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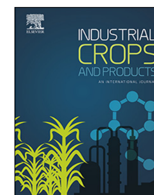

Application of a combined fungal and diluted acid pretreatment on olive tree biomass

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ABSTRACT

A biological pretreatment of olive tree biomass (OTB) was carried out. First, seven white-rot fungi (WRF) were screened on solid-state fermentations by analyzing the substrate composition, ligninolytic enzymes production and enzymatic hydrolysis yields at three different pretreatment times (15, 30 and 45 days). Glucose released by enzymatic hydrolysis of OTB pretreated with *Irpex lacteus* for 45 days doubled that obtained with the control (non-inoculated). In addition, to enhance this yield, the combination of fungal pretreatment with a chemical pretreatment was studied. It was also found that the order of the pretreatment combination has a relevant effect on the glucose yield. Thereby, the best option determined, fungal pretreatment with *I. lacteus* after 28 days of culture followed by diluted acid pretreatment (2% w/v H_2SO_4, 130 °C and 90 min), enhanced 34% the enzymatic hydrolysis yield compared with the acid pretreatment alone. Applying the best pretreatment combination, the overall sugar yield of the whole process (sequential pretreatment plus enzymatic hydrolysis) was 51% of the theoretical yield.

1. Introduction

Olive tree biomass (OTB) is a lignocellulosic residue generated yearly as a consequence of the pruning carried out to remove old branches and prepare the tree for the next crop. With an estimation of 1.5 tons per ha per year (Ruiz et al., 2017), more than 3 million tons OTB are generated yearly in Spain. Most part of OTB is eliminated by burning or gridding and spreading across the fields with environmental risks without generating any added value (Martínez-Patiño et al., 2017). OTB composition and its availability allow to propose this biomass as a raw material to produce second-generation bioethanol and other high added-value products, developing a multiproduct industry (Romero-García et al., 2014; Ruiz et al., 2017).

Lignocellulosic biomass has a very complex structure that hinders the accessibility of enzymes to cellulose during enzymatic hydrolysis. For this reason, pretreatment is an essential step in the biorefinery process (Sindhu et al., 2016). Different pretreatments such as liquid hot water (Cara et al., 2007; Requejo et al., 2012), steam explosion (Cara et al., 2008), phosphoric acid (Martínez-Patiño et al., 2015), inorganic salts (López-Linares et al., 2013) and organosolv pretreatment (Díaz et al., 2011; Toledano et al., 2011) have been applied to OTB. However, these pretreatments require high energy demand and high capital cost. Moreover, they often generate toxic compounds and cause corrosion problems, which make the process commercially uncompetitive with a negative impact on the environment (Alvira et al., 2010).

To overcome these inconveniences, over the last years the biological pretreatment has gained great attention by researchers (Deswal et al., 2014). The potential of biological pretreatment is explained by the ability of certain microorganisms to degrade lignin from lignocellulosic biomass. White-rot fungi (WRF), a small group of basidiomycetes, are the most effective microorganisms in breaking down and mineralizing lignin due to the extracellular secretion of oxidative enzymes, namely manganese peroxidase (MnP), versatile peroxidase (VP), lignin peroxidase (LiP) and lacasse (Lac). Fungal pretreatment avoids the use of chemicals and the formation of inhibitory compounds. Besides, other advantages of the biological pretreatments are their low capital cost and energy requirement. On the contrary, the main disadvantages are long pretreatment times, carbohydrates loss and low hydrolysis rates compared with other pretreatments (García-Torreiro et al., 2016; Martín-Sampedro et al., 2015).

A strategy to overcome the drawbacks of biological pretreatment can be the combination of fungal pretreatment with another physical or chemical pretreatment such as diluted acid (Gui et al., 2013; Ma et al., 2010), alkali (Zhong et al., 2011), steam explosion (Taniguchi et al.,

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2010), autohydrolysis (Martín-Sampedro et al., 2015), organosolv (Monrroy et al., 2010), mineral salt (Wang et al., 2013b) or ultrasound (Kadimaliev et al., 2003). The main effect of dilute acid pretreatment is the solubilization of the hemicellulose fraction. The combination of fungal and dilute acid pretreatment would decrease the severity of acid pretreatment and therefore would reduce the formation of inhibitory compounds. On the other hand, it could also reduce the biological pretreatment time.

The aim of this study was to evaluate the effectiveness of the biological pretreatment on OTB. In a first part of this work, seven white-rot fungi in solid-state fermentation were tested at different pretreatment times. Substrate composition, ligninolytic enzyme production and enzymatic hydrolysis of pretreated OTB were evaluated to select the most adequate fungus and pretreatment time. In a second part, a combination of the fungal pretreatment and dilute acid pretreatment was investigated to obtain higher sugar yields using the fungus and the pretreatment time selected previously. In addition, the structural changes in OTB after different pretreatments were also investigated by FTIR.

2. Material and methods

2.1. Raw material

Olive tree biomass was collected in the province of Jaén (Spain) after fruit-harvesting, air-dried to an equilibrium moisture content of about 7%, milled to a particle size of 4 mm and stored in a dry place until use.

2.2. Fungal strains and inoculum preparation

Seven strains of white-rot fungi were used to pretreat OTB. Six of them were obtained from the culture collection of the Chemical Engineering Department of the University of Santiago de Compostela (Spain): anamorph R1 of *Bjerkandera* sp. (Taboada-Puig et al., 2011), *Irpex lacteus* (Fr.238 617/93), *Lentinus tigrinus* (PW 94-2), *Pleurotus eryngii* (ATCC 90787), *Polyporus ciliatus* (ONO 94-1), and *Stereum hirsutum* (PW 93-4). *Ganoderma lucidum* was isolated from mushroom spent substrate, kindly provided by Hifas da Terra S.L. (Pontevedra, Spain). Strains were maintained on slants culture tubes of 2% (w/v) malt extract agar at 4 °C.

Inoculum was prepared by growing the fungal strains at 30 °C for 7 days on malt extract plates composed of: 5 g/L glucose; 15 g/L agar and 3.5 g/L malt extract. Then, six plugs of about 5 × 5 mm were added to a Fernbach flask containing 200 mL of Kimura medium (20 g/L glucose, 5 g/L peptone, 2 g/L yeast extract, 1 g/L KH_2PO_4, and 0.5 g/L $MgSO_4$). After 7 days of static growth, the mycelium was separated from the liquid medium by filtration and homogenized with 200 mL of sterile distilled water in a blender.

2.3. Fungal pretreatment of biomass

Biological pretreatment was carried out in 250 mL Erlenmeyer flasks plugged with hydrophobic cotton where 10 g OTB (dried basis) plus 30 mL tap water were sterilized at 120 °C for 15 min. Then, flasks were inoculated by adding 0.5 mL blended inoculum per gram of dry biomass and maintained at 30 °C during the solid-state fermentation. Three different pretreatment times (15, 30, 45 days) were evaluated. Non-inoculated flasks were used as control.

After pretreatment, the whole pretreated OTB was washed by adding 60 mL distilled water in each flask and kept at 150 rpm for 1 h in an orbital shaker. Then, solid and liquid fractions were separated by filtration. The activity of ligninolytic enzymes in the liquid fraction were measured as described in Section 2.4. Pretreated biomass was characterized by using the method described in Section 2.7, and then subjected to enzymatic hydrolysis tests to determine its cellulose digestibility.

2.4. Estimation of ligninolytic activities

Enzyme activities of laccase (Lac), manganese peroxidase (MnP) and lignin peroxidase (LiP) were determined in the filtered liquids from biological pretreatment by spectrophotometry taking into account the washing dilution. Lac activity was measured by monitoring the oxidation of 5 mM 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in 100 mM sodium acetate buffer (pH 5.0; $\varepsilon_{436} = 29300\ M^{-1}\ cm^{-1}$). MnP activity was evaluated by monitoring the formation of Mn^{+3}-tartrate complex during the oxidation of 0.1 mM $MnSO_4$ in 100 mM tartrate buffer in the presence of 0.1 mM H_2O_2 (pH 5.0; $\varepsilon_{238} = 6500\ M^{-1}\ cm^{-1}$). LiP activity was determined as 2 mM veratryl alcohol oxidation by formation of veratraldehyde ($\varepsilon_{310} = 9300\ M^{-1}\ cm^{-1}$) in 100 mM sodium tartrate (pH 3.0) in the presence of 0.4 mM H_2O_2. All enzymatic activities were measured at room temperature. One unit of enzyme activity was defined as the amount of enzyme forming 1 μmol of product per minute. After measuring the enzymatic activity in the liquid fraction (in U/L), it was related to the biomass from which it was extracted and expressed as U/g. Each assay was carried out in triplicate, and the mean value was calculated.

2.5. Enzymatic hydrolysis

Pretreated OTB was enzymatically hydrolysed using a cellulolytic complex (Celluclast 1.5 L) and a xylanase complex (NS 50030) supplemented with β-glucosidase (NS 50010). All enzymes were kindly provided by Novozymes A/S, Denmark. Experiments were performed in 100 mL Erlenmeyer flasks with 5% (w/v) biomass suspension in 0.05 M sodium citrate buffer (pH 5.0). Flasks were maintained at 50 °C and 150 rpm for 72 h in a rotatory shaker. Enzyme loading per g of substrate was 15 FPU cellulase, 15 IU β-glucosidase and 30 U/g xylanase. The following enzyme activities were considered for calculating the volume of each enzyme cocktail added to the flasks: 70 FPU cellulase/mL Celluclast 1.5, 810 IU β-glucosidase/mL NS50010, and 924 U xylanase/mL NS50030. Tetracycline (200 μg/mL) was also added to avoid bacterial growth. All experiments were conducted by triplicate and glucose concentration in samples was determined by high-performance liquid chromatography (HPLC). Enzymatic hydrolysis yields were determined considering the glucose production after 72 h (Eqs. (1) and (2)):

$$EHyield = \frac{gglucose\ by\ enzymatic\ hydrolysis}{gglucose\ in\ pretreated\ OTB}100 \quad (1)$$

$$Glucose\ recovery\ by\ EH = \frac{gglucose\ by\ enzymatic\ hydrolysis}{gglucose\ in\ raw\ OTB}100 \quad (2)$$

2.6. Combination of fungal and dilute acid pretreatment

In this work, sequential pretreatments, chemical plus biological and vice versa, were conducted to assess their effectiveness on olive tree biomass. Thereby, a dilute sulfuric acid pretreatment was tested before and after the biological pretreatment with a selected fungus.

Sulfuric acid pretreatment was carried out at mild conditions based on previous experiences. It was conducted in an autoclave at 130 °C for 90 min with 2% (w/v) sulfuric acid using 250 mL glass bottles with a solid/liquid ratio of 1:5 (w/v). The amount of raw OTB or fungal pretreated OTB (depending of the pretreatment order) used in the acid pretreatment step was 20 g dried biomass for bottle. After acid treatment, solid and liquid fractions were separated by filtration. Fibers were washed with distilled water until neutral pH and then they were characterized according to the methodology indicated in Section 2.7. Likewise, the composition of the liquid fractions in sugars and inhibitors was determined as described also in Section 2.7.

Concerning to biological pretreatment, raw OTB or acid pretreated OTB (depending of the pretreatment order) was pretreated by the

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