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Fungal pretreatment: An alternative in second-generation ethanol from wheat straw

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ABSTRACT

The potential of a fungal pretreatment combined with a mild alkali treatment to replace or complement current physico-chemical methods for ethanol production from wheat straw has been investigated. Changes in substrate composition, secretion of ligninolytic enzymes, enzymatic hydrolysis efficiency and ethanol yield after 7, 14 and 21 days of solid-state fermentation were evaluated. Most fungi degraded lignin with variable selectivity degrees, although only eight of them improved sugar recovery compared to untreated samples. Glucose yield after 21 days of pretreatment with *Poria subvermispora* and *Irpex lacteus* reached 69% and 66% of cellulose available in the wheat straw, respectively, with an ethanol yield of 62% in both cases. Conversions from glucose to ethanol reached around 90%, showing that no inhibitors were generated during this pretreatment. No close correlations were found between ligninolytic enzymes production and sugar yields.

1. Introduction

Lignocellulosic materials are the major component of biomass and represent the most abundant renewable energy resource available on earth (Lin and Tanaka, 2006). Among them, agricultural wastes are the most extended and the cheapest, especially wheat straw which is the most plentiful in Europe and the second one worldwide after rice straw (Kim and Dale, 2004). Moreover, wheat straw is a residue that does not compete with human food resources constituting an auspicious alternative to generate renewable biofuels.

Second-generation bioethanol production from wheat straw includes three main steps: (i) pretreatment, (ii) enzymatic hydrolysis of cellulose and hemicellulose, and (iii) ethanol fermentation (Talebnia et al., 2010). The aim of the pretreatment is the disruption of the lignocellulose structure, improving cellulose and hemicellulose accessibility. Nowadays, steam explosion, which requires high pressures and temperatures, is the most common and effective pretreatment for this purpose, although the severity of this process generates by-products that affect adversely subsequent steps (Alvira et al., 2010; Jurado et al., 2009). An alternative to avoid these problems is the use of biological pretreatments, which present additional advantages as being cheaper, safer, less energy-consuming and more environmentally friendly.

Biopretreatment is based on the capacity of some organisms of degrading lignin to gain access to cellulose and hemicellulose. Nevertheless, the literature shows a big controversial about this topic, since the largest lignin degradations not always correspond with the best sugar recoveries (Capelari and Tomás-Pejó, 1997; Shi et al., 2008). Biological degradation of lignocellulose is a complex process where many factors, as fungal strain, culture conditions, fungal enzymatic secretion and oxidative mechanisms, are implicated (Guillén et al., 2000; Wan and Li, 2010). Therefore, analysis of the whole process is relevant to understand the mechanisms of fungal degradation and to get the best fungi and optimum growth conditions for obtaining the maximum amount of fermentable sugars.

The pretreatment of wheat straw by using basidiomycetes to produce ethanol has been barely studied. Moreover, the rates of this type of pretreatment are still far from industrial purposes besides of presenting disadvantages, as long storage times or extended cellulose and hemicellulose consumptions (Galbe and Zacchi, 2007). Nevertheless, it could be used alone or combined with other pretreatments to result more effective. Alkali pretreatments are well known to improve sugar recovery because they cause lignin solubilisation (Kumar et al., 2009; Talebnia et al., 2010) but only a few investigations have complemented it with a biological pretreatment (Hatakka, 1983; Yu et al., 2010). In the present study, a fungal screening using 21 basidiomycetes has been carried out, combined with a very mild alkali washing, to select the best fungal strains to be included in the pretreatment step for second generation bioethanol production from wheat straw. The relationship among different variables, such as lignin degrada-

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tion, cellulose and hemicellulose digestibility, and ethanol production was analysed, and the role of the ligninolytic enzymes in the process is discussed.

2. Methods

2.1. Fungal strains and culture media

The strains of basidiomycetes used in the present study were obtained from different fungal collections: Centraalbureau voor Schimmelcultures (CBS; Baarn, The Netherlands), Instituto Jaime Ferrán de Microbiología (IJFM; Centro Investigaciones Biológicas, Madrid, Spain) and Colección Española de Cultivos Tipo (CECT; Burjassot, Valencia, Spain). Most of the fungi used in this study were white-rot fungi: *Bjerkandera* anamorph IJFM A757, *Bjerkandera adusta* CBS 595.78, *Coriolopsis rigida* CECT 20449, *Fomes fasciatus* IJFM A772, *Fomes fomentarius* IJFM A166, *Ganoderma australe* IJFM A130, *Irpex lacteus* IJFM A792, *Lentinus tigrinus* IJFM A790, *Panus tigrinus* (a synonym of *L. tigrinus*) IJFM A768, *Phanerochaete chrysosporium* CBS 481.73, *Phellinus robustus* IJFM A788 , *Phlebia radiata* CBS 184.83, *Phlebiopsis gigantea* CBS 935.7, *Pleurotus eryngii* CBS 613.91, *Pleurotus ostreatus* CBS 411.71, *Polyporus alveolaris* IJFM A794, *Poria subvermispora* (a synonym of *Ceriporiopsis subvermispora*) IJFM A718, *Pycnoporus coccineus* IJFM A780, *Stereum hirsutum* IJFM A793, and *Trametes versicolor* IJFM A136. In addition one brown-rot fungus was used, *Postia placenta* IJFM A781. Strains were maintained on 2% malt extract agar (w/v) and preserved at 4 °C.

Fungal strains were individually cultured at 28 °C for 7 days on MEA plates. Four agar plugs of about 1 cm^2 were cut from actively growing mycelium and inoculated into 250 mL Erlenmeyer flasks with 30 mL of growth medium (pH 5.6) and incubated at 28 °C, and 180 rpm for 7 days. The growth medium contained (L^{-1}): glucose, 40 g; $FeSO_4{\cdot}7H_2O$, 0.4 g; $(NH_4)_2SO_4$, 9 g; KH_2PO_4, 4 g; corn steep solids, 26.3 g; $CaCO_3$, 7 g; soybean oil, 2.8 mL. Each culture was aseptically homogenised (Omnimixer, Sorvall), and 2.5 mL were used to inoculate second generation cultures, which were incubated for 5 days as described above. These cultures will be used as inocula for solid state fermentation (SSF) experiments.

2.2. Pretreatment of wheat straw

2.2.1. Fungal screening

Wheat (*Triticum aestivum*) straw was harvested from Galicia fields (Spain) and chopped (<1 cm). Two grams of dry grinded wheat straw plus 6 mL of distilled water were autoclaved at 121 °C for 15 min into 100 mL Erlenmeyer flasks capped with hydrophobic cotton. These flasks were inoculated with 5-day-old mycelia (2 mL) and incubated at 28 °C. Triplicate flasks of each fungal culture were sampled after 7, 14 and 21 days. Samples were washed with distilled water (15 mL) at 28 °C and 180 rpm for 1 h, and filtered under vacuum to remove most of the water-soluble components, which were stored at 4 °C. The solid fractions from biopretreated wheat straw were dried in an aeration oven at 65 °C and weighted. This value was used to calculate weight loss of the samples. Non-inoculated samples were incubated and treated under the same conditions, being used as control.

2.2.2. Mild alkali treatment

Solid fractions (300 mg, dry weight) were subjected to a mild alkali treatment with a final concentration of 0.1% sodium hydroxide (5% w/v), at 50 °C and 165 rpm for 1 h. The alkali-treated material was filtered and washed, until neutrality, with distilled water at 50 °C. Total reducing sugars were measured (Somogyi, 1945) in the filtrates, and the solid residue was dried at 60 °C and weighted. The effect of the alkali treatment on subsequent enzymatic hydrolysis was investigated by comparison of different samples with and without alkali washing.

2.3. Enzymatic hydrolysis and sugar yield estimation

Solid residues or solid fractions, with or without alkali treatment respectively, were hydrolysed in duplicate at 5% (w/v) by enzyme complexes (Novozymes Bagsvaerd, 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. Tetracycline (200 μg mL^{-1}) was also added to avoid bacterial growth during enzymatic treatments. After hydrolysis, 0.5 mL of treated material were centrifuged at 5000 rpm for 5 min, and glucose and xylose content were measured in the supernatants. The "Glucose-TR" kit (Spinreact) was used to quantify glucose. Xylose content was calculated as the difference between total reducing sugars (Somogyi, 1945) and glucose. A set of samples was chosen to quantify glucose and xylose also by gas chromatography (GC) (Prieto et al., 2008), to compare both methodologies.

To probe enzymatic hydrolysis efficiency, cellulose (D_c) and hemicellulose (D_h) digestibilities were evaluated and expressed, according to Eq. (1), as the quotient between the percentage of glucose (G_r) and xylose (X_r) released from either the solid fraction or the solid residue from alkali washing and the theoretical maximum amount of glucose (G_s) and xylose (X_s) in the solid fraction, respectively.

$$D_c \text{ or } D_h\,(\%) = (g\,G_r \text{ or } X_r / g\,G_s \text{ or } X_s) \times 100 \quad (1)$$

To calculate sugar yields, glucose (G_i) and xylose (X_i) per gram of dry wheat straw, glucose (G_f) and xylose (X_f) remaining in pretreated wheat straw, and cellulose (D_g) and hemicellulose (D_h) digestibilities were taken into account as shown in Eq. (2). Thus, this yield considers both, the sugar digestibility (D_c or D_h) and the percentage of sugar loss during the biological pretreatment (G_f/G_i or X_f/X_i).

$$\text{Sugar yield}\,(\%) = [(G_f \times D_g) + (X_f \times D_h)]/(G_i + X_i) \quad (2)$$

Glucose and xylose recovery yields were separately estimated applying Equations (3) and (4), respectively.

$$\text{Glucose yield}\,(\%) = (G_f \times D_c)/G_i \quad (3)$$

$$\text{Xylose yield}\,(\%) = (X_f \times D_h)/X_i \quad (4)$$

2.4. Fermentation to ethanol

To evaluate the potential inhibitory effect of fungal pretreatment and alkali washing on yeast growth, solid fractions or solid residues, hydrolysed with cellulases and xylanases as previously indicated, were subsequently fermented with *Saccharomyces cerevisiae* (Fermentis LPA 3035). The yeast (0.5 g L^{-1} inoculum) was grown at 32 °C and 200 rpm for 24 h in 250 mL Erlenmeyer flasks containing 50 mL of a liquid medium. The medium was composed of (L^{-1}) glucose (20 g), yeast extract (3 g), peptone (5 g), and tetracycline (200 mg). Seven millilitres of hydrolysed wheat straw samples were inoculated with 350 μL of a yeast suspension (OD 625 nm = 2) in 10 mL glass tubes. Tubes were sealed with rubber plugs and incubated at 32 °C and 200 rpm for 72 h. Then, tubes were centrifuged for 2 min at 7000 rpm and 5 mL of the supernatant were extracted with ethanol-free chloroform (0.5 mL) to determine the ethanol content in the organic phase by gas chromatography (GC). Methanol (1%) was added in the samples before chloroform extraction, as internal standard. GC analyses were performed on an Agilent 7890A instrument equipped with a flame

ionisation detector (FID), using a HP5-MS capillary column (30 m × 0.25 mm, 0.25 μm film thickness) and helium (25 psi) as the carrier gas. Separation was carried out isothermically at 28 °C, and injector and detector were maintained at 100 °C. Peaks were identified on the basis of sample coincidence with retention times of commercial standards, and quantified using peak areas and the corresponding response factors. All the experiments were carried out by triplicate. Finally, the ethanol conversion yield was calculated as shown in Eq. (5) taking into account the glucose recovery after enzymatic hydrolysis. The value 0.511 corresponds to the conversion factor from glucose to ethanol (Maiorella et al., 1981).

$$\text{Glucose conversion } (\%) = \frac{\text{gL}^{-1} \text{ ethanol produced in fermentation broth}}{\text{gL}^{-1} \text{ initial glucose in fermentation broth} \times 0.511} \times 100 \quad (5)$$

2.5. Substrate characterisation and analysis methods

Weight loss was calculated as the percentage of total solids lost after each biopretreatment. Klason lignin content and polysaccharide composition of untreated and biopretreated wheat straw were determined on hydrolysates according to standard Tappi methods (Tappi, 1974, 1975). Glucose and xylose were also measured as described in Section 2.3. The content of cellulose was calculated from glucose while hemicellulose was calculated from xylose, using an anhydro correction of 0.90 and 0.88 for both sugars, respectively. Total reducing sugars in the water-soluble extracts were determined using the Somogyi method (Somogyi, 1945).

2.6. Estimation of ligninolytic activities

Enzymatic activities were evaluated in water-soluble extracts of biopretreated wheat straw (Section 2.2.1). Laccase activity was assayed using 5 mM 2,6-dimethoxyphenol (DMP) in 100 mM sodium citrate buffer (pH 5.0; $\varepsilon_{469} = 27{,}500\ M^{-1}\ cm^{-1}$, referred to DMP concentration). Mn^{2+}-oxidising peroxidase was estimated by measuring the formation of Mn^{+3}-tartrate complex ($\varepsilon_{238} = 6500\ M^{-1}\ cm^{-1}$) during the oxidation of 0.1 mM $MnSO_4$ in 100 mM sodium tartrate buffer (pH 5.0) in the presence of 0.1 mM H_2O_2. Lignin peroxidase was assayed by veratraldehyde formation from 2 mM veratryl alcohol (3,4-dimethoxybenzyl alcohol) in 100 mM sodium tartrate buffer (pH 3), in the presence of 0.4 mM H_2O_2. International enzymatic units (μmoles per minute) were used.

3. Results and discussion

3.1. Fungal pretreatment of wheat straw

3.1.1. Cell wall components degradation

The objective of this study was to select fungal species, in a wide screening, to produce second generation bioethanol from biopretreated wheat straw. The selected strain should be the one giving the highest amount of fermentable sugars from wheat straw in the shortest period of time. A screening of fungi was carried out to evaluate lignin and polysaccharides degradation from wheat straw after 7, 14 and 21 days of SSF. Most fungi colonised the substrate appropriately, except *F. fasciatus* which presented a poor evolution.

Polysaccharide content was first estimated from GC analysis. Untreated wheat straw used as control (0 and 21 days of incubation), was composed of 36.9% cellulose and 23% hemicellulose (18% xylan, 3.4% arabinan, 1.1% mannan, and 0.5% galactan). Cellulose and hemicellulose content were also evaluated by colorimetric methods. Glucose, assayed by the "Glucose-TR" kit (Spinreact), and xylose, determined by difference between total sugars and glucose, gave values not significantly different to those detected by GC. In the case of xylose this result could be explained because it is the major hemicellulose component in wheat straw (as shown by GC). In addition, this lignocellulosic material contained 24% lignin (22.8% acid-insoluble lignin and 1.2% acid-soluble lignin).

Weight loss of the cultures gives an estimation of the extent of substrate degradation. The greatest weight losses, after 21 days of SSF, were caused by *B. adusta*, *F. fomentarius* and *P. coccineus* (up to 35%), and the lowest by *P. eryngii*, *P. gigantea* and *P. placenta* (down to 6%). Composition of wheat straw after fungal treatment was analysed (Table 1) and different degradation patterns were appreciated among the studied basidiomycetes. Some fungi, as *P. chrysosporium* and *P. gigantea*, as well as the brown-rot *P. placenta*, were not able to degrade lignin under the assayed culture conditions, showing a polysaccharidic preferential degradation. Other fungi, as *P. tigrinus* and *P. radiata*, degraded lignin and sugars simultaneously. Finally, basidiomycetes as *P. eryngii* and *P. robustus* were able to remove lignin selectively and faster than the carbohydrate components in wheat straw.

On the other hand, sugar degradation can be balanced or preferential from cellulose or hemicellulose. *B. adusta* degraded both polymers equitably (54% and 43% respectively). *S. hirsutum* only altered cellulose (43%) and *P. coccineus* degraded almost all hemicellulose (98%) but less cellulose (31%). These data give information about the amount of glucose and xylose (from cellulose and hemicellulose, respectively) available for alcohol fermentation, since largest degradations should imply a performance decrease.

3.1.2. Water-soluble fraction analysis

To evaluate the total sugar recovery, water-soluble sugars from the hydrosoluble fraction were also analysed, since they could also be potentially fermented. The greatest recovery reached only 6% after 21 days of pretreatment with *P. radiata*. Significantly less water-soluble sugars were quantified at 7 or 14 days with this fungus, and even at 21 days with the remaining fungi screened (data not shown). These results are in agreement with previous studies which showed that the two first weeks of incubation correspond to an early delignification stage with low water-soluble sugars content because fungi consume monosaccharides and disaccharides to grow (Valmaseda et al., 1991). Because of its low amount of sugars, the water-soluble fraction was not enzymatically hydrolysed for further ethanol production by yeasts. Consequently, this fraction was not taken into account in the final process yield.

3.2. Enzymatic hydrolysis

3.2.1. Digestibility

Digestibility represents the yield of conversion of the raw material into fermentable sugars. To increase this value in biopretreated wheat straw, samples are subjected to a very mild alkali treatment before enzymatic hydrolysis with cellulases and xylanases (Kumar et al., 2009; Yu et al., 2010, 2009). Preliminary studies in our laboratory showed that alkali treatment with only 0.1% NaOH at 50 °C during 1 h does not affect xylose recovery, probably because hemicellulose does not form packed crystalline structures like cellulose, becoming a substrate more accessible to fungi and enzymatic hydrolysis (Xu et al., 2009). However, this step is crucial to improve glucose release from cellulose, since digestibility at 21 days increased more than twice in several biopretreated samples (data not shown). Recently the use of a combined biological and mild chemical pretreatment of cornstalks has been reported (Yu et al., 2010). They obtained values of glucan digestibility comparable to those found in the present study, using similar temperatures and

Table 1
Degradation of wheat straw components (% of the initial content) produced by 21 basidiomycetes after incubation periods of 7, 14, and 21 days. Data are means of triplicates (±SD). CEL, cellulose; HEM, hemicellulose; LIG, lignin. *Bjerkandera** = *Bjerkandera* anamorph.

Fungi	Loss (%)								
	7 days			14 days			21 days		
	CEL	HEM	LIG	CEL	HEM	LIG	CEL	HEM	LIG
*Bjerkandera**	14 ± 0	7 ± 1	4 ± 3	16 ± 0	14 ± 3	1 ± 0	21 ± 1	12 ± 1	6 ± 0
B. adusta	27 ± 0	0 ± 0	9 ± 0	42 ± 2	16 ± 2	28 ± 1	54 ± 6	43 ± 5	37 ± 0
C. rigida	18 ± 0	28 ± 6	15 ± 0	22 ± 1	52 ± 10	24 ± 1	36 ± 2	42 ± 12	34 ± 1
F. fasciatus	0 ± 0	0 ± 1	0 ± 0	0 ± 0	0 ± 3	0 ± 0	0 ± 0	0 ± 1	0 ± 0
F. fomentarius	17 ± 1	2 ± 0	0 ± 0	30 ± 1	28 ± 4	17 ± 0	45 ± 1	51 ± 27	35 ± 1
G. australe	4 ± 0	13 ± 0	0 ± 0	4 ± 0	16 ± 1	9 ± 0	15 ± 0	23 ± 3	25 ± 1
I. lacteus	9 ± 0	13 ± 7	11 ± 0	17 ± 1	13 ± 4	27 ± 1	17 ± 0	26 ± 7	34 ± 2
L. tigrinus	24 ± 1	46 ± 10	0 ± 0	28 ± 1	68 ± 12	15 ± 1	40 ± 1	58 ± 9	23 ± 1
P. tigrinus	12 ± 0	24 ± 7	17 ± 1	20 ± 1	41 ± 7	32 ± 1	24 ± 1	60 ± 26	47 ± 4
P. chrysosporium	23 ± 0	36 ± 8	0 ± 0	31 ± 2	22 ± 2	0 ± 0	35 ± 0	70 ± 24	0 ± 0
P. robustus	4 ± 0	0 ± 1	0 ± 0	6 ± 0	0 ± 0	21 ± 1	8 ± 1	3 ± 0	25 ± 2
P. radiata	13 ± 1	36 ± 11	8 ± 0	20 ± 2	40 ± 10	29 ± 1	24 ± 3	41 ± 5	40 ± 2
P. gigantea	8 ± 0	0 ± 0	0 ± 0	9 ± 1	0 ± 0	0 ± 0	7 ± 0	9 ± 1	0 ± 0
P. eryngii	0 ± 0	0 ± 0	2 ± 0	0 ± 0	4 ± 0	14 ± 0	0 ± 0	8 ± 1	17 ± 1
P. ostreatus	10 ± 1	14 ± 1	2 ± 0	14 ± 1	38 ± 9	18 ± 0	22 ± 1	52 ± 13	27 ± 1
P. alveolaris	14 ± 1	8 ± 1	18 ± 1	18 ± 0	28 ± 9	34 ± 1	28 ± 2	42 ± 8	43 ± 2
P. subvermispora	1 ± 0	33 ± 10	8 ± 0	4 ± 0	35 ± 6	25 ± 1	13 ± 0	36 ± 6	30 ± 1
P. placenta	3 ± 0	11 ± 2	0 ± 0	0 ± 0	15 ± 3	0 ± 0	2 ± 0	9 ± 3	1 ± 0
P. coccineus	12 ± 0	74 ± 43	11 ± 0	26 ± 2	77 ± 20	26 ± 1	31 ± 1	98 ± 1	36 ± 1
S. hirsutum	24 ± 0	0 ± 1	15 ± 1	37 ± 4	1 ± 0	30 ± 1	43 ± 2	2 ± 0	37 ± 2
T. versicolor	12 ± 0	5 ± 1	24 ± 1	18 ± 1	23 ± 3	33 ± 0	23 ± 1	21 ± 6	46 ± 1

incubation times but with a NaOH concentration tenfold higher. There are several advantages of using low amounts of alkali for the chemical treatment. First of all, the effect of the biological pretreatment can be clearly observed, since it is not masked as a result of more aggressive alkali pretreatments. In addition, the process is cheaper and the generation of inhibitors for downstream steps of the process is diminished or even avoided.

Cellulose and hemicellulose conversion to fermentable sugars from biopretreated wheat straw is depicted in Fig. 1. No differences were found between controls analysed at the beginning (0 days) and at the end of the incubation time (21 days). In both cases, the conversion of cell wall polysaccharides to glucose and xylose was around 36% and 35%, respectively. Only eight of the fungal strains studied increased digestibilities at 14 and 21 days of biopretreatment with respect to the controls, and only one of them, *P. tigrinus*, was able to improve them after 7 days of SSF. The greatest values for glucose and xylose recovery were 82% and 78% in samples pretreated for 21 days with *I. lacteus* and *P. tigrinus*, respectively. Our results show higher increases in wheat straw digestibility in shorter incubation times than those reported in previous studies (Capelari and Tomás-Pejó, 1997; Dias et al., 2010; Valmaseda et al., 1991).

Lignin polymers are the main obstacle to the efficient utilisation of lignocellulosic materials. Consequently, a preferential delignifi-

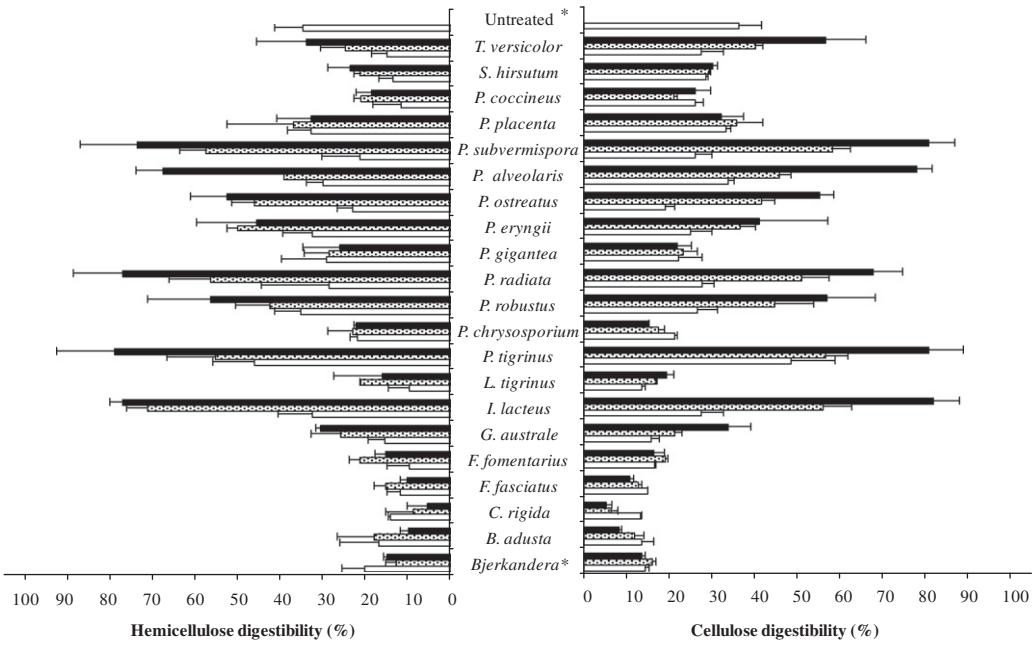

Fig. 1. Cellulose and hemicellulose digestibility (%) from pretreated wheat straw. White, dotted and black bars correspond to 7, 14, and 21 day cultures respectively. Control corresponds to non-inoculated wheat straw. Data are means of triplicates. *Bjerkandera** = *Bjerkandera* anamorph.

cation would improve the process performance because it would facilitate the access of hydrolytic enzymes to polysaccharides (Camarero et al., 1994; Kuhar et al., 2008; Valmaseda et al., 1991) maintaining at the same time a good level of fermentable sugars, which would be only slightly consumed for fungal growth. With the aim to correlate both variables, lignin degradation and digestibility were compared. The two fungi that generated the highest lignin degradation, *T. versicolor* (46%) and *P. tigrinus* (47%), gave pretreated wheat straw with significant differences in cellulose and hemicelluloses digestibilities. After treatment with *T. versicolor*, cellulose digestibility was 25% less than after pretreating with *P. tigrinus*. Otherwise, hemicellulose digestibility was not improved by *T. versicolor* treatment as compared with untreated wheat straw while, after growing of *P. tigrinus*, a 78% of conversion was reached. In contrast, fungi as *P. eryngii* and *P. robustus*, which did not produce high lignin losses, were able to raise cellulose and hemicellulose digestibility of the substrate. According to these data, although lignin attack is essential to the efficiency of the enzymatic hydrolysis of cell wall polysaccharides, the highest lignin degradation is not always positively correlated with the highest levels of cellulose and hemicellulose digestibility. These results agree with previous reports (Capelari and Tomás-Pejó, 1997; Müller and Trösch, 1986), which remark that the level of delignification cannot be considered as the only parameter to assess if a microorganism is a valid candidate for biological pretreatment.

3.2.2. Fermentable sugar yields

Digestibility values and carbohydrate losses during biopretreatment were essential to quantify the amount of potentially fermentable sugars. Sugars were not found in the liquid fraction after alkali washing what indicates that only lignin was removed in this step.

The treatments which improved the recovery of fermentable sugars, compared to untreated wheat straw, are presented in Fig. 2. Glucose yields increased in most cases after 21 days of fungal treatment, especially with *P. subvermispora* (69%) and *I. lacteus* (66%). In addition, only these two fungi led to a significant increase in glucose yield in samples pretreated for 14 days. During the first 2 weeks of incubation, fungi consume a huge amount of glucose and energy for their own growth. After this time, they continue on wheat straw degradation consuming less sugars, which increases the final sugar recovery (Valmaseda et al., 1991). In the present work we have obtained the highest yields reported so far from wheat straw combining a 21 days-biological pretreatment with a very mild chemical reagent. Recent studies described similar glucose recoveries by using corn stover treated with *C. subvermispora* during 35 days (Wan and Li, 2010) and rice straw treated with *P. chrysosporium* for 15 days (Bak et al., 2009) although in this case the raw material was autoclaved twice, before and after biopretreatment.

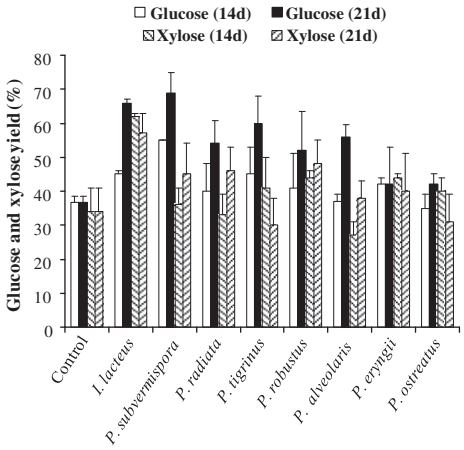

Fig. 2. Glucose and xylose yield (%) from 14 and 21 days pretreated wheat straw. Fungi are listed in order of the best sugar yields (left to right). Control corresponds to non-inoculated wheat straw. Data are means of triplicates.

Concerning xylose yields, the differences between untreated and pretreated samples were not very significant, excluding *I. lacteus* pretreatment which reached 62% and 47% at 14 and 21 days, respectively. This finding set out that, among the studied basidiomycetes, this fungus would be the best candidate to recover xylose from biological pretreatment. At the present time, xylose cannot be fermented at industrial scale; however it is important to know the extent of hemicellulose conversion to xylose to be taken into account to improve process yields in the future.

Considering the total sugar yield (data not shown), the best results were obtained after 21 days of incubation with *I. lacteus* (62%) and *P. subvermispora* (61%). On the contrary, treatment with *P. ostreatus* did not increase significantly this yield and was not included in further ethanol fermentation experiments.

3.2.3. Relationship between fungal enzymes and sugar yield

Differences in wheat straw degradation have been related to variations in the pattern and levels of ligninolytic enzymes (Camarero et al., 1996; Manubens et al., 2007; Pedersen and Meyer, 2009). Consequently, those enzymes could be used as markers of the process yield. In this study, Mn^{2+}-oxidising peroxidases, laccase and LiP activities, as models of enzymes closely related to lignin degradation, were analysed in the water-soluble extracts from the 21 fungal strains during the 3 weeks of incubation (Fig. 3). LiP was not detected in any case.

The largest Mn^{2+}-oxidising peroxidase activities (per gram of dry wheat straw) were detected in the seven cultures that gave an improved sugar recovery, with the maximum values in 14-days cultures of *P. radiata* (6.9 U g^{-1}) and *P. robustus* (6.7 U g^{-1}). In any case, these high activities were not correlated with the best sugar recoveries. On the other hand, the highest laccase activities were found in *P. robustus* (3.3 U g^{-1}) and *P. eryngii* (2.4 U g^{-1}) at 7 and 14 days, respectively. Both fungi displayed a preferential degradation of lignin, which could be related to the high laccase secretion detected in these fungi. Fungi as *I. lacteus* and *P. subvermispora*, which showed low laccase activity (<0.25 U g^{-1}) and not very high Mn^{2+}-oxidising peroxidase activity (<3.6 U g^{-1}), gave the best sugar yields after wheat straw biopretreatment. These species showed a simultaneous degradation of all lignocellulosic components. Alternatively, other fungi which produced high lignin degradation presented very low ligninolytic activities (as *B. adusta* and *C. rigida*).

These results corroborate again that it is not easy to find a direct correlation among enzyme production, lignin degradation, and sugar yield in biopretreated wheat straw. As previously stated, lignin degradation is an oxidative and rather nonspecific process where extracellular ligninolytic enzymes participate, but also low molecular-weight extracellular oxidant compounds (e.g. Mn^{3+} and oxygen free radicals), which can be generated during the process, having a very important role (Guillén et al., 2000; Hammel et al., 2002).

3.3. Ethanol production

Evaluation of ethanol production is necessary to quantify the process final performance. At industrial level, only glucose is being fermented with high ethanol production yields while xylose fermentation, which is also essential for the economical success of lignocellulosic ethanol, continues being investigated to raise the low yields obtained so far (Gírio et al., 2010; Lee, 1997).

Glucose fermentations by *S. cerevisiae* were carried out on the seven enzymatic hydrolysates which gave significantly improved

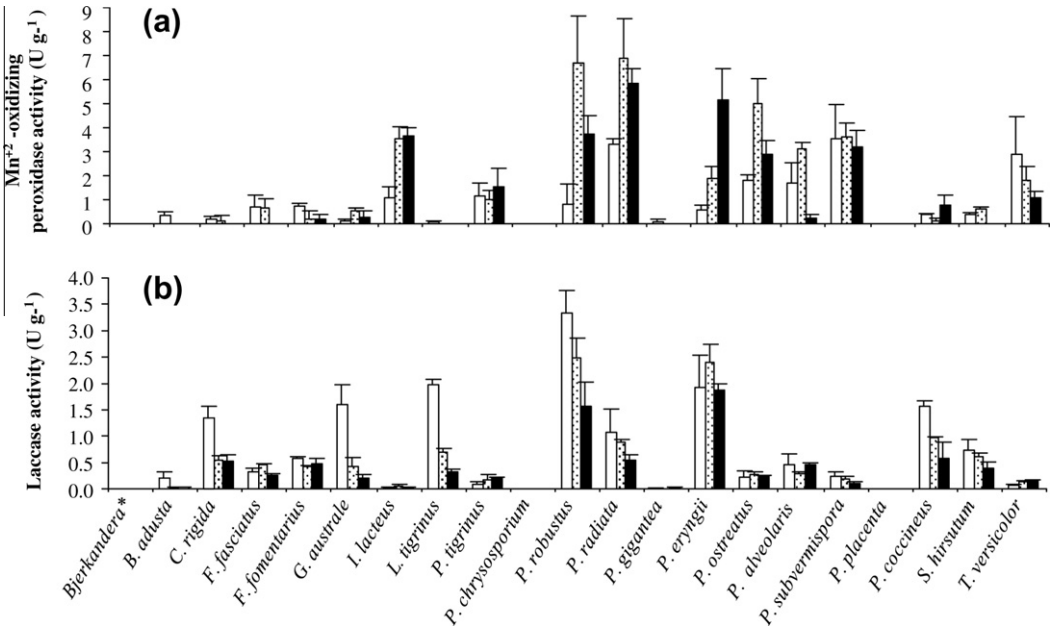

Fig. 3. Ligninolytic enzymes secreted by fungi during wheat straw biopretreatment: (a) Mn^{2+}-oxidising peroxidase and (b) laccase. White, dotted and black bars correspond to 7, 14, and 21 day cultures, respectively. Data are means of triplicates. *Bjerkandera** = *Bjerkandera* anamorph.

sugar recoveries as compared to control samples. Most conversion yields from available glucose to ethanol were superior to 90% except those coming from pretreatments with *P. tigrinus*, *P. eryngii* and *P. robustus*, which showed conversions of 84%, 81% and 79%, respectively. These results indicate that the fungal plus alkali washing pretreatment of wheat straw does not generate significant inhibitors of yeast growth. Based on the dry weight of wheat straw (1 g), the glucose availability (0.41 g per gram of dry wheat straw) and the stoichiometry of the reaction (1 glucose → 2 ethanol + 2 CO_2), and estimating that 5% of glucose is used for yeast metabolism, the theoretical maximum ethanol production is approximately 0.2 g per gram of dry wheat straw (Eq. (5)). In this study, after checking total glucose consumption for yeast growth, the largest ethanol production found, which corresponds to the highest process yield, was around 62% of the theoretical maximum in samples pretreated with *I. lacteus* and *P. subvermispora* for 21 days (Table 2). These results were slightly higher than those reported for 35 day-pretreated corn stover with *P. subvermispora* (Wan and Li, 2010) and similar to those obtained from rice straw, autoclaved twice and pretreated for 15 days with *P. chrysosporium* (62.7%) (Bak et al., 2009). In contrast, much lower ethanol production has been obtained from 14 days pretreated cotton stalks with *P. chrysosporium* (13%) (Shi et al., 2009).

The complete process from wheat straw to ethanol with the fungi which improved sugar recoveries, as compared to biologically untreated wheat straw is summarised in Table 2. It has been previously stated that to increase the final sugar yields it would be desirable to have a low consumption of sugars during biopretreatment. However, it can be highlighted that this is not the most important variable. It can be observed that although *P. eryngii* and *P. robustus* (fungi which degraded selectively the lignin) consumed less glucose for their growth than the other fungi, enzymatic hydrolysis after biopretreatments released the lowest amounts of glucose. On the other hand *P. alveolaris,* which consumed the highest amount of glucose from wheat straw, gave a glucose recovery after enzymatic hydrolysis slightly higher than those found in the above mentioned species. Finallly, *I. lacteus* and *P. subvermispora,* showed intermediate levels of glucose consumption but gave the best glucose recoveries after enzymatic hydrolysis and also the best yields of ethanol production. Then, they can be considered as the best species to be potentially used for wheat straw biopretreatment. Since conversions from glucose to ethanol were high in all cases, it can be guessed that the main differences in the whole process should arise from biopretreatment. Lignocellulose degradation mechanisms are very difficult to predict because of their complexity and variety, and influence

Table 2
Monitoring of glucose content (GLC) and ethanol production per gram of dry wheat straw (g WS). Wheat straw samples were biopretreated (B) during 21 days. A mild alkali washing (AW) was done after biopretreatment. Data are means of triplicates (±SD). EH = enzymatic hydrolysis.

Process step	Initial WS	Pretreatment (B + AW)	EH	GLC fermentation	Process yield (%)
	GLC (mg/g WS)	GLC (mg/g WS)	GLC (mg/g WS)	Ethanol (mg/g WS)	
Theoretical maximum	410	410	410	199	100
Control[a]	410 ± 9	410 ± 9	133 ± 7	68 ± 2	35
I. lacteus	410 ± 9	340 ± 2	254 ± 8	123 ± 5	62
P. subvermispora	410 ± 9	357 ± 1	260 ± 14	122 ± 8	62
P. radiata	410 ± 9	311 ± 13	202 ± 13	97 ± 9	49
P. tigrinus	410 ± 9	311 ± 3	227 ± 18	97 ± 4	49
P. alveolaris	410 ± 9	295 ± 10	207 ± 7	94 ± 8	47
P. robustus	410 ± 9	377 ± 5	193 ± 21	78 ± 0	39
P. eryngii	410 ± 9	410 ± 0	151 ± 22	62 ± 9	31

[a] Control is a biologically untreated sample, only subjected to alkali washing.

the subsequent enzymatic hydrolysis step. Then, a complete study of the process is required for each fungal treatment, in order to analyse the efficiency of the biological pretreatment on ethanol production.

Chen et al. (2007) reported on a chemical pretreatment of wheat straw using acid and alkaline reagents, which allowed the recovery of more glucose (>10%) but with an ethanol production only 3% higher than the maximum reached in this study. Probably this is because, as stated above, biopretreatment does not generate toxic by-products affecting yeast growth, while steam explosion does (Alvira et al., 2010). Our results are still far from the high yields obtained using combined steam explosion and alkaline peroxide pretreatments (Chen et al., 2008), but suggest that the biological pretreatment with *I. lacteus* or *P. subvermispora*, complemented with a very mild alkali washing, could be an alternative to replace certain current chemical pretreatments without generating inhibitors of the fermentation step.

4. Conclusions

Our data showed that very few fungi are suitable to increase sugar recoveries from wheat straw. The combination of a biological pretreatment by *I. lacteus* or *P. subvermispora* with a mild alkali pretreatment did not produce inhibitors for downstream processes, improving significantly ethanol production. These results turn both methods into possible and environmentally friendly alternatives in the production of second-generation ethanol. At the present time, pretreatments of wheat straw with the selected fungi are being carried out to scale up the process and check its viability at industrial level.

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