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Fungal pretreatment of agricultural residues for bioethanol production

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ABSTRACT

The biological pretreatment of lignocellulosic biomass for the production of ethanol is an environmentally friendly process that needs to be evaluated with different feedstocks in order to avoid dependence on a single feedstock. In this study, four agricultural residues (corn stover, barley straw, corncob and wheat straw), selected in terms of their composition and geographic availability, were pretreated using the white-rot fungus *Irpex lacteus*. After the fungal pretreatment, the biggest reduction in lignin content (45.8 ± 3.5%), lowest sugars consumption (11.5 ± 1.4%) and highest lignin selectivity removal (2.1 ± 0.15) were achieved with corn stover. Moreover, total holocellulose digestibility was significantly increased after the biological pretreatment with all the substrates (37–103%), excepting corncob. In this study, fungal pretreatment was successfully applied to three of the most common agricultural residues available in Europe for producing ethanol, demonstrating that it is capable of handling feedstocks of variable origin.

1. Introduction

In the European Union, agricultural residues are abundant and cheap lignocellulosic resources that can be used to obtain valuable products such as bioethanol. This raw material has the advantage of not being competitive in food markets, like other substrates derived from plants such as cereals or sugarcane, used as well in the bioethanol industry. Nevertheless, the challenge to obtain the sugar fractions from these substrates, due to its complex and heterogeneous structure, is the main bottleneck of the second generation bioethanol production.

Several pretreatments based on physical, chemical or physicochemical mechanisms have been extensively studied (Alvira et al., 2010). They are frequently based on the use of chemicals, extreme conditions of temperature or pressure and special equipment to deal with these extreme conditions. The sugar yield reported with these treatments is used to be very high; nevertheless inhibitory compounds are also produced in different amounts depending on the severity or the chemicals used (Jönsson et al., 2013). In this context, the biological pretreatment appears as an environmental friendly alternative. It is based on the special abilities of a small group of filamentous fungi belonging to the phylum basidiomycota, known as white-rot fungi (WRF), which have evolved the capability of degrading lignin, the most recalcitrant component of plant cell wall (Hatakka, 1983), by using a set of extracellular ligninolytic enzymes. With the partial removal of lignin, an easier degradability of the substrate is achieved, without producing inhibitors as the commonly found with the other pretreatments (Salvachúa et al., 2011). In particular, the white-rot basidiomycete *Irpex lacteus* presents a great biodegradation capability (Novotný et al., 2009; Salvachúa et al., 2011). The advantages of the biological pretreatment include low energy requirement and mild conditions. However, there is an inevitable dependence between lignin removal and sugars consumption, since the fungal strategy consists of degrading the lignin fraction in order to reach easily the cellulose and hemicellulose. The other main disadvantage, compared to physicochemical pretreatmets, is the prolonged time needed to reach similar digestibility improvements, which can be as long as 4–8 weeks (Sarkar et al., 2012). This time can be reduced to 2–3 weeks by combining the biological treatment with an alkaline wash at soft conditions and optimizing operational conditions (Salvachúa et al., 2011; López-Abelairas et al., 2013a).

In order to be considered a feasible alternative, the biological pretreatment should be evaluated using different feedstocks to demonstrate its flexibility. There are several previous research works that studied the effect of using different lignocellulosic raw materials on the performance of chemical and/or physicochemical pretreatments (Martin et al., 2007; Njoku et al., 2012; Srivastava et al., 2014; Wang et al., 2014), but only one previous work

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(Wan and Li, 2011) have studied the effect of using a fungal biological pretreatment with another WRF (*Ceriporiopsis subvermispora*) on different types of feedstocks.

In the present study, the biological pretreatment of four lignocellulosic agricultural residues (corn stover, barley straw, corncob and wheat straw) was carried out. These agricultural residues were chosen according to their composition and their availability in Europe. Despite the fact that the main composition of the raw materials is basically the same, a different fungal behavior during the growth on the different plant species could be expected. The efficiency of the fungal process was studied in detail in order to clarify if the fungal performance presents any dependence with the substrate origin.

2. Materials and methods

2.1. Microorganisms and inocula preparation

The basidiomycete *I. lacteus* (Fr. 238 617/93) was obtained from the culture collection of the Chemical Engineering Department of the University of Santiago de Compostela (Spain). Mycelium production in static cultures and fungal inoculum preparation was carried out as described by Salvachúa et al. (2011).

The microorganism used for fermenting the lignocellulosic hydrolysates was the yeast *Pachysolen tannophilus* (CETC 1426). The yeast was maintained in YPD medium plates, composed of (per L): peptone, 10 g; yeast extract, 20 g; glucose 10 g and agar 15 g. For inoculum preparation, yeast from a fresh plate, was transferred to 100 mL of liquid media composed of (per L): malt extract, 20 g; glucose, 20 g and peptone 1 g. Inoculum was incubated in an orbital shaker at 150 rpm and 32.5 °C for 16 h or when an optical density of approx. 1.5 was reached.

2.2. Fungal pretreatment

Lignocellulosic residues procured from the local market (Santiago de Compostela, Spain) were used as the lignocellulosic feedstock source for ethanol production after fungal pretreatment. The moisture content of the raw biomasses was in the range of 7.3–8.5%, these values were taking into account for the composition calculations in dry basis. The biological pretreatment parameters and conditions were optimized in a previous work (López-Abelairas et al., 2013a). In brief, 6 g of blended (approx. 0.5 cm of particle size) lignocellulosic material were mixed with 20 mL of tap water in 250 mL Erlenmeyer flasks and sterilized by autoclave at 121 °C during 20 min. After sterilization, 3 mL of fungal inoculum (as pellets) were added to each flask and maintained at 30 °C during 21 d. The biologically pretreated biomass, with its liquid fraction, was freeze-dried. Total weight (TW) loss was calculated as the percentage of total solids lost after pretreatment and part of the dried solid was used for digestibility and composition measures. All the experiments were carried out in triplicate, data in tables and figures are presented with the mean and its standard deviation.

2.3. Ethanol production

After the fungal pretreatment, the different substrates were subjected to an alkali recycling treatment as described by García-Torreiro et al. (2016). In this protocol, the solid phases accumulated during up to three successive alkali washes with NaOH (0.36 g NaOH g^{-1} substrate) were mixed with the remaining liquid phase and citrate buffer 0.1 M. This buffer was added to adjust the solids percentage to 10% (w/v). Then, H_2SO_4 was used to adjust the pH between 4.8–5, and 200 μg mL^{-1} of tetracycline was finally added to avoid contamination.

Simultaneous saccharification and fermentation (SSF) configuration, as previously described by López-Abelairas et al. (2013b), was chosen to convert the resulting sugars into ethanol. The SSF configuration consists on a short enzymatic hydrolysis followed by the fermentation of the resulting sugars while the hydrolysing enzymes are still acting. The enzymatic doses used were previously optimized for biopretreated biomass subjected to alkali recycling treatment (García-Torreiro et al., 2016). Cellulase and beta-glucosidase were provided by the enzyme mixtures Cellic CTec2 and NS50010, respectively, while beta-xylosidase and xylanase by Cellic Htec2 (Novozymes). The cocktail doses applied were 96, 18 and 5 μL g^{-1} raw substrate of Cellic CTec2, Cellic Htec2 and NS50010, respectively. The hydrolysis was carried out in citrate buffer 0.1 M (pH 4.8) with a solid load of 10% (w/v). After 24 h of incubation at 150 rpm and 50 °C, the yeast *P. tannophilus* was inoculated using a ratio of 10% v/v of hydrolyzate. The fermentation and simultaneous saccharification was carried out at 37 °C and 150 rpm. Samples were taken periodically during the fermentation. The concentration of total reducing sugars, glucose and ethanol was determined in these samples.

2.4. Analytical protocols

2.4.1. Composition determination

An adapted protocol from NREL (National Renewable Energy Laboratory, Golden, USA) was used for the determination of the biomass composition in glucan, xylan and lignin. Acid hydrolysis allows lignin separation from sugar fraction and it can be determined by weight. The concentration of xylan and glucan, were calculated from the concentration of the corresponding monomeric sugars using an anhydro correction of 0.88 and 0.90 for C5 and C6 sugars, respectively.

2.4.2. Glucan and xylan digestibilities

Glucan (D_g) and xylan (D_x) digestibilities were evaluated and expressed, according to Eqs. (1) and (2), respectively.

$$D_g(\%) = \frac{G_r(g)}{G_s(g)} \times 100 \quad (1)$$

$$D_x(\%) = \frac{X_r(g)}{X_s(g)} \times 100 \quad (2)$$

where G_r and X_r are the glucose and xylose released from the biomass after pretreatment (including the enzymatic hydrolysis step), repectively and the G_s and X_s are the theoretical maximum amount of glucose and xylose available in the raw material, respectively. Total digestibility was calculated using the total amount of reducing sugars, instead of glucose or xylose. This parameter gives an idea of how available the fermentable sugars are.

2.4.3. Determination of substrate and product concentrations

Glucose content was measured using HPLC (Sluiter et al., 2008) and an enzymatic colorimetric method (GOD-PAP/Trinder, Spinreact, GI, Spain). No significant differences were found between HPLC and enzymatic method, for that reason the latter was used as routine method for glucose determination. Total reducing sugars (TRS) were determined by the dinitrosalicylic acid (DNS) method (Miller, 1959). The concentration of ethanol was determined using an HPLC system (Hewlett Packard 1100, USA) with an IR 1047 detector and an Aminex HPX-87H column. H_2SO_4 (5 mM) was used as the mobile phase with a flow of 0.6 mL min^{-1} at 60 °C. Samples were filtered through 0.22 μm syringe filters prior to HPLC.

2.4.4. FTIR analysis

FTIR was used to provide complementary information to understand the structural differences due to the effect of fungal