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Sequential white-rot and brown-rot fungal pretreatment of wheat straw as a promising alternative for complementary mild treatments

Edward Hermosilla [a,b], Olga Rubilar [b,c], Heidi Schalchli [b], Ayla Sant'Ana da Silva [d], Viridiana Ferreira-Leitao [d,e], María Cristina Diez [b,c,*]

[a] Doctoral Program in Sciences of Natural Resources, Universidad de La Frontera, Temuco, Chile
[b] Biotechnological Research Center Applied to the Environment (CIBAMA-BIOREN), Universidad de La Frontera, Temuco, Chile
[c] Chemical Engineering Department, Universidad de La Frontera, P.O. Box 54-D, Temuco, Chile
[d] Biocatalysis Laboratory, National Institute of Technology, Ministry of Science, Technology, Innovation and Communication, Rio de Janeiro, RJ, Brazil
[e] Federal University of Rio de Janeiro, Chemistry Institute, Department of Biochemistry, Postgraduate Program in Biochemistry, Rio de Janeiro, RJ, Brazil

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ABSTRACT

White-rot and brown-rot fungi have complementary mechanisms to selectively degrade lignin and holocellullose, respectively. Thereby, a fungal co-culture of a white-rot and a brown-rot fungal could result in efficient strategy for a mild lignocellulosic biomass pretreatment. In this work, single, sequential and co-inoculation of the selective-lignin degrading white-rot fungus *Ganoderma lobatum* and the brown-rot fungus *Gloeophyllum trabeum* were evaluated as biological pretreatments of wheat straw to enhance enzymatic hydrolysis of cellulose. The single cultures of *G. lobatum* and *G. trabeum* exhibited preferential degradation of lignin and hemicellulose, respectively. The total crystallinity index decreased in samples pretreated with *G. trabeum* but not with *G. lobatum*. The pretreatment with single cultures of *G. lobatum* or *G. trabeum* increased glucose yields by 43.6% and 26.1% respectively compared to untreated straw. Although co-inoculation resulted in higher yields of glucose when compared with single cultures, only a slight synergistic effect between fungi was observed. Contrary, the sequential inoculation of *G. lobatum* incubated for 10 days followed by *G. trabeum* incubated for 10 days more showed a strong synergic effect on enzymatic hydrolysis. This sequential culture showed the highest glucose yield (191.5 mg g^{-1} wheat straw), 2.8-fold higher than untreated wheat straw, and 140–150% higher than the single-cultures of *G. lobatum* and *G. trabeum*, respectively.

1. Introduction

Lignocellulosic biomass derived from agricultural waste has been established as a renewable and socially acceptable alternative raw material for biofuel production. Due to the high cellulosic content (35–50% of dry weight) in these materials, enzymatic hydrolysis can be used to convert cellulose into glucose that can subsequently be fermented into ethanol (Yu et al., 2018; Silveira et al., 2015) or used for the production of other chemicals in a bio-refinery concept. However, the native state of lignocellulosic biomass is highly recalcitrant to enzymatic hydrolysis because the cellulosic fraction is sheathed by lignin and hemicellulose; this physical barrier hinders the access of cellulase enzymes during hydrolysis (Tezcan and Atıcı, 2017). In addition, cellulose fibers present intra-chain and inter-chain hydrogen bonds forming highly resistant crystalline regions. A feasible process for obtaining cellulose-derived products (biofuels or chemicals) from lignocellulosic biomass therefore requires a pretreatment step to increase biodegradability and access to cellulose (Wan and Li, 2010; Mosier et al., 2005). In biological pretreatment, white-rot fungi have been used for their ability to selectively degrade lignin (Hermosilla et al., 2017; Saha et al., 2016; Wan and Li, 2010), whereas brown-rot fungi have been used for their ability to cause rapid, extensive degradation and depolymerization of cellulose and hemicellulose, with minimal assimilation of degradation products (Rafieenia et al., 2018; Machado and Ferraz, 2017; Rasmussen et al., 2010; Lee et al., 2008). Currently, the biological pretreatment of lignocellulosic biomass has focused on the use of single-culture white-rot fungi, while a few works report the use of brown-rot and soft-rot fungi. In nature however, the degradation of lignocellulosic biomass is carried out by the interaction of various wood-rotting fungi. The use of a fungal co-culture that mimics this type of

* Corresponding author at: Chemical Engineering Department, Universidad de La Frontera, P.O. Box 54-D, Temuco, Chile.
E-mail address: cristina.diez@ufrontera.cl (M.C. Diez).

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interaction could therefore shorten the pretreatment time or increase its efficiency. To date, studies of co-cultures of wood-rotting fungi in lignocellulosic biomass have focused on the production of ligninolytic enzymes (Iakovlev and Stenlid, 2000; Kannaiyan et al., 2015) or facilitating the pulping process through partial degradation of lignin (Chi et al., 2007). On the other hand, the effect of the interaction of white-rot and brown-rot fungi in co-culture or sequential inoculation of fungi on lignocellulosic biomass pretreatment has been poorly studied.

In our previous studies, the white-rot fungus *G. lobatum* CCCT16.03 showed higher selectivity to degrade lignin in wheat straw (Hermosilla et al., 2017) compared with the extensively studied selective lignin-degrading fungi, *Pleurotus ostreatus*, *Ceriporiopsis suvermispora* and *Irpex lacteous* (Wan and Li, 2010; Thakur et al., 2012; Salvachúa et al., 2013). *G. trabeum* is one of the most commonly studied brown-rot fungus, which showed potential to improve the efficiency of organosolv pretreatment and enzymatic hydrolysis yield in wood chips (Schilling et al., 2009; Monrroy et al., 2011).

The aim of this study was to evaluate the effect of single cultures of *G. lobatum* (white-rot fungus) and *G. trabeum* (brown-rot fungus) and co-cultures using sequential and co-inoculation of these fungi for the pretreatment of wheat straw.

2. Materials and methods

2.1. Fungal strains

The fungal strains *G. lobatum* CCCT16.03 and *G. trabeum* CCCT16.04 were collected from a temperate forest in the Antuco area, located in the Bio-Bio Region, Chile (38°39′S; 72°35′W). The fungal isolates were obtained by placing small fragments of fungal fruiting bodies on glucose malt-extract agar plates (per litre: 15 g of agar, 3.5 g of malt extract, and 10 g of glucose) and kept at 25 ± 1 °C. The pure cultures were kept in slanted culture tubes with malt-extract agar medium at 4 °C and periodically sub-cultured. The strains were identified by molecular methods and deposited in the Chilean Culture Collection of Type Strains – CCCT/UFRO (WDCM 1111) of Scientific and Technological Bioresource Nucleus at Universidad de La Frontera, Temuco, Chile. For molecular identification, the DNA of fungal strain was extracted using E.Z.N.A.® SP Fungal DNA Mini Kit D5524-01 and the sequence PCR amplification was carried out using universal primers ITS4 5′-TCCTCCGCTTATT GATATGC-3′ and ITS1 5′-TCCGTAGGTGAACCTGCGG-3′ (White et al., 1990). PCR products were sequenced by dye Terminator Cycle Sequencing Kit and an ABI 3730XL DNA Sequencer (Applied Biosystems) by Macrogen, Seul, South Korea (http://www.macrogen.com/eng/macrogen/macrogen_main.jsp). The ITS-1 and ITS-4 DNA sequences were compared with the compilation available in the GenBank/EMBL/DDBJ database using BLASTN (http://www.ncbi.nlm.nih.gov/blast), according Horisawa et al. (2013). The nucleotide sequences identified in this study were deposited in the NCBI nucleotide sequence database (GenBank/NCBI) under accession numbers KU645997 (*G. lobatum*) and MH287513 (*G. trabeum*).

2.2. Intraspecific and interspecific mycelial interactions under co-culture

The intraspecific and interspecific interactions of *G. lobatum* and *G. trabeum* were evaluated in solid medium malt extract agar (per litre: 15 g of agar, 3.5 g of malt extract) with pH adjusted to 4.5. The plates were inoculated with agar-mycelia plugs (6 mm diameter) cut from the actively growing margin of fungal cultures on malt extract agar plates. To evaluate interspecific interactions, two agar plugs of *G. lobatum* and *G. trabeum*, respectively, were placed on opposite sides (40 mm apart) of each assay plate. In addition, self-pairings of each species were included to evaluate intraspecific interactions. Three replicate plates were carried out for each fungal pairing studied to check the reproducibility of the interaction. The assay plates were incubated in the dark at 25 ± 1 °C for 3 weeks. The interactions between opposing fungal colonies were evaluated visually at least once every 3–4 days and classified according to Boddy (2000).

2.3. Fungal inoculum preparation

The inoculum preparation for wheat straw pretreatment was carried out in Erlenmeyer flasks (500 mL) containing 100 mL of modified Kirk medium containing per litre: 10 g of glucose, 2 g of peptone, 2 g of KH_2PO_4, 0.5 g of $MgSO_4$, 0.1 g of $CaCl_2$, 84 mg of $MnSO_4{\cdot}H_2O$, 2 mg of thiamine, and 10 mL of mineral salts (per litre: 0.1 g of $FeSO_4{\cdot}7H_2O$, 0.1 g of $CoCl_2$, 0.1 g of $ZnSO_4{\cdot}7H_2O$, 0.1 g of $CuSO_4$, 10 of mg $AlK(SO_4)_2{\cdot}12H_2O$, 10 mg of H_3BO_3 and 10 mg of $Na_2MoO_4{\cdot}2H_2O$). Each flask was inoculated with five agar disks (6-mm diameter) of active mycelia of *G. lobatum* or *G. trabeum* from a five-day-old culture on malt-extract agar cultivated in Petri dishes and incubated at 30 ± 1 °C for 10 days. Then each fungal culture was homogenized in a sterilized blender for 1 min and used as a source for the inoculum of wheat straw pretreatments.

2.4. Wheat straw pretreatments assays

Wheat straw was collected from crop residues at Maquehue experimental field of Universidad de La Frontera, Temuco, Chile. The pretreatments were carried out in Erlenmeyer flasks (250 mL) containing 5 g (dry material) of chopped wheat straw (particle size ~50 mm long) and moistened with 25 mL of water. The wheat straw was supplemented with NO_3^-, Fe^{2+}, and Mn^{2+} at different concentrations established in previous studies from our laboratory, which depended on the fungal inoculum being evaluated (Hermosilla et al., 2017) (Table 1). The flasks were stoppered with hydrophobic cotton and gauze and autoclaved for 25 min at 121 °C. Sterilized wheat straw was inoculated with 0.5 mL of blended fungal mycelia (10 mg of dry weight fungal mycelia) and kept in the dark at 25 ± 1 °C without shaking for 10–40 days. In parallel, uninoculated flasks were incubated under the same conditions (untreated wheat straw) as negative controls. Each pretreatment assay was carried out in triplicate and the samples were taken by destructive sampling.

The effect of co-culture of fungal strains for the pretreatment of wheat straw was evaluated considering the results obtained from the assays of single cultures. Firstly, both *G. lobatum* (white-rot) and *G. trabeum* (brown-rot) were co-inoculated (CI) in the same flask and incubated for 20 days. Afterwards, sequential inoculation of the fungi was also evaluated as follows: first strategy (S1), *G. lobatum* was inoculated in the wheat straw for 10 days, and then *G. trabeum* was added to the flasks and the treatment was incubated for another 10 days; second strategy (S2) was similar to S1, except that *G. trabeum* was inoculated after 20 days; third strategy

Table 1
Conditions of fungal pretreatments of wheat straw.

Pretreatment	Conditions
G. lobatum	0.18 M $NaNO_3$; 0.73 mM $FeSO_4$; 1 mM $MnSO_4^*$
G. trabeum	0.24 M $NaNO_3$; 0.95 mM $FeSO_4$; 0.85 mM $MnSO_4^{**}$
G. lobatum plus *G. trabeum*	0.24 M $NaNO_3$; 0.74 mM $FeSO_4$; 1 mM $MnSO_4^{**}$

According to Hermosilla et al. (2017) (*) and unpublished data (**).