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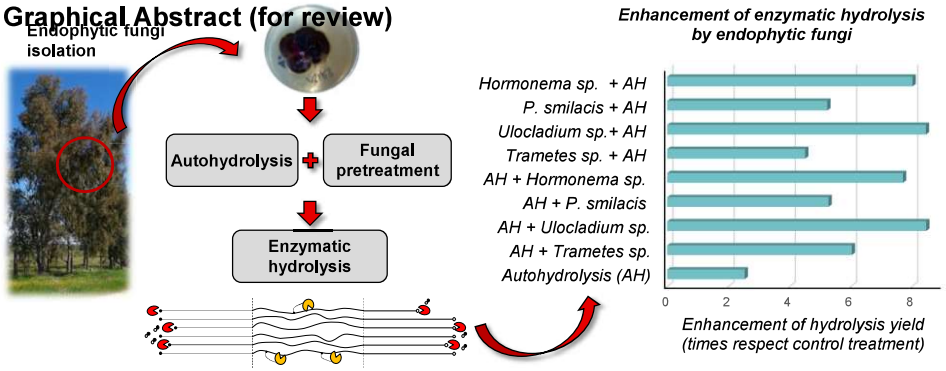
Abstract: New endophytic fungi are assessed for the first time as pretreatment to enhance saccharification of Eucalyptus globulus wood. The fungi are all laccase-producing ascomycetes and were isolated from eucalyptus trees in Spain. After five endophytes had been assayed alone or in combination with white-rot fungus Trametes sp. I-62, three were pre-selected. To improve sugar production, an autohydrolysis pretreatment was performed before or after fungal treatment. Pretreatment increased sugar production 2.7 times compared to non-pretreated wood. When fungal and autohydrolysis pretreatments were combined, a synergistic increase in saccharification was observed in all cases. Endophytic fungi Ulocladium sp. and Hormonema sp. produced greater enhancements in saccharification than Trametes sp. I-62 (increase in sugar yields of 8.5, 8.0 and 6.0 times, respectively), demonstrating the high potential of these new endophytic fungi for saccharification enhancement.

Graphical Abstract (for review)



HIGHLIGHTS

1) Endophytic fungi are applied for the first time to enhance saccharification.
2) Mild autohydrolysis pretreatment boosted the fungal effect.
3) *Ulocladium* sp. produced an 8.5-fold increase in sugar yield.
4) *Hormonema* sp. produced an 8-fold increase in sugar yield.
5) *Trametes* sp. I-62 produced a 6-fold increase in sugar yield.

USE OF NEW ENDOPHYTIC FUNGI AS PRETREATMENT TO ENHANCE ENZYMATIC SACCHARIFICATION OF EUCALYPTUS GLOBULUS

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ABSTRACT

New endophytic fungi are assessed for the first time as pretreatment to enhance saccharification of *Eucalyptus globulus* wood. The fungi are all laccase-producing ascomycetes and were isolated from eucalyptus trees in Spain. After five endophytes had been assayed alone or in combination with white-rot fungus *Trametes* sp. I-62, three were pre-selected. To improve sugar production, an autohydrolysis pretreatment was performed before or after fungal treatment. Pretreatment increased sugar production 2.7 times compared to non-pretreated wood. When fungal and autohydrolysis pretreatments were combined, a synergistic increase in saccharification was observed in all cases. Endophytic fungi *Ulocladium* sp. and *Hormonema* sp. produced greater enhancements in saccharification than *Trametes* sp. I-62 (increase in sugar yields of 8.5, 8.0 and 6.0 times, respectively), demonstrating the high potential of these new endophytic fungi for saccharification enhancement.

Keywords: Endophytes; enzymatic saccharification; Ascomycetes; Dothideomycetes; Pretreatment.

1. INTRODUCTION

Production of liquid fuels, chemicals, materials and energy relies primarily on non-renewable resources and is unsustainable from both a social and an environmental point of view. In this context, the search for alternative and renewable energy sources has brought increasing attention to the use of lignocellulosic biomass in fuel and chemical production (Himmel et al., 2007).

A major rate-limiting step in biochemical processing of lignocellulose is the enzymatic depolymerisation of biomass into sugars that are subsequently fermented and processed to obtain fuels (such as second generation bioethanol) or other chemicals (Pauly & Keegstra, 2008). Significant research is under way to develop efficient pretreatment technologies to break down the complex and recalcitrant structure of lignocellulose, which greatly inhibits enzymatic saccharification of cellulose and hemicellulose. These pretreatments are intended to improve sugar formation and prevent degradation or loss of carbohydrates and formation of inhibitory products, and should also be cost-effective (Alvira et al., 2010). They can be classified into biological, physical, chemical and physicochemical pretreatments, depending on the type of forces or energy consumed in the pretreatment process (Alvira et al., 2010).

Biological pretreatments are based on the use of microorganisms (such as brown-, white-, and soft-rot fungi) capable of degrading lignin and hemicelluloses and a small amount of cellulose. White-rot fungi are the most effective due to their ability to produce enzymes (laccases and peroxidases) which can partially degrade lignin and modify its molecular structure (Alvira et al., 2010; Guillén et al., 2005). Basidiomycetes such as *Trametes velutina, Pycnoporus* sp. *SYBC-L3, Pleurotus eryngii, Irpex lacteus, Ceriporiopsis subvermispora, Trametes versicolor* and *Phanerochaete chrysosporium*

have been used to treat various lignocellulosic biomasses and have enhanced hydrolysis yields (Cianchetta et al., 2014; Gui et al., 2013; Liu et al., 2013; López-Abelairas et al., 2013; Salvachúa et al., 2011; Wang et al., 2013a; Zhong et al., 2011). However, endophytic fungi (mainly ascomycetes) have not previously been used for this purpose. These fungi inhabit asymptomatic plant tissues, living in symbiosis with their hosts. The extent of the enormous diversity of these fungi remains unknown, making them potential sources of biotechnological tools for multiple uses. The highly specific activity of endophytic fungi as primary degraders of lignocellulosic substrates to which they are adapted by evolutionary processes probably improves the efficiency of biological pretreatments.

The advantages of fungal pretreatments are the low capital cost, low energy and no chemical requirements, mild environmental conditions and no emission of inhibitors to fermentation. Drawbacks include the long treatment time required and the low hydrolysis rate obtained compared to other technologies, indicating that recalcitrant barriers still exist in the fungi-pretreated residues (Alvira et al., 2010; Wang et al., 2013a). The combination of fungal and physical or chemical pretreatments can potentially overcome recalcitrance, improving the yields of end products. Pretreatments with mild acid (Gui et al., 2013), alkali (Salvachúa et al., 2011; Yang et al., 2013; Zhong et al., 2011), organosolv (Muñoz et al., 2007), hydrogen peroxide (Yu et al., 2009), $FeCl_3$ (Wang et al., 2013b) and thermal treatments (López-Abelairas et al., 2013) have been combined with fungal pretreatments to increase saccharification yields. These combinations would also diminish the severity of chemical and/or physical pretreatments, minimising some of the disadvantages, such as the formation of inhibitory compounds (López-Abelairas et al., 2013; Wang et al., 2013a), and/or

reducing fungal treatment times (Yu et al., 2009; Zhong et al., 2011).

This study tests five new endophytic fungi for their application as a pretreatment to enhance saccharification of *Eucalyptus globulus* wood. The effect of these fungi was compared with that of white-rot fungus *Trametes* sp. I-62, used as a reference. A mild alkaline treatment was carried out after all fungal pretreatments to increase digestibility of the samples without masking the effect of the biological pretreatment (Salvachúa et al., 2011). Fungal pretreatment was combined with mild autohydrolysis to improve the fungal effect and increase sugar yields.

2. MATERIALS AND METHODS

2.1. Chemicals and Raw Material

All chemicals were reagent-grade, obtained from Merck (Barcelona, Spain), Panreac (Barcelona, Spain) and Sigma-Aldrich (Madrid, Spain). Enzymes were Celluclast 1.5L and Novozym 188, donated by Novozymes (Bagsvaerd, Denmark). *E. globulus* chips were provided by La Montañanesa pulp mill (Torraspapel - Lecta Group, Spain). The material was air dried then homogenised into a single stock by conditioning inside polyethylene bags to avoid differences in composition and water content. Chips were ground and sieved to select pieces 0.5-1.5 cm long and 1-2 mm wide. Test pieces were stored in polyethylene bags at 25º C. Raw material composition was 2.0% ethanol extractives, 22.0% Klason lignin, 47.8% glucose, 15.7% xylose, 0.8% arabinose and 1.3% manose.

2.2. Fungal strains and inoculum preparation

2.2.1. Saprophytic fungus

The saprophytic white-rot fungus used as a reference was *Trametes* sp. I-62, obtained from the collection of *Instituto Jaime Ferrán de Microbiología-CIB* (Madrid, Spain).

2.2.2. Isolation of endophytic fungi

More than 100 strains of endophytic fungi were isolated from *Eucalyptus* sp. trees in five Spanish provinces: Cantabria (coded CA at the beginning of the strain name), Asturias (AS), Seville, (SE), Extremadura (EX) and Toledo (TO). For endophyte isolation, four twigs approx. 2 cm in diameter were collected from 1-3 trees from each sampling location. After surface sterilisation with 70% ethanol and 4% bleach and removal of the outermost bark, five explants per twig were cultured in the following media: Malt Extract Agar (MEA), Potato Dextrose Agar, Rose Bengal Chloramphenicol Agar, Yeast Extract Agar, and Sapwood Agar (coded M, P, R, Y, and S in the penultimate position of the strain name). The first four media were prepared following the manufacturer's instructions (Cultimed, Panreac, Barcelona, Spain). Sapwood agar was prepared by mixing 50 g ground, dried and autoclaved eucalyptus twigs with 7.5 g agar in 500 mL water. Explants were axial twig slices (approx. 2 mm thick), including phloem and xylem tissue. After two weeks of incubation, growing fungal strains were transferred to independent plates containing MEA.

2.2.3. Screening of endophytic fungi in solid media

Isolated endophytic strains were tested for their ability to produce ligninolytic enzymes in agar-plate medium containing 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (Niku-Paavola et al., 1988). The solid medium comprised 1g L^{-1} KH_2PO_4, 0.5g L^{-1} $C_4H_{12}N_2O_6$, 0.001g L^{-1} $CuSO_4 \cdot 5H_2O$, 0.5g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.001 g L^{-1} $Fe_2(SO_4)_3$, 0.01 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0.001 g L^{-1} $MnSO_4 \cdot H_2O$, 0.01 g L^{-1} yeast

extract, 4 g L^{-1} glucose, 16 g L^{-1} agar and 0.5 g L^{-1} ABTS. Plates were inoculated with an agar disk of active mycelia cultured in malt extract agar. *Trametes* sp. I-62 was used as a positive control. After three days at 22 ºC, fungal strains producing extracellular ABTS-oxidising enzymes (such as laccases and peroxidases) showed a dark green halo around the mycelia colony due to oxidation of ABTS to its cation radical ($ABTS^{+}$). Positive reactions were classified as high, medium or low oxidation according to a [diameter of green halo / diameter of the colony] ratio higher than 1.5, 1.2 or 1.0 after three days. Endophytic fungi showing high oxidation were selected for preliminary enzymatic hydrolysis assays.

2.2.4. Identification of endophytic fungi producing ligninolytic enzymes

Endophytic strains showing ligninolytic activity during screening in solid media were identified by their specific Internal Transcribed Spacer regions, ITS1 and ITS2, of ribosomal DNA. The online programme BLAST (MEGA Blast algorithm), by GenBank (NCBI, USA), was used to find the most similar sequence (highest bit score) within this database. Species assignment should be taken with caution, as it is based on taxonomic proximity.

2.2.5. Inoculum preparation

Saprophyte *Trametes* sp. I-62 and the strains of endophytic fungi showing high oxidation of ABTS in solid medium were grown on 2% malt extract media plates supplemented with 2% agar at 23 °C for 7-15 days and stored at 4 °C. Media plugs overgrown with mycelium were used to start a pre-inoculum in sterilised liquid medium (plugs from 1 plate of 90 mm Ø per 200 mL distilled water). Pre-inocula were incubated at 23 ºC on a rotary shaker at 100 rpm for two days and used to inoculate the *E. globulus* wood chips.

2.3. Fungal pretreatment: pre-selection of endophytic fungi

Ten grams of dry sieved chips (0.5-1.5 cm long and 1-2 mm wide) were autoclaved in 100 mL Erlenmeyer flasks plugged with hydrophobic cotton wool. Flasks were inoculated with 10 mL of the individual fungi pre-inocula prepared as described above. Consistency was adjusted to 17% w/w by adding autoclaved distilled water. Six flasks of each individual fungus were prepared and incubated at 23 ºC for 28 days. In three of the flasks inoculated with each endophytic fungus, *Trametes* sp. I-62 inoculum was added on day 10 of incubation to test an endophyte-saprophyte sequential treatment, simulating the wood degradation sequence occurring in nature. Control samples were prepared following the same procedure, adding autoclaved distilled water instead of fungal pre-inoculum. After 28 days of incubation, 100 mL acetate buffer (pH 5.2) was added to each flask and flasks were stirred at 150 rpm for 1 h to remove superficial mycelium. Decayed wood samples were filtered under vacuum and air dried at room temperature and their moisture contents were determined. Weight loss was calculated from the initial and final dry weight of chips.

Laccase and peroxidase activities were determined in the filtered liquid by UV-spectroscopy using 2.6-dimethoxyphenol (DMP) (λ=468nm, pH 5.2 at room temperature, ε_o = 49600 M^{-1} cm^{-1}). The final concentration of DMP was 10 mM in 100 mM acetate buffer. When peroxidase activities were determined, hydrogen peroxide was added in a final concentration of 0.01 mM. Endoglucanase, exoglucanase and xylanase activities were also evaluated by applying the Somogyi-Nelson procedure (Nelson, 1944; Somogyi, 1952), using carboxymethyl cellulose, crystalline cellulose (avicel) and xylan as substrates. All enzymatic activities were expressed per gram of treated wood.

After fungal treatment, all solid samples were subjected to a mild alkali treatment before enzymatic hydrolysis to improve saccharification yields (Salvachúa et al., 2011). Alkali treatment was performed at 50 ºC and 165 rpm for 1 h with a final concentration of 0.1% sodium hydroxide and 5% w/w consistency. Samples were then filtered and washed with distilled water until neutral pH.

Enzymatic hydrolysis of pretreated and control samples was conducted as described in section 2.5. Based on the increases in hydrolysis yields observed, three endophytic fungi were selected for further analysis.

2.4. Autohydrolysis and fungal pretreatment combinations

After pre-selection of the three endophytic fungi which produced the greatest enhancement of enzymatic hydrolysis, the pretreatments were altered to improve sugar production and maximise the fungal effect. Samples were ground in a Wiley mill and sieved to select a size of 0.25-0.20 mm before pretreatment. An autohydrolysis (AH) pretreatment was carried out before (AH + Fungal) or after (Fungal + AH) the fungal plus mild alkaline treatment. Autohydrolysis was performed in an autoclave (Trade Raypa S.L.) at 135 ºC for 30 min with a liquid to solid ratio of 6:1. Conditions for fungal and mild alkaline treatments are described in section 2.3. and enzymatic hydrolysis was conducted according to section 2.5. Samples were not air dried between treatments or before enzymatic hydrolysis. A control subjected to autohydrolysis pretreatment but not to fungal or mild alkaline treatment was prepared (Control-AH).

The severity factor of the autohydrolysis pretreatment was calculated according to the following equation (Eq. 1) defined by Overend and Chornet (1987),

$$S_0 = \log\left(e^{\frac{T-100}{14.75}\cdot t}\right) \qquad \text{(Eq. 1)}$$

in which T is the temperature (ºC) and t the duration of the treatment (min).

2.5. Enzymatic hydrolysis

The solid samples of biopretreated *E. globulus* wood were subjected to enzymatic hydrolysis. All experiments were conducted in triplicate. A cellulolytic complex (Celluclast 1.5L) supplemented with β-glucosidase (Novozym 188) was added to a 5% w/w wood suspension in 50 mM sodium citrate buffer (pH 4.8). Celluclast 1.5L is a cellulase preparation (92.5 FPU/ml) with some xylanase activity but practically no β-glucosidase activity. Therefore, supplementation with Novozym 188, which mainly presents β-glucosidase activity (1274 UI/ml), is typically used in saccharification processes. Enzyme doses were 15 FPU Celluclast 1.5L and 15 UI β-glucosidase per gram of dry sample. Enzymatic hydrolysis was performed in a thermostatic rotary shaker at 50 ºC and 120 rpm for 72 h. Samples of 1.5 mL were taken after 24, 48 and 72 h of hydrolysis to evaluate the release of reducing sugar using 3.5-dinitrosalicylic acid (DNS) reagent (Miller, 1959) (in pre-selection assays) or glucose and xylose concentration by High Pressure Liquid Chromatography (HPLC) (in assays combining AH and fungal treatment). Before determination of sugar concentration by each method, liquid samples were heated in boiling water for 10 min and filtered through a 0.45 μm nylon syringe filter after cooling.

Glucose, xylose and total sugar yields were calculated according to equation Eq. 2:

$$\text{Sugar yield (\%)} = \frac{\text{g of sugar in liquid phase}}{\text{g of sugar in initial material}} \times 100 = \frac{C_h \times V_h}{(m_i \times C_i)/Y_{AH.F}} \times 100 \quad \text{(Eq. 2)}$$

where: C_h is the concentration of sugar (glucose, xylose or total sugar) in the hydrolysate after 72 h of enzymatic hydrolysis, expressed in g L^{-1}; V_h is the volume of hydrolysate in L^{-1}; m_i are the g of initial material subjected to the first pretreatment (fungal treatment or AH); C_i is the concentration of sugar (glucose, xylose or total sugar) in the raw material, expressed as a percentage; and $Y_{AH,F}$ is the solid yield of the autohydrolysis and fungal pretreatments, when applied, expressed as a percentage.

2.6. Analytical methods

The composition of the raw material and the solid fractions obtained in the pretreatments was determined by standard analytical methods (NREL, 2011). Carbohydrate content was quantified by high-performance liquid chromatography (HPLC) in an Agilent Technologies 1260 HPLC fitted with a refractive index detector (Agilent, Waldbronn, Germany) using an Agilent Hi-PlexPb column operated at 70 ºC with Milli-Q water as a mobile phase pumped at a rate of 0.6 mL min^{-1}. The solid residue remaining after acid hydrolysis is referred to as acid insoluble lignin (Klason lignin). Acid soluble lignin was quantified using UV-spectrophotometry at 205 nm.

Reducing sugar content of the hydrolysates obtained during enzymatic hydrolysis in the pre-selection assays was determined using DNS reagent. A 0.5 mL measure of sample was mixed with 1 mL 50 mM sodium citrate and 3 ml DNS reagent in an assay tube. The mixture was boiled for 5 min then cooled in an ice bath. 0.5 ml of the coloured solutions was centrifuged at 10,000 g for 3 min and the supernatant was

used to determine absorbance at 540 nm in a UV- spectrophotometer (Jasco V-530). A calibration sugar curve was obtained using glucose standard solutions to correlate absorbance with glucose (reducing sugar) content.

Glucose and xylose concentrations were determined in the hydrolysates obtained during enzymatic hydrolysis after the combined AH and fungal pretreatments. Hydrolysate samples were used to determine monosaccharides by means of an Agilent Technologies 1260 HPLC fitted with a refractive index detector (Agilent, Waldbronn, Germany) and an Agilent Hi-PlexH column operated at 65 ºC with a mobile phase containing 5 mmol L^{-1} sulfuric acid pumped at a rate of 0.6 mL min^{-1}.

2.7. FTIR analysis

Samples subjected to fungal pretreatment and autohydrolysis were analysed by FTIR. Spectra were obtained using a JASCO FT/IR 460 Plus spectrometer equipped with an accessory single reflection diamond, operating with a resolution of 1 cm^{-1}, 400 scans and a spectral range of 4000-650 cm^{-1}. FTIR bands were assigned by comparison with those reported in the literature (Dorado et al., 1999; Faix, 1991; Ibarra et al., 2004; Pandey & Pitman, 2003; Yuan et al., 2011).

3. RESULTS AND DISCUSSION

3.1. Pre-selection of endophytic fungi

3.1.1. Screening in solid media

Of the more than 100 endophytic fungi isolated and screened for production of ligninolytic enzymes, 17 were able to oxidise ABTS to varying degrees (table 1). Five strains produced high ABTS oxidation ([diameter of green halo / diameter of the colony]

ratio higher than 1.5) and were selected for the preliminary enzymatic hydrolysis assays. These strains are ascomycete fungi of the Dothideomycetes class, and the closest species correspond to *Neofusicoccum luteum*, *Ulocladium* sp., *Pringsheimia smilacis, Hormonema* sp. and *Neofusicoccum australe*. The predominance of Dothideomycetes in this selection could be because (i) they are more abundant in endophytic flora, (ii) they are more easily isolable than the others or (iii) they present more oxidoreductase activity. However, this predominance was not observed in the endophytic composition of eucalyptus twigs (unpublished data). Only strains with ABTS oxidative activity were preserved and identified and therefore it is impossible to determine whether the abundance of Dothideomycetes is due to the ease of isolation of this class or to a higher oxidoreductase capability.

3.1.2. Lignocellulose substrate degradation

The five endophytic fungi selected and saprophyte *Trametes* sp. I-62 were tested as a pretreatment for improving enzymatic hydrolysis. As mentioned in materials and methods, in these preliminary assays small chips (0.5-1.5 cm long and 1-2 mm wide) were inoculated either with one fungus or with an endophyte and the saprophyte to test the endophyte-saprophyte sequential treatment as well. When only an endophyte was used, weight loss of 0.8-2.5% was observed. However, when *Trametes* sp. I-62 was used alone or in combination with an endophyte, weight loss increased 1.6-5.2%, probably due to greater degradation by the saprophytic fungus. Cianchetta et al. (2014) treated wheat straw with various basidiomycete fungi for four weeks and reported similar or higher weight losses: 2-12% for *C. subvermispora*, *T. versicolor*, *Cyathus stercoreus* and *Pleurotus ostreatus* and 25% for *P. chrysosporium*.

3.1.3. Fungal enzymes

In the enzymatic activities at the end of the fungal pretreatment, neither cellulase (endo- or exoglucanase) nor xylanase activities were detected. However, ligninolytic activities (laccase and peroxidase) were found in all cases and laccase activity was predominant. The highest ligninolytic activities were observed when *Hormonema* sp. was applied in combination with *Trametes* sp. I-62: 53 mU/g and 7.0 mU/g laccase and peroxidase activities, respectively. Nevertheless, high ligninolytic activities were also detected when *N. australe* and *Trametes* sp. I-62 were used alone (laccase activity of 17.2 mU/g and 42.8 mU/g, respectively; peroxidase activity of 6.5 mU/g and 2.8 mU/g, respectively) or in combination (23.0 mU/g and 8.4 mU/g laccase and peroxidase activities, respectively). Liu et al. (2013) also found higher laccase activity than manganese peroxidase (MnP) or lignin peroxidase (LiP) activities, and no cellulose or xylanase activities when switchgrass was treated with white-rot fungus *Pycnoporus* sp. SYBC-L3. Similar results were obtained by Deswal et al. (2014) during fungal treatment of sugarcane bagasse with three white-rot fungi, *Pleurotus florida*, *Coriolopsis caperata* RCK 2011 and *Ganoderma* sp. rckk-02. Dias et al. (2010) reported that MnP was the predominant ligninolytic enzyme secreted during solid state fermentation of wheat straw with white-rot fungi *I. lacteus* and Euc-1, probably due to the high level of manganese (50 ppm) found in this raw material. However, the level of ligninolytic activities depends not only on the substrate, but also on the specific fungal strain and pretreatment conditions (Cianchetta et al., 2014).

3.1.4. Enzymatic hydrolysis

Figure 1a shows the reducing sugar concentration at various enzymatic hydrolysis times for samples pretreated with only one fungus. Figure 1b shows data for endophyte-saprophyte sequential pretreatments. All samples (including the control)

were subjected to a mild alkali treatment before enzymatic hydrolysis to improve digestibility of the samples without masking the effect of the biological pretreatment (Salvachúa et al., 2011). Pretreatment with saprophyte *Trametes* sp. I-62 produced the highest reducing sugar concentration. However, pretreatment with *P. smilacis*, *Hormonema* sp. and *Ulocladium* sp. also enhanced sugar production at 72 h of enzymatic hydrolysis by 55.4%, 33.3% and 30.9%, respectively, compared to the control sample. This saccharification enhancement could be due to the increase of cellulose accessibility via breakdown of the lignin-carbohydrate complex (Dias et al., 2010) and improvement in porosity of plant material because of biological pretreatment, leading to an increase in the initial adsorption of cellulase to cellulose (Deswal et al., 2014). When sequential pretreatments were conducted, only slight increases in sugar production were observed for *N. luteum*, *P. smilacis,* *Ulocladium* sp. and *N. australe* (16.9%, 13.7%,12.7% and 4.1%, respectively), probably due to fungal competition. These results indicate no relation between extracellular ligninolytic activities in the pretreatment and the enhancement of enzymatic hydrolysis, as other authors have reported (Salvachúa et al., 2011).

3.1.5. Substrate composition of fungal pretreated and control samples

To determine whether there is a relation between composition and enzymatic hydrolysis enhancement, the composition of the four highest sugar yielding pretreated samples (fungal plus mild alkali treatment) and of the control sample (subjected to mild alkali treatment, Control AE) were compared (Table 2). Cellulose and hemicellulose contents were similar in pretreated and control samples but Klason lignin content decreased: 22.3% in the control sample versus 20.0%, 21.0%, 21.0% and 20.9% for *Ulocladium* sp., *P. smilacis*, *Hormonema* sp. and *Trametes* sp., respectively. This

decrease could be the cause of the higher sugar yield observed for the pretreated samples, as lignin content is known to inhibit enzymatic hydrolysis of lignocellulosic biomass (Berlin et al., 2006; Martín-Sampedro et al., 2013; Rahikainen et al., 2013). The improved accessibility of cellulose after fungal pretreatment (due to partial delignification) and the increase in the carbohydrate/lignin ratio in pretreated samples allow better action of cellulolytic enzymes (Dias et al., 2010). However, a comparison of pretreated samples shows no relation between lignin content and sugar yield. These findings concur with those of other authors (Cianchetta et al., 2014; Salvachúa et al., 2011) who observed that 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. Wang et al. (2013a) reported that when lignin is degraded to a threshold level, lignin would have no further impact on cellulose conversion, and complete delignification is not necessary during pretreatment.

On the basis of the results of the preliminary assays, *Ulocladium* sp., *P. smilacis* and *Hormonema* sp. were selected because of their potential for enhancing enzymatic hydrolysis. These fungi were applied alone in further experiments, as the endophyte-saprophyte sequential treatment produced lower sugar yields. However, it should be taken into account that the sugar productions obtained in all the preliminary assays were very low compared to other raw materials such as wheat straw or switchgrass due to the low accessibility of *E. globulus*. Therefore, samples were milled and an autohydrolysis pretreatment was performed before enzymatic hydrolysis in subsequent assays, not only to increase sugar production, but also to maximise the fungal effect.

3.2. Autohydrolysis and fungal pretreatment combinations

3.2.1. Enzymatic hydrolysis

Figure 2 shows glucose, xylose and total sugar production during enzymatic hydrolysis of samples subjected to autohydrolysis before or after fungal pretreatments. A control with no pretreatment (Control) and a control subjected to autohydrolysis but not to fungal pretreatment (Control AH) are also included. Comparison of the control samples shows that autohydrolysis pretreatment increased glucose yield from 3.9% to 10.5% and xylose yield from 3.4% to 6.8%. This effect has been attributed to increased porosity and the removal of hemicelluloses, which increase the accessibility of the pretreated material (Alvira et al., 2010; Yang & Wyman, 2004). Other authors reported higher sugar yields after enzymatic hydrolysis of *E. globulus* subjected to autohydrolysis pretreatment using more severe pretreatment conditions. Inoue et al. (2008), Martin-Sampedro et al. (2014) and Romaní et al. (2010) observed glucose yields of 23.3-50.5% after autohydrolysis pretreatment with a severity factor S_0 of 3.1-3.8. Even higher glucose yields (65-97%) were reported when the severity factor was increased to 3.8-4.3 (Inoue et al., 2008; Romaní et al., 2010), proving the strong relation between the severity of the autohydrolysis pretreatment and enhancement of the subsequent enzymatic hydrolysis. In this study, a mild autohydrolysis pretreatment was selected ($S_0 = 2.5$) because the objective of the pretreatment was to increase the hydrolysis yield without masking the fungal effect as a result of more aggressive autohydrolysis pretreatment. A severe hydrothermal pretreatment could have hindered fungal colonisation due to the presence of inhibitory substances generated during thermal pretreatment or by modification of the raw material morphology at microscopic level (López-Abelairas et al., 2013).

When fungal pretreatment was conducted before or after autohydrolysis pretreatment, enhancement of enzymatic hydrolysis was observed in all cases. Interestingly, a synergistic effect of both pretreatments was found. This effect was more prominent when *Ulocladium* sp. or *Hormonema* sp. were used: increases of 8.5 and 8.0 times in total sugar yields for both fungi (31.0-34.2% glucose and 23.6-29.0% xylose yields) compared to the control sample with no pretreatment (3.9% glucose and 3.4% xylose yield). Compared to control AH (10.5% glucose and 6.8% xylose yield), the increases in total sugar yields were 2.9-3.3 times higher for both fungi. When white-rot fungus *Trametes* sp. I-62 was applied after autohydrolysis pretreatment, total sugar yield was 6.0 and 2.3 times higher than the yields of untreated and autohydrolised *E. globulus* wood, respectively. Enhancement of enzymatic hydrolysis by this white-rot fungus was even lower than the controls when autohydrolysis treatment was conducted after fungal treatment. The endophytic fungi therefore produced a greater increase in saccharification of *E. globulus* wood, indicating their high potential for this application.

Other authors combined fungal pretreatment with physical or chemical methods to improve saccharification yields and mitigate some of the disadvantages of these pretreatments, such as the formation of inhibitory compounds during physical or chemical methods (López-Abelairas et al., 2013; Wang et al., 2013a) and/or the reduction of fungal pretreatment times from 60 to 15-18 days (Yu et al., 2009; Zhong et al., 2011). However, most reported lower increases in hydrolysis yields than those obtained with endophytic fungi. López-Abelairas et al. (2013) observed that when a thermal pretreatment was performed before fungal treatment with *P. eryngii* or *I. lacteus*, glucose yields were 3.9 and 4.6 times higher than in untreated wheat straw. Gui et al. (2013) combined a fungal treatment using *P. chrysosporium* with 2.5% sulfuric acid

treatment and obtained glucose yields 1.08-1.71 times higher than in acid-treated *Glycyrrhiza uralensis* under the same conditions. Wang et al. (2013b) reported that fungal pretreatment of poplar wood with *Trametes orientalis* (white-rot fungus) or *Fomitopsis palustris* (brown-rot fungus) followed by $FeCl_3$ treatment increased sugar yields 1.4 and 1.6 times more than $FeCl_3$ treatment alone. Only Yang et al. (2013) observed a similar enhancement of bioconversion to that found in this study through synergistic treatment of poplar with a four-week white-rot fungus *Trametes velutina* D10149 pretreatment and alkaline fractionation (1% NaOH, 75 ºC, 3h), obtaining a glucose yield of 38.8%.

No significant differences in hydrolysis yields were observed when the sequential order of the pretreatments was altered, except for *Trametes* sp. I-62. The saprophytic fungus therefore provided better results when it was applied after autohydrolysis treatment. These results seem to indicate that although the saprophytic fungus is favoured when a pretreatment is used due to improved accessibility of the material and removal of some wood compounds (such as extractives and hemicelluloses), the endophytic fungi are able to act in both untreated and autohydrolised materials with similar intensity. The reason for this could be that the endophytic fungi are adapted to *E. globulus* by evolutionary processes that enable them to produce greater enhancement of enzymatic hydrolysis than the saprophyte assayed.

3.2.2. Substrate composition of samples subjected to autohydrolysis before or after fungal pretreatments

Lignin and sugar composition of pretreated and control samples before enzymatic hydrolysis are shown in table 3. A decrease in Klason lignin and hemicellulose (mainly xylose) contents is observed when autohydrolysis was performed.

The decrease was greater when this pretreatment was combined with fungal pretreatment. The lower lignin content would partially reduce inhibition due to unproductive binding of enzymes onto lignin, thus improving sugar production in pretreated samples, as observed. Although the highest lignin decreases were found when *E. globulus* was treated with *P. smilacis* before or after autohydrolysis pretreatment, these combinations of pretreatment did not produce the greatest enhancement of enzymatic hydrolysis. These results once again indicate that the extent of lignin removal is not always correlated with the enhancement of saccharification yields (Cianchetta et al., 2014; Salvachúa et al., 2011). As in the preliminary assays without autohydrolysis pretreatment, no relation was found between ligninolytic activities (data not shown) and hydrolysis yields, concurring with other authors (Salvachúa et al., 2011).

3.3. FTIR characterisation

Figure S1 shows the FTIR spectra of control and fungal + alkali pretreated samples (with selected endophytes *Ulocladium* sp., *P. smilacis* and *Hormonema* sp. and saprophyte *Trametes* sp. I-62). The spectra were clearly dominated by lignin and carbohydrate (cellulose and hemicelluloses) bands, including a wide band observed around 3330 cm^{-1} representing the O–H stretching vibration in aromatic and aliphatic structure, and the band at 2847 cm^{-1} due to a C–H vibration sketch in the CH_3 group. The bands at 1730 cm^{-1} and 1648 cm^{-1} represented the C=O bonds in lignin of non-conjugated and conjugated ketone groups, respectively. However, the band at 1730 cm^{-1} is also associated with carboxylic acid ester in hemicelluloses. The bands at 1591, 1502 and 1420 cm^{-1} were attributed to aromatic skeleton vibrations in lignin, whereas the band at 1457 cm^{-1} corresponded to the C–H asymmetric vibrations and deformations

(asymmetric in methyl and methylene). The band at 1366 cm^{-1} was assigned to the C–H deformation in cellulose and hemicelluloses and the bands at 1323 cm^{-1} (aromatic ring breathing) and 836 cm^{-1} (out-of-plane C–H bending in position 2 and 6) corresponded to the syringyl lignin units, typical of S-type lignins such as eucalyptus (Ibarra et al., 2004). A set of bands at 1230, 1156, 1107, 1028 and 896 cm^{-1} was assigned to hemicellulose and cellulose. The absorption at 1230 is caused by the C–O stretching of acetyl groups present in hemicellulose molecular chains. The remaining bands corresponded to cellulose, including the C–O asymmetric band observed at 1156 cm^{-1}, the C–OH skeletal vibration associated with 1107 cm^{-1}, the C–O stretching vibration attributed to 1028 cm^{-1}, and the C–H deformation at 896 cm^{-1}.

Despite the preferential lignin removal reported above for the strains studied, no significant changes were observed in FTIR spectra of fungal pretreated samples compared to the control. A decrease in the relative intensity of the lignin band around 1502 cm^{-1} was reported in beech (*Fagus sylvatica* L.) and Scots pine (*Pinus sylvestris* L.) treated with *Coriolus versicolor* and *P. chrysosporium* (Pandey & Pitman, 2003), fungi which preferentially decay lignin. The decrease was accompanied by increased intensity at 1648 cm^{-1}, corresponding to conjugated ketone groups. In addition to the increment at 1648 cm^{-1}, Dorado et al. (1999) observed an increment at 1730 cm^{-1} assigned to non-conjugated ketone groups when wheat straw was treated with ligninolytic fungi *P. chrysosporium*, *Pleurotus eryngii*, *Phlebia radiata* and *C. subvermispora*. These carbonyl group increments point to oxidative alteration of aromatic lignin moieties. However, the mild alkaline extraction carried out in this study immediately after the biological treatments could have released the altered lignin, including oxidised lignin (Dorado et al., 1999), preventing possible alterations in lignin by fungal pretreatment.

When autohydrolysis pretreatment was performed on fungal pretreated samples, several spectral changes were found compared to the control (Figure S2). Intensity of carbohydrate bands at 1730, 1366 and 1230 cm^{-1} decreased significantly in all fungal pretreated samples. In contrast, intensities of absorption bands resulting from lignin at 1591, 1502, 1457, 1420 and 1323 cm^{-1} increased. A shoulder at 1268 cm^{-1}, not observed in the control spectrum, appeared in all fungal pretreated samples at 1230 cm^{-1}. This band is assigned for guaiacyl ring breathing and its appearance may be mainly due to a decrease in the intensity of the 1230 cm^{-1} band resulting from xylan degradation. Similar observations were made when the fungal pretreatment with the selected endophytes and the saprophyte strain was conducted on autohydrolised samples (spectra not shown). Enhanced hemicellulose dissolution by the combination of fungal pretreatment and autohydrolysis was observed.

4. CONCLUSIONS

Endophytic fungi were applied for the first time to enhance saccharification of *E. globulus* and their high potential for this biotechnological application was demonstrated. Two of the selected fungi, *Ulocladium* sp. and *Hormonema* sp., produced greater enhancement in saccharification than various white-rot fungi mentioned in the literature and the one assayed in this work (*Trametes* sp. I-62). This could be due to the highly specific activity of these fungi as primary degraders of the lignocellulosic substrates to which they are adapted by evolutionary processes. The combination of mild autohydrolysis and fungal pretreatment caused a synergistic increase in sugar yields.

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Supplementary material

FTIR spectra of control and fungal pretreated samples without (Figure S1) and with autohydrolysis pretreatment (Figure S2) are provided as supplementary material.

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Figure Caption

Figure 1. Reducing sugar concentration at various enzymatic hydrolysis times for samples pretreated with a) one fungus and b) endophyte-saprophyte sequential pretreatment. Tr: *Trametes* sp. I-62

Figure 2. Glucose (a), xylose (b) and total sugar (c) concentrations during enzymatic hydrolysis of the samples subjected to autohydrolysis before or after fungal pretreatments, and control samples with no pretreatment (Control) or with autohydrolysis but no fungal pretreatment (Control AH).

Table 1. Positive ABTS-oxidation in solid medium by strains isolated from eucalyptus trees.

Strain	ABTS oxidation [a]	Identity [b] (%)	Family	Closest species
AS1M1	+++	98	*Dothioraceae*	*Pringsheimia smilacis*
AS2S4	+	98	*Incertae sedis*	*Phaeomoniella niveniae*
AS3R3	+++	98	*Dothioraceae*	*Hormonema sp*
CA1R5	+	100	*Montagnulaceae*	*Paraconiothyrium variabile*
CA3R3	+++	99	*Botryosphaeriaceae*	*Neofusicoccum luteum*
EX3M2	+++	100	*Pleosporaceae*	*Ulocladium sp.*
EX3S1*	++	91	*Amphisphaeriaceae*	*Leiosphaerella praeclara*
EX3S2*	++	90	*Amphisphaeriaceae*	*Leiosphaerella praeclara*
SE1M1	+++	100	*Botryosphaeriaceae*	*Neofusicoccum australe*
TO1M1§	++	99	*Botryosphaeriaceae*	*Dothiorella sarmentorum*
TO1Y1§	+	99	*Botryosphaeriaceae*	*Dothiorella sarmentorum*
TO2M1§	++	98	*Botryosphaeriaceae*	*Dothiorella iberica*
TO2M2	+	99	*Pleosporaceae*	*Pyrenochaeta cava*
TO2Y1	++	97	*Coniothyriaceae*	*Coniothyrium carteri*
TO3M2	+	97	*Incertae sedis*	*Phaeomoniella niveniae*
TO3M4	+	99	*Pleosporaceae*	*Pyrenochaeta cava*
TO3P1	+	95	*Incertae sedis*	*Tumularia aquatica*

Strains sharing symbols (,§) possessed identical ITS sequences but differed in length.*

[a] *+++ (high oxidation), ++ (medium oxidation) and + (low oxidation).*

[b] *Identity percentage between the strain sequence and the most similar identified accession in GenBank.*

Table 2: Lignin and sugar composition (%) of no autohydrolyzed fungal pretreated and control samples, after alkaline extraction.

	Klason lignin	Glucose	Xylose	Arabinose	Manose
Control AE	22.3 ± 0.2	49.7 ± 0.3	15.8 ± 0.2	0.9 ± 0.1	1.4 ± 0.1
***Trametes* sp.**	20.9 ± 0.4	49.1 ± 0.2	15.4 ± 0.2	0.8 ± 0.1	1.9 ± 0.1
***Ulocladium* sp.**	20.0 ± 0.6	49.6 ± 0.5	15.7 ± 0.4	0.6 ± 0.1	1.9 ± 0.1
P. smilacis	21.0 ± 0.7	49.3 ± 0.3	15.8 ± 0.2	0.7 ± 0.1	1.9 ± 0.2
***Hormonema* sp.**	21.0 ± 0.5	49.1 ± 0.7	15.4 ± 0.5	0.8 ± 0.0	1.9 ± 0.1

Table 3: Lignin and sugar composition (%) of pretreated and control samples before enzymatic hydrolysis, when fungal pretreatment was combined with autohydrolysis pretreatment.

	Klason lignin	Glucose	Xylose	Arabinose	Manose
Control	22.0 ± 0.7	47.8 ± 0.2	15.7 ± 0.2	0.8 ± 0.1	1.3 ± 0.1
Control AH	19.0 ± 0.8	44.4 ± 0.8	14.9 ± 0.1	0.5 ± 0.0	1.3 ± 0.1
AH + *Trametes* sp.	17.8 ± 0.3	47.2 ± 0.3	14.7 ± 0.1	0.6 ± 0.1	1.2 ± 0.1
AH + *Ulocladium* sp.	17.8 ± 0.7	47.5 ± 0.6	13.3 ± 0.4	0.6 ± 0.1	1.2 ± 0.2
AH + *P. smilacis*	15.5 ± 0.8	45.7 ± 0.2	13.8 ± 0.1	0.5 ± 0.0	1.2 ± 0.1
AH + *Hormonema* sp.	18.7 ± 0.4	48.1 ± 0.4	14.6 ± 0.2	0.5 ± 0.1	1.3 ± 0.1
***Trametes* sp. + AH**	16.3 ± 0.5	47.5 ± 0.6	15.4 ± 0.7	0.6 ± 0.1	0.8 ± 0.2
***Ulocladium* sp. + AH**	17.3 ± 0.7	49.7 ± 0.4	14.9 ± 0.6	0.4 ± 0.0	1.3 ± 0.1
***P. smilacis* + AH**	14.7 ± 0.4	46.6 ± 0.8	14.6 ± 0.6	0.5 ± 0.0	1.2 ± 0.1
***Hormonema* sp. + AH**	18.4 ± 0.2	48.1 ± 0.8	13.6 ± 0.5	0.6 ± 0.0	1.3 ± 0.1

Figure 1

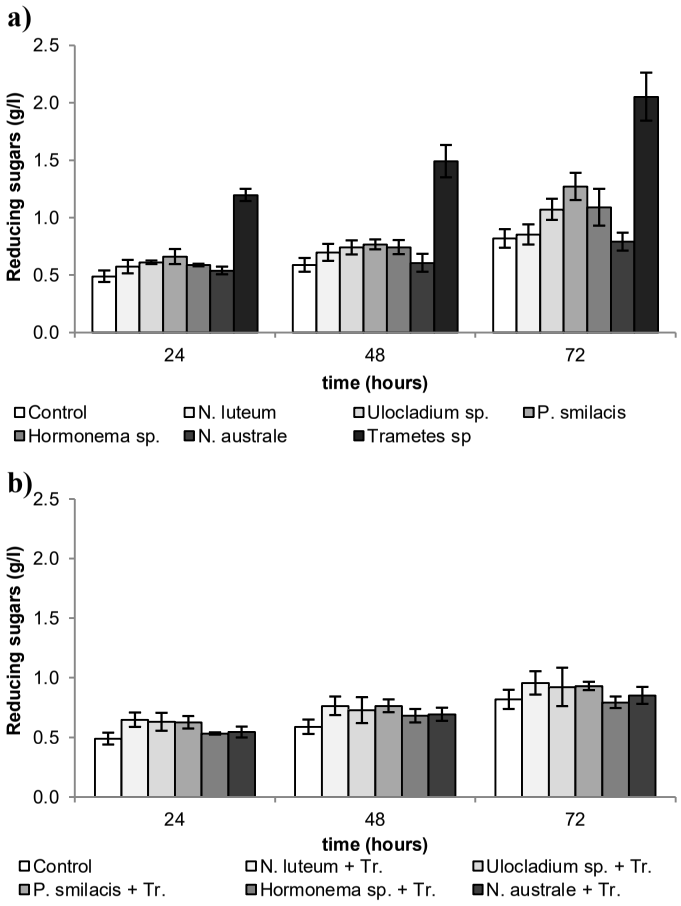
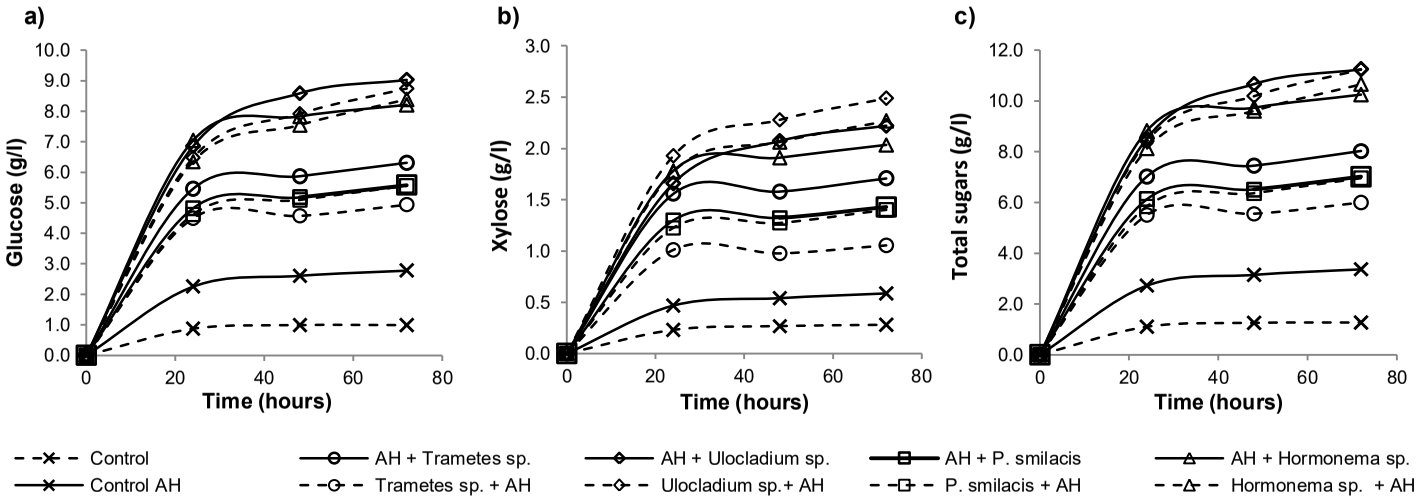

a)
Reducing sugars (g/l)
2.5
2.0
1.5
1.0
0.5
0.0
24
48
72
time (hours)
Control
N. luteum
Ulocladium sp.
P. smilacis
Hormonema sp.
N. australe
Trametes sp
b)
Reducing sugars (g/l)
2.5
2.0
1.5
1.0
0.5
0.0
24
48
72
time (hours)
Control
N. luteum + Tr.
Ulocladium sp. + Tr.
P. smilacis + Tr.
Hormonema sp. + Tr.
N. australe + Tr.

Figure 2


a)
Glucose (g/l)
Time (hours)
b)
Xylose (g/l)
Time (hours)
c)
Total sugars (g/l)
Time (hours)
Control
Control AH
AH + Trametes sp.
Trametes sp. + AH
AH + Ulocladium sp.
Ulocladium sp.+ AH
AH + P. smilacis
P. smilacis + AH
AH + Hormonema sp.
Hormonema sp. + AH

Supplementary Interactive Plot Data (CSV)

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