot fungus *I. lacteus* (IJFM A792), deposited in the Fungal Culture Collection of the Centro de Investigaciones Biológicas (Madrid, Spain), was maintained on 2% malt extract agar (w/v) at 4 °C and cultured on plates containing the same medium at 28 °C for one week before being used.

2.2. Pre-inoculum production

2.2.1. Culture media screening

Four 1-cm^{-2} agar plugs were cut from actively growing mycelium on agar plates and used to inoculate 250-mL Erlenmeyer flasks with 30 mL of growth medium (CSS) (Salvachúa et al., 2011) that were incubated at 28 °C and 180 rpm for 7 days. These cultures were aseptically homogenized (Omnimixer, Sorvall), and 2.5 mL were used to inoculate 30 mL of different liquid culture media in 250-mL flasks. The screened media were: (i) CSS medium, (ii) K medium pH 5.5 (glucose, 20 g L^{-1}; $MgSO_4 \times 7H_2O$, 0.5 g L^{-1}; KH_2PO_4, 1 g L^{-1}; yeast extract, 2 g L^{-1}; peptone, 5 g L^{-1}), (iii) wheat mush pH 5.5 diluted until the glucose concentration was 40 g L^{-1}, (iv) wheat thin stillage pH 4 (glucose, 4 g L^{-1}), (v) wheat thin stillage pH 4 supplemented with glucose to a final concentration of 40 g L^{-1}, (vi) wheat thin stillage pH 4 supplemented with nitrogen (0.3 g L^{-1}) from ammonium tartrate, and (vii) wheat thin stillage pH 4 with both glucose and nitrogen at the concentrations listed under (v) and (vi). Wheat mush and wheat thin stillage were obtained from first-generation bioethanol production at Bioetanol Galicia S.A. (Abengoa Bioenergy, Spain).

Cultures were collected at 3, 5, 7, and 10 days of incubation and vacuum-filtered through filter paper to separate the solids to measure biomass, and to determine total reducing sugars in the filtrate. The pH influence on biomass values was studied in wheat thin stillage medium, adjusted to pH 4, 4.5, 5, 5.5, and 6 with NaOH. These cultures were analyzed at 24, 48, and 72 h. All cultures were grown in triplicate and incubated at 28 °C and180 rpm.

2.2.2. Inoculums for solid-state fermentation (SSF) experiments

Pre-inoculums were grown in wheat thin stillage medium pH 5 as described in Section 2.2.1 and 2.5 mL of an aseptically homogenized culture was used to inoculate the stillage. The cultures were incubated at 28 °C and 180 rpm, and 2 mL of 1-day-old mycelium was used as inoculums for SSF experiments.

2.3. Wheat straw pretreatment

Wheat straw was harvested from Galician fields (Spain), dried, and chopped (<1 cm). *I. lacteus* basal cultures (ILC) with 2 g of wheat straw and 6 mL of water were prepared and cultured under SSF conditions as previously described (Salvachúa et al., 2011). These basal conditions were modified in other *I. lacteus* cultures to test the effect of: (i) wheat straw particle size by using milled straw (MWS, <0.5 mm), (ii) maintaining moisture content at 75%, either by replacing lost water daily or by increasing the initial moisture content to 86%, and (iii) the addition of 0.3 mM $MnSO_4$, $CuSO_4$ and $FeSO_4$, peptone (20 g L^{-1}), and wheat thin stillage (diluted so as to reach 2 g L^{-1} of glucose). To maintain the original moisture content, the lost weight in control cultures was attributed to water evaporation and this amount was added to all treatments. Additives (salts and others) were dissolved in distilled water (6 mL) before autoclaving. Untreated wheat straw of both particle sizes was incubated under the same conditions as the treatments and used as controls. Assays were performed in triplicate.

Biopretreated and untreated wheat straw collected after 7, 14, and 21 days of incubation were washed with distilled water (15 mL) for 1 h, at 28 °C and 180 rpm, and vacuum-filtered to extract water-soluble compounds. Solid fractions were dried in an aeration oven at 65 °C and weighed. After calculating weight loss and analyzing the main remaining components in the wheat straw (see Section 2.6.), solid fractions were subjected to mild alkali treatments with 0.1% sodium hydroxide (5% w/v), at 50 °C and 165 rpm for 1 h. This alkali mixture was then filtered, washed until neutral with distilled water at 50 °C, dried at 65 °C, and total reducing sugars were analyzed in the alkali filtrates.

2.4. Enzymatic hydrolysis, digestibility and sugar yield estimations

Pretreated fractions were hydrolyzed in duplicate at 5% (w/v) by commercial enzyme cocktails (from Novozymes, Denmark) as 15 FPU g^{-1} of cellulases (Celluclast and NS50010) and 30 U g^{-1} of xylanases (NS50013 and NS50030) in 100 mM sodium citrate buffer (pH 4.8) at 50 °C, and 165 rpm for 60 h and analyzed for fermentable sugar release (Salvachúa et al., 2011). The digestibility of cellulose (D_c) and hemicellulose (D_h) was calculated according to Eq. (1), as the ratio between the percentage of glucose (G_r) or xylose (X_r) released from pretreated fractions and the estimated glucose (G_p) or xylose (X_p) in the fraction prior to enzymatic hydrolysis, respectively. Glucose (G_y) and xylose (X_y) yields were determined by taking into account glucose (G_i) and xylose (X_i) content per gram of dry wheat straw, glucose (G_f) and xylose (X_f) remaining after wheat straw pretreatment, and the digestibility of cellulose (D_c) and hemicellulose (D_h), respectively, as shown in Eq. (2).

$$D_c \text{ or } D_h\ (\%) = (g\ G_r \text{ or } X_{r/g}\ G_p \text{ or } X_p) \times 100 \quad (1)$$

$$G_y \text{ or } X_y\ (\%) = (G_f \text{ or } X_f \times D_c \text{ or } D_h)/G_i \text{ or } X_i \quad (2)$$

2.5. Substrate characterization

Wheat straw weight loss for each fungal pretreatment was calculated as the percentage of total solids lost after water washing. Total hydrolysis of wheat straw was performed to determine Klason lignin (Tappi, 1974) and sugar composition.

2.6. Sugar and protein determination

Sugars from wheat mush and wheat thin stillage were analyzed by gas chromatography (GC) as previously reported (Prieto et al., 2008). Total reducing sugars were estimated by the Somogyi–Nelson method (Somogyi, 1945), using glucose as standard. Glucose, xylose, cellulose, and hemicelluloses estimations were calculated as described elsewhere (Salvachúa et al., 2011). Protein concentrations from wheat mush and wheat thin stillage were determined using the Bradford reagent (Bio-Rad), with bovine serum albumin as standard.

2.7. Enzyme assays

Enzymes were evaluated in the water-soluble fractions from SSF experiments and expressed in international enzyme units (micro moles per minute) per gram of wheat straw. Lignin peroxidase (LiP), laccase, and Mn(II)-oxidizing peroxidase (MnP) were

analyzed in all treatments as previously described (Salvachúa et al., 2011). In addition, Mn(II)-independent peroxidase (MiP) activity was measured using 5 mM 2,6-dimethoxyphenol (DMP) in 100 mM sodium tartrate buffer (pH 5; $\varepsilon 469 = 27{,}500\ M^{-1}\ cm^{-1}$ referred to DMP concentration) in the presence of 0.1 mM H_2O_2.

2.8. Estimation of fungal growth

Liquid cultures were vacuum-filtered and the mycelia dried in an aeration oven at 65 °C for 24 h and weighted.

During SSF experiments, fungal growth was estimated by measuring ergosterol content. First, the percentage of ergosterol in fungal cell walls was determined in lyophilized 5-day old mycelium obtained from CSS medium. Pulverized mycelium (5, 10, 20, 40, and 80 mg) was extracted as described below to calculate ergosterol content as micro gram per milli gram of fungal mycelium, which was taken as the reference value. To evaluate fungal growth in SSF cultures, 100 mg of untreated and biopretreated wheat straw samples were extracted according to the method of Seitz et al. (1979) with some modifications. Samples were placed in glass tubes with petroleum ether (1 mL) and 10% KOH in methanol (4 mL), and sonicated for 15 min. The mixture was left to settle for 45 min and then samples were incubated at 70 °C for 90 min. Milli- Q water (1 mL) and petroleum ether (2 mL) were added to the cooled samples and stirred for 30 s. Tubes were centrifuged at 470*g* for 5 min and the organic phase was removed and allowed to dry at room temperature. Samples were resuspended in methanol and analyzed by HPLC-MS/MS. The system was equipped with a Surveyor HPLC with a Spheri-5 PTH column (Applied Biosystems; 220 mm × 2.1 mm × 5 μm) coupled to a Finnigan TM LXQ TM Linear Ion Trap Mass Spectrometer (Thermo). Samples were analyzed isocratically using methanol (100%) as mobile phase, with a flow of 300 $\mu L\ min^{-1}$. Ergosterol was identified and quantified by comparing the retention time and response factor with those of ergosterol standards analyzed under the same conditions. Fungal growth was estimated by comparing the ergosterol detected in each SSF culture (ED) with its percentage in pulverized mycelium (EP), according to Eq. (3).

$$\textit{I. lacteus}\ \text{biomass}\ (\mu g/g\ \text{wheat straw}) = (\text{ED}\ (\mu g/g\ \text{wheat straw})/\text{EP}) \times 100 \quad (3)$$

3. Results and discussion

3.1. Medium selection for inoculums production

Prior to media screening, characterization was carried out of the wheat mush and wheat thin stillage from first-generation bioethanol production processes. The wheat mush contained glucose (240 $g\ L^{-1}$), xylose (6 $g\ L^{-1}$), and protein (3.6 $g\ L^{-1}$), and the wheat thin stillage included glucose (4 $g\ L^{-1}$), xylose (1 $g\ L^{-1}$), galactose (0.5 $g\ L^{-1}$), mannose (0.6 $g\ L^{-1}$), and protein (0.2 $g\ L^{-1}$). Taking into account these values, dilutions or glucose supplementations were carried out in wheat mush or wheat thin stillage, respectively, to reach a glucose content similar to that in the other media (maximum 40 $g\ L^{-1}$).

Biomass of *I. lacteus* was monitored in different liquid media for 10 d of incubation (Fig. 1). Wheat mush and K media had the lowest fungal biomass content ($\leqslant 10\ mg\ mL^{-1}$) while the highest values (30 $mg\ mL^{-1}$) were found in media containing wheat thin stillage. Wheat thin stillage, the main by-product of ethanol-producing industries, is rich in organic matter and macronutrients (Bustamante et al., 2008), but its glucose content is low. In spite of the low glucose content, the initial growth of *I. lacteus* was significantly faster than that attained when wheat thin stillage was supplemented with glucose, which suggests that the glucose concentration was not the only crucial component for triggering fungal growth. Nitrogen supplementation of wheat thin stillage medium (by adding peptone) did not improve *I. lacteus* growth, but the addition of both glucose and nitrogen to the medium did produce a slow increase in biomass, reaching a maximum at 10 d of incubation instead of at 3 or 5 days.

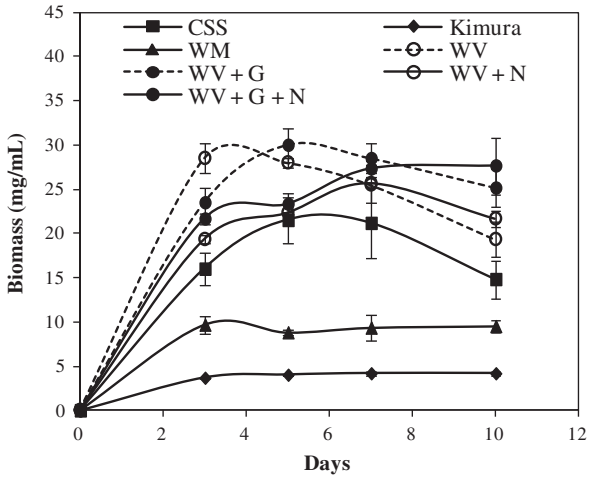

Fig. 1. *I. lacteus* biomass in different aqueous media for 10 days of incubation. CSS medium (CSS); WM = wheat mush; WV = wheat thin stillage; G = glucose; N = nitrogen.

To test the effect of pH on *I. lacteus* growth, the pH of wheat thin stillage medium was adjusted to different values. Fungal growth rate increased when the initial pH was $\geqslant 5$, reaching similar biomass levels in one day as those obtained in the previous experiment, at pH 4, in 3 days (data not shown). Therefore, it can be concluded that growth is more dependent on specific medium components than on its initial pH, although the optimal pH value should be experimentally adjusted for a given medium. Considering the convenience of short process times and decreased costs, wheat thin stillage (pH 5) was selected for producing 1-day old inoculums. Wheat thin stillage contains polyphenols with potential phytotoxic and antimicrobial effects (Bustamante et al., 2008), as well as high levels of recalcitrant organic matter. The ability of *I. lacteus* to grow quickly and efficiently in this medium has been demonstrated in the present work, confirming the resistance of this species to toxic compounds (Novotny et al., 2009). Thus, a suitable cheap medium has been found for producing *I. lacteus* pre-inocula with high fungal biomass content and reduced process costs.

3.2. I. lacteus biopretreatment in different SSF conditions

3.2.1. Effect on wheat straw degradation

I. lacteus is a basidiomycete capable of improving sugar recovery from wheat straw without promoting significant changes in its microstructure and structural integrity (Fig. S1). Initial wheat straw consisted of cellulose (37%), hemicelluloses (23%), and lignin (24%). As xylan was the principal component in hemicellulose (18%) it was included in all subsequent estimations.

During SSF, the main weight losses occurred during the first 2 weeks, with similar results from 14 to 21 days in most treatments. High moisture content and peptone supplementation produced the lowest and highest weight losses, respectively, corresponding to approximately 20% and 38% at 21-d biopretreatment (data not shown).

Hemicellulose degradation increased by a factor of two after 14 and 21 days of fungal growth when straw with a particle size of ⩽0.5 mm was used, although cellulose loss and lignin degradation diminished under these conditions (Table 1). These findings can be explained by the reduction in the ordered structure of smaller particles, especially of hemicellulose, which is not as well-packed as cellulose (Xu et al., 2009). Due to the accessibility of this component the fungus makes preferential use of xylose and hardly consumes or degrades other wheat straw components, like cellulose or lignin.

Regardless of the tested moisture contents maintained during the 3-week SSF period, cellulose and lignin losses were similar to those from control cultures (Table 1). In contrast, hemicellulose degradation was either negligible with increased moisture or delayed under constant wetness. It has previously been observed that xylanase production can be reduced by water excesses (Mohana et al., 2008), resulting in those low hemicellulose losses. Those results could be explained by too much water possibly obstructing inter-particle spaces, thereby inhibiting gas circulation, compacting the substrate and making fungus action difficult (Reid, 1989), thus changing the degradation pattern relative to ILC.

While Cu(II) and Fe(II) supplementation resulted in low hemicellulose degradation (>11%) and fast cellulose consumption at 14 days (~29%), less cellulose (17%) and more hemicellulose (~30%) degradation was observed with Mn(II) (Table 1). During the third week, cellulose content did not change significantly relative to previous weeks, but parallel increases in lignin and hemicellulose degradation were detected in all cases, with lignin reaching the highest degradation (45.9%) with Fe(II). Fe(II) favors the production of hydroxyl radicals, strong oxidants formed in Fenton reactions, which are considered to be one of the low-molecular weight agents implicated in the initial lignocellulose attack (Evans et al., 1994).

Lignin degradation was higher when peptone and wheat stillage was added compared to ILC at 14 and 21 days (Table 1). Therefore, peptone addition resulted in elevated cellulose (34%) and hemicellulose (50%) consumption at 21 days. In contrast, wheat thin stillage supplementation provoked significantly less hemicellulose loss. Enzyme production in SSF is dependent on the N source and the fungus and, in the case of the ligninolytic system, activation normally occurs at low nitrogen concentrations (Kachlishvili et al., 2006). Nevertheless, considering that this high lignin loss was accompanied by substantial differences in the composition of the wheat straw recovered after these SSF treatments, the action of other lignin degradation mechanisms, based on alternative oxidative processes (Tanaka et al., 1999), should be considered.

By comparing all treatments (Fig. 2), a positive correlation ($r = 0.721$) was found between lignin and cellulose loss during the 21-d biopretreatment. It seems that as lignin is being degraded, accessibility to cellulose increases and consequently, *I. lacteus* consumes this component more extensively, resulting in a decrease in glucose yields. In contrast, hemicellulose losses were not dependent on cellulose ($r = 0.141$) or lignin ($r = 0.385$) degradation.

3.2.2. Effect on water-soluble sugars recovery

The sugar content of the water-soluble fraction was less than 1% in all cases, with the exception of the treatment with peptone where slightly higher values of 1.4 and 1.1% were detected at 14 and 21 days respectively (data not shown). This result parallels the highest sugar losses observed in treatments with peptone. Since most of the sugars released by fungal action are being consumed by the fungus itself for its own growth and survival and because of their low content the soluble sugars were not included in the final sugar yield calculations.

3.2.3. Effect on digestibility and sugar yields

To evaluate enzymatic hydrolysis efficiency, cellulose and hemicellulose digestibility were calculated after the wheat straw had been subjected to mild alkali treatment to improve digestibility (Salvachúa et al., 2011; Zhong et al., 2011) and sugar losses were not detected after alkali-washing and major increases were quantified in cellulose (around 20–30%) but not in hemicellulose digestibility (data not shown).

Neither an initial content of 86% nor maintaining 75% wetness was satisfactory, so this parameter merits further optimization for the SSF of wheat straw by *I. lacteus*. Particle size reduction (MWS), boosted hemicellulose digestibility in all samples (Fig. 3). A slight increase in cellulose digestibility at 7 days of biopretreatment was observed only with MWS (5% more than non-milled wheat straw), but sugar yields after this short period were poor, suggesting a need for longer biopretreatments to enhance fermentable sugar recoveries relative to untreated straw, as reported by Pedersen and Meyer (2009).

After 14 days of fungal treatment, cellulose digestibility improved in most cases compared with untreated samples, except in the case of cultures with moisture variations, Cu(II), and peptone supplementations. In contrast, better hemicellulose digestibility was only found in ILC, cultures with MWS, and cultures with added Mn(II). Fermentable sugar yields were generally similar to those of untreated wheat straw cultures, although glucose recovery reached 47% in ILC, 49% using MWS as the substrate, and 48% with Mn(II) supplementation. Xylose recovery (Fig. 4) was higher in ILC (57%) and wheat thin stillage (52%) than in non-biopretreated samples.

At 21 days all treatments improved digestibility compared with non-biopretreated samples, with the exception of hemicellulose digestibility from samples with added peptone. In fact, significant differences in hemicellulose digestibility were found between both

Table 1
Loss of wheat straw components after biopretreatment with *I. lacteus* after 7, 14, and 21 days of incubation under different SSF conditions. Values are calculated taking into account the initial content of each component in WS. Data are means of triplicates and standard deviations are provided. CEL = cellulose; HEM; hemicellulose; LIG = lignin; ILC = *I. lacteus* basal cultures; MWS = milled wheat straw.

	Loss (%)								
	7 days			14 days			21 days		
	CEL	HEM	LIG	CEL	HEM	LIG	CEL	HEM	LIG
ILC	12.5 ± 1.0	16.9 ± 1.3	18.5 ± 0.6	17.3 ± 0.4	19.4 ± 1.9	28.2 ± 0.5	20.7 ± 2.0	22.5 ± 1.5	36.3 ± 0.5
MWS	12.3 ± 0.4	26.0 ± 4.8	9.8 ± 0.0	12.7 ± 0.5	43.3 ± 10.9	22.9 ± 0.0	12.1 ± 1.3	49.1 ± 5.7	25.8 ± 0.1
Constant moisture	14.6 ± 0.5	2.4 ± 0.3	19.4 ± 0.9	23.0 ± 0.3	16.1 ± 3.0	23.8 ± 0.8	25.6 ± 0.4	39.4 ± 9.2	37.5 ± 0.5
High moisture	15.9 ± 0.2	0.0 ± 1.0	15.3 ± 0.4	21.8 ± 0.8	0.0 ± 0.1	26.6 ± 0.7	27.1 ± 0.7	2.3 ± 0.0	29.8 ± 0.7
Mn (II)	14.9 ± 0.7	0.0 ± 0.0	22.2 ± 0.5	16.8 ± 0.5	29.7 ± 3.4	31.5 ± 0.9	17.9 ± 0.9	45.1 ± 5.6	37.9 ± 0.7
Cu (II)	11.8 ± 0.1	4.3 ± 0.1	12.5 ± 0.6	28.3 ± 1.5	10.6 ± 1.2	29.4 ± 1.3	26.2 ± 0.7	11.1 ± 0.1	42.8 ± 1.8
Fe (II)	9.8 ± 0.0	6.4 ± 0.1	14.5 ± 0.2	29.5 ± 1.6	8.5 ± 0.1	29.5 ± 1.6	27.2 ± 0.9	24.3 ± 0.8	45.9 ± 0.1
Peptone	11.2 ± 0.3	16.2 ± 0.2	18.5 ± 0.6	22.5 ± 0.7	21.4 ± 1.5	31.5 ± 0.6	34.2 ± 0.7	50.4 ± 0.4	42.3 ± 0.3
Wheat thin stillage	12.5 ± 0.0	2.0 ± 1.9	18.0 ± 0.0	17.2 ± 0.5	2.6 ± 0.2	32.7 ± 0.8	19.2 ± 1.4	14.4 ± 1.3	39.9 ± 1.1

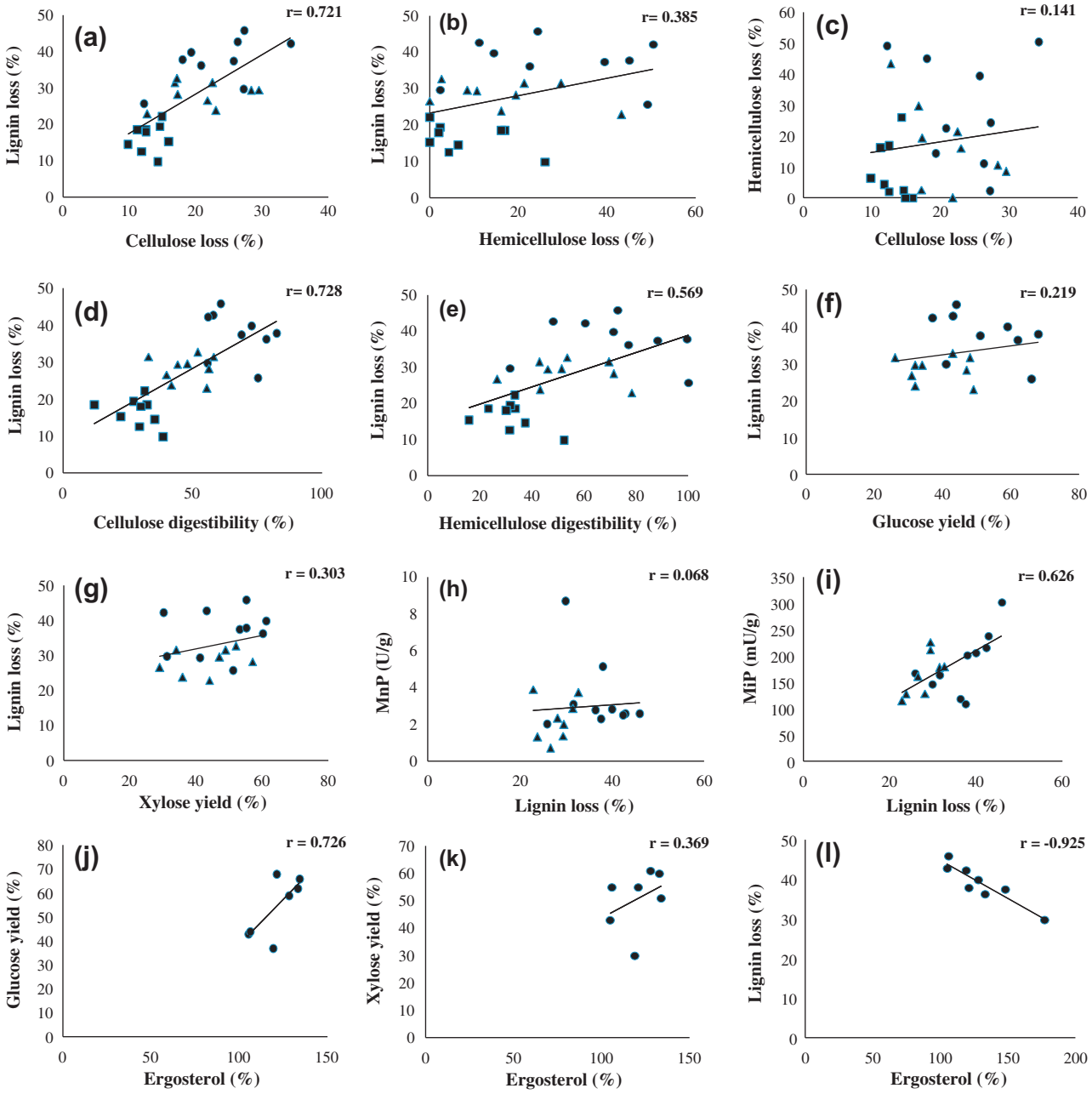

Fig. 2. Scatter plots comparing several variables analyzed under different SSF conditions during *I. lacteus* biopretreatment: (a–c) wheat straw components loss at 7, 14 and 21 days; (d–e) lignin loss with digestibility at 7, 14 and 21 days; (f–g) lignin loss with sugar yields at 14 and 21 days; (h–i) lignin loss with peroxidase activities at 14 and 21 days; and (j–k) ergosterol with both sugar yields and lignin loss at 21 days. Squares, triangles, and circles represent samples from 7, 14, and 21 days respectively.

MWS and Mn(II) supplemented treatments and control cultures, the hemicellulose being completely digested after enzymatic hydrolysis. Nevertheless, despite these significant improvements, hemicellulose was extensively degraded during fungal growth, decreasing the final xylose yield. Only ILC, and Mn(II) and wheat thin stillage-supplemented treatments increased xylose yields up to 55–61%, while untreated samples gave 33% and 45% for wheat straw and MWS, respectively, with no significant differences among them (Fig. 4). These xylose recoveries are very high as compared with the 20% reported by Wan and Li (2011) after wheat straw biopretreatment with *Ceriporiopsis subvermispora* for 35 days. Concerning glucose (Fig. 4), five treatments (ILC, MWS, maintained moisture, Mn(II), and wheat thin stillage) improved recovery compared with untreated samples (33% and 37% for wheat straw and MWS, respectively), but only the Mn(II) treatment gave a better yield (68%) than ILC (62%). In view of these results, Mn(II) supplementation seems to be appropriate to significantly enhance glucose yields from 21-d SSF cultures of *I. lacteus* on wheat straw. As previously reported, Mn(II) addition to SSF cultures probably affects the induction of MnP thus increasing the efficiency of the process (Camarero et al., 1996).

According to the present data, Mn(II) supplementation produced total sugar recoveries 91% higher than those from untreated wheat straw. In comparison, a 62% increase in total reducing sugar recoveries was recently reported with *I. lacteus* in 21-d SSFbcultures on wheat straw (Pinto et al., 2012). Moreover, significantly lower recoveries than those presented in this study were reached after wheat straw pretreatments using other fungi, such as

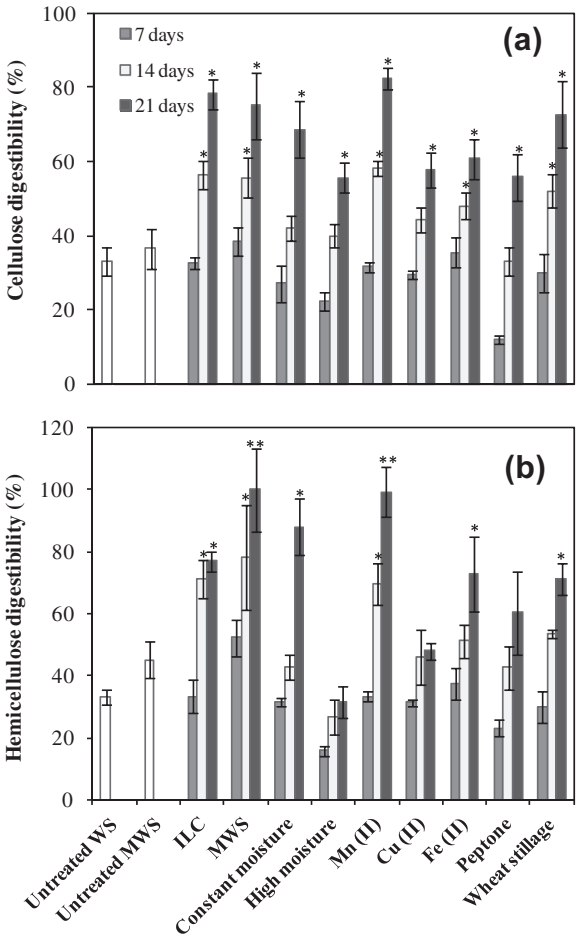

Fig. 3. Percentage of (a) cellulose and (b) hemicellulose digestibility from pretreated wheat straw (WS) during 7, 14 and 21 days of SSF. Untreated samples (white bars) correspond to non-biopretreated WS. Data are means of triplicates and standard deviations are provided. Asterisks over the bars represent samples with significantly higher values than non-biopretreated WS (*) and *I. lacteus* basal cultures (ILC) (**). MWS = milled wheat straw.

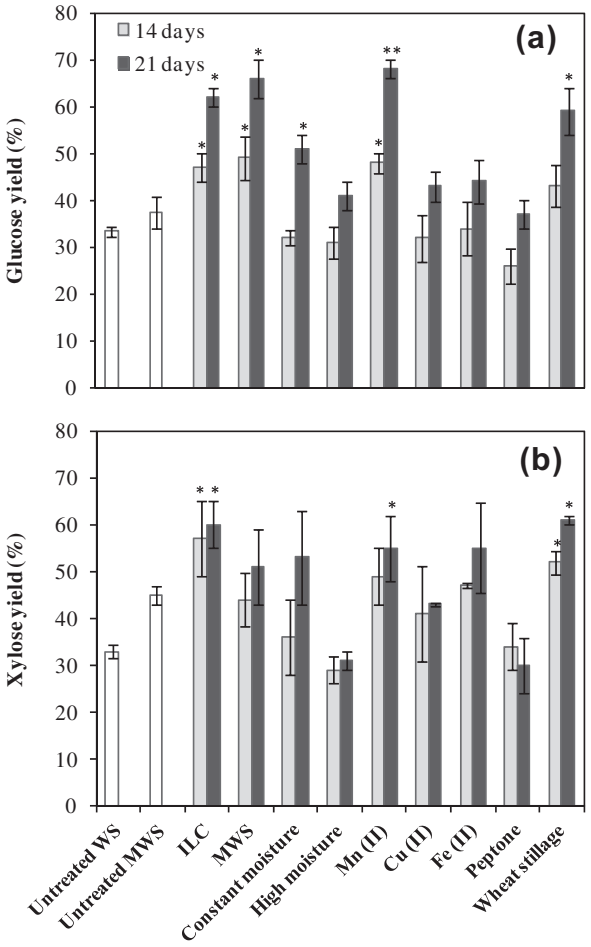

Fig. 4. Percentage of (a) glucose and (b) xylose yield from pretreated wheat straw (WS) after 14 and 21 days of SSF. Untreated samples (white bars) correspond to non-biopretreated WS. Data are means of triplicates and standard deviations are provided. Asterisks on bars represent samples with significantly higher values than non-biopretreated WS (*) and *I. lacteus* basal cultures (ILC) (**). MWS = milled wheat straw.

C. subvermispora (Wan and Li, 2011) and Euc-1 (Dias et al., 2010). The efficiency of *I. lacteus* for biopretreating lignocellulosic material has already been demonstrated on other substrates. Zhong et al. (2011) described 50% yields after 15-d of SSF using corn stalks and Xu et al. (2010) obtained a 66.7% glucose yield from corn stover in 25-d treatments. Better glucose recoveries (82%) have also been reported by Du et al. (2011) with a 28-d biopretreatment of corn stalks with *I. lacteus*, after supplementing the enzymatic hydrolysis broth with by-products secreted by the same fungus.

3.3. Search for efficiency indicators of I. lacteus biopretreatment

3.3.1. Relationship between lignin degradation, digestibility, and sugar yields

A moderate positive correlation was found between lignin loss and digestibility of cellulose ($r = 0.728$) and hemicellulose ($r = 0.569$), but no such correlation was found between lignin loss and glucose ($r = 0.219$) or xylose ($r = 0.303$) yields (Fig. 2). These results confirm that lignin is the main barrier preventing efficient enzymatic hydrolysis; however, lignin degradation is not directly correlated with the final yields of fermentable sugars since better sugar availability can also lead to their use as a carbon source for fungal growth.

3.3.2. Relationship between extracellular ligninolytic enzymes and sugar yields

Although the search for ligninolytic activities revealed the absence of LiP and laccase activity under the assayed conditions, their activity was found in *I. lacteus* cultures growing on different substrates such as wheat straw and corn stalks (Dias et al., 2010; Du et al., 2011; Gupte et al., 2007). Conversely, peroxidase activity whether dependent on or independent of Mn(II) was considerable (Fig. 5) even though it was not found by Gupte et al. (2007) with the same fungus growing on wheat straw. According to the current data, the release of peroxidases seemed to play an important role during the first weeks of incubation. The levels of MnP were significantly higher, compared to ILC, only after 7 days of SSF on MWS, and after 21 days with either high moisture content or Mn(II) supplementation. On the other hand, MiP activity was, in general, notably superior when organic or inorganic compounds were added as supplements. As an exception, in 21-d cultures only the addition of Cu(II) and Fe(II) produced enhanced activities compared with ILC. Both minerals can induce the production of ligninolytic enzymes (Manubens et al., 2007).

No relationship was found between peroxidase activities and sugar yields, and no correlation ($r = 0.068$) was found between MnP activity and lignin loss, even so a moderate positive correlation ($r = 0.626$) was established between MiP and lignin loss

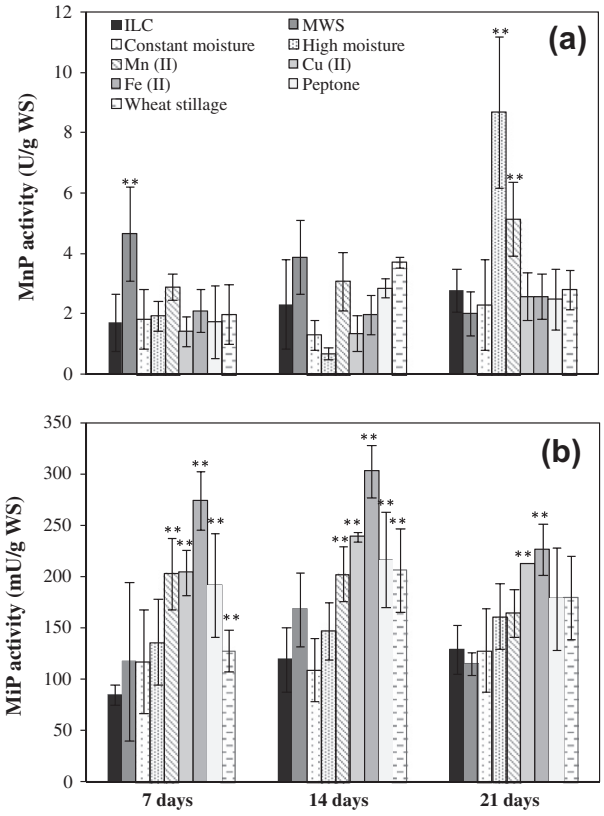

Fig. 5. (a) Mn(II)-oxidizing peroxidase (MnP) activity and (b) Mn(II) independent peroxidase (MiP) activity (U g^{-1} WS) detected in fungal cultures after 7, 14 and 21 days of SSF. Data are means of triplicates and standard deviations are provided. Asterisks on bars represent samples with significantly higher values than *I. lacteus* basal cultures (ILC) (**). WS = wheat straw; MWS = milled wheat straw.

(Fig. 2). This result suggests that MiP is probably more implicated in lignin degradation than MnP under the assayed conditions.

3.3.3. Relationship between fungal biomass, sugar yields, and lignin degradation

Ergosterol, an exclusive component of fungal cellular membranes (Pitt and Hocking, 2009), was selected as a fungal growth indicator. This sterol was analyzed in freeze-dried fungal mycelium to calculate its approximate percentage in the fungal cells (0.46% ± 0.06). With this value, *I. lacteus* biomass estimations were carried out in wheat straw cultures. Basal amounts of ergosterol were detected in untreated samples, probably because of the presence of endogenous fungi on wheat straw taken from the field, so *I. lacteus* biomass estimations were not done in these samples (Table S1).

ILC experienced an increase in fungal biomass throughout the incubation period, though this biomass did not grow significantly from 14 to 21 days reaching 29.2 ± 2.1 mg g^{-1} wheat straw at 21 days. Since the best sugar yields were obtained at 21-d of SSF, ergosterol was analyzed only at that time. The lowest fungal growth was found in media supplemented with Cu(II), Fe(II) and peptone (23.2 ± 2.4, 23.3 ± 2.3, and 26.2 ± 0.8 mg g^{-1} wheat straw, respectively) (Table S1). Furthermore, those three treatments also gave the lowest glucose yields, thus a moderate correlation (r = 0.726) was found between both variables but not with respect to xylose yield (r = 0.369) (Fig. 2).

Lignin degradation and fungal biomass were also correlated, and a tight negative correlation was found between both variables (r = −0.925) (Fig. 2). Cultures with Cu(II) or Fe(II) had the lowest fungal biomass content but the highest levels of lignin degradation, which could be attributed to an overproduction of free radicals and/or to increased enzyme-releasing activity of the mycelium. When peroxidase production was expressed per mg of fungal biomass (Table S1) to determine if the increase in enzyme secretion could be correlated with the extent of fungal growth as previously reported (Kachlishvili et al., 2006), for MiP, the most active mycelia were found in treatments with Cu(II) and Fe(II), while MnP production was not linked to fungal growth.

Few ligninolytic activities were detected in *I. lacteus* SSF cultures on wheat straw, suggesting that with this fungus, lignin degradation could be mostly driven by oxidation mechanisms where the enzymes secreted play an important role in its production, according to data previously reported (Gómez-Toribio et al., 2001). Consequently, future studies will focus on the search for and characterization of potential oxidative species that could be strongly correlated with the efficiency of the process, such as hydroxyl radicals or low-molecular-weight substances released by *I. lacteus*.

4. Conclusions

Irpex lacteus is capable of modifying wheat straw to produce enhanced sugar yields at 14 and 21 days of SSF. Results obtained in this work demonstrate the high complexity of the fungal wheat straw degradation process, since a single modification of culture conditions can produce noteworthy differences in its efficiency. Digestibility was significantly improved by particle size reduction and Mn(II) supplementation in the cultures, the latter yielding the highest glucose recoveries. To gain an insight into lignocellulose breakdown mechanisms and optimize approaches for sugar exploitation, a proteomic study of the *I. lacteus* enzyme system is currently in progress.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2012.11.089.

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