



Biopulping of wood chips with *Phlebia brevispora* BAFC 633 reduces lignin content and improves pulp quality

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ABSTRACT

The white-rot fungus *Phlebia brevispora* BAFC 633 produces laccases in large proportions. In this work *P. brevispora* BAFC 633 was grown on *Pinus taeda* wood chips in 10-L bioreactors. To select the biopulping experimental conditions, we analyzed the variables affecting enzymatic laccase activity in the culture supernatants, indicating that the suitable incubation temperature was 30 °C in order to promote enzyme stability. *Phlebia brevispora* BAFC 633 secreted 744 U/g of laccase, selectively removing lignin during biotreatment of wood chips, causing a reduction in Kappa number and 10% weight loss, and creating a more open structure and better access to the pulping liquor, which would require less chemical consumption, thus diminishing the environmental impact of the chemical pulping process.

These results support the biotechnological potential of *P. brevispora* BAFC 633 for biopulping processes and enhance the potential for bioprospecting native isolates of the microflora of our country's natural environment.

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1. Introduction

Pulp and paper constitute one of the major manufacturing activities in Misiones, Argentina. The strong demand for paper as a commodity leads to a steady expansion of paper industries in our country. The main objective of the paper manufacturing processes is the removal of lignin from wood; this is the first step in chemical pulping. Kraft pulping is the most widespread process (Da Re et al., 2010). Kraft pulping and bleaching stages use large amounts of chlorine and chloride chemicals (Polaina and MacCabe, 2007; Selvam et al., 2011). The derived products of these chemicals are chlorinated organic substances, some of which are toxic, mutagenic, persistent and can cause numerous harmful disturbances in biological systems (Bajpai and Bajpai, 1997).

In this sense filamentous fungi of the phylum Basidiomycota have been widely studied for their ability to degrade wood (Kirk and Cullen, 1998); these fungi are unique in their ability to degrade most components of wood due to their ability to synthesize the relevant hydrolytic and oxidative extracellular enzymes (Poojary et al., 2012). Within the basidiomycetes, white-rot fungi (WRF) have received special attention because they are the only organisms capable of mineralizing lignin to CO₂ and H₂O (Martinez, 2002) by secreting oxidative enzymes, such as peroxidases and laccases, which have a broad range of substrates (Field et al., 1993). Some white-rot fungi have the ability to selectively remove extensive amounts of lignin with only insignificant losses of cellulose and moderate to low losses of hemicellulose (Blanchette et al., 1985; Otjen and Blanchette, 1986). One of the less harmful and more promising alternatives to improve conventional pulp and paper processes is the use of microorganisms (such as WRF) and enzymes as a treatment for wood chips to reduce lignin content. This alternative process is known as biopulping (Otjen and Blanchette, 1987; Blanchette and Burnes, 1988; Villalba et al., 2006). This process can save substantial amounts of energy,

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improves paper quality, reduces the environmental impact of pulping, and enhances economic competitiveness.

The objective of this study was to evaluate the WRF *Phlebia brevispora* BAFC 633 for biotechnological application in the biopulping of *Pinus taeda* chips.

2. Materials and methods

2.1. Fungal strain and wood chips

Phlebia brevispora BAFC 633 was provided by the Mycological Culture Collection of the Department of Biological Sciences, Faculty of Exact and Natural Sciences, University of Buenos Aires, Argentina. Stock cultures were maintained on malt agar (12.7 g l⁻¹), agar (2% w/v) (MEA) slants at 4 °C with periodic transfer.

Wood chips of *Pinus taeda* were selected for this study because they have regional importance in the production of cellulose, and they are fast-growing trees. There are previous studies using white-rot fungi for the treatment of this substrate (Otjen and Blanchette, 1987; Blanchette and Burnes, 1988; Villalba et al., 2006).

2.2. Preliminary studies of laccase temperature and pH optimum, and enzyme thermo-stability on culture supernatants

Preliminary tests were performed using culture supernatants from 50 ml of ME medium (12.7 g l⁻¹ malt extract and 5 g l⁻¹ corn steep liquor) at 10 days of incubation, pH 4.5, in static conditions to establish optimal conditions for laccase activity. The optimum temperature of laccase was determined between 30 and 80 °C. The optimum pH for laccase was determined using sodium acetate buffer 0.1 M at a pH range of 3.6–5.6.

For thermo-stability evaluation during the enzymatic bioprocess, culture supernatants were incubated continuously for 7 h at optimum pH and temperature. The effect of temperature on stability of laccase on culture supernatants was determined for 7 h at 20, 30, 40, 50, and 60 °C.

2.3. Preparation of fungal inocula

One agar plug (0.5 mm²) of fungus grown on an MEA plate at 29 °C for 5 days was added to 50 mL of ME medium and incubated at 29 °C statically for 10 days. For the inoculation experiments, the mycelium was removed under aseptic conditions, washed in sterile distilled water three times, mashed for 5 s to achieve a homogenous mixture, and finally added to the substrate.

2.4. Wood preparation

Ideal chip size for commercial pulping is 1 × 2.5 × 0.3 cm, so in order to achieve these dimensions, *P. taeda* chips from the sawmill were carefully selected and separated manually. After classification, they were thoroughly mixed and placed in dishes. Three aliquots of the initial chips were taken to determine the moisture content. Wood chips were autoclaved for 30 min at 121 °C to prevent contamination by microorganisms that can antagonize or inhibit the growth of fungus. Subsequently, 400 g (on a dry weight basis) under sterile conditions were poured into the 10-L bioreactors.

2.5. Inoculation procedure

Crushed fungus mycelium was used as inoculum (0.5 mg mycelium/g chips) and poured over the wood chips with extensive mixing. Sodium acetate buffer 0.1 M pH 3.6 was added to the system to reach 65% moisture content. The incubation step was carried

out at bioreactors maintained at 29 °C in an environmentally controlled chamber. The experiments were conducted in duplicate.

After 30 days, the incubation period was completed. The chips were air-dried and stored at –20 °C in plastic bags to stop fungal activity before pulping.

2.6. Weight loss measurement

Before incubation, the wood chips were dried to constant weight at 80 °C, and the initial weight was calculated. After the incubation, the wood chips were washed with sterile distilled water and filtered to remove residual mycelium. The washed chips were dried at 80 °C to constant weight, and the weight loss was calculated based on the initial and final dry weights.

2.7. Microscopic characteristics

Optical microscopy was used to visualize morphological changes in the wood as a result of fungal degradation. To obtain thin slices (0.1 mm thickness) for microscopic analysis, the commercial chip samples were taken from each of the treatments and controls, boiled for 3 h with distilled water, and then sliced by hand using a scalpel. The wood sections were stained according to the technique described by Isenberg (1967).

2.8. Enzyme assays

Laccase (EC 1.10.3.2) activity was measured at 30 °C using 5 mM of 2,6-dimethoxyphenol (DMP) in 0.1 M sodium acetate buffer pH 3.6. Absorbance was monitored at 469 nm ($E_{469} = 27.5 \text{ mM}^{-1} \text{ cm}^{-1}$) in a Shimadzu UV-3600 spectrophotometer. One laccase activity unit was defined as the amount of enzyme required to oxidize 1 µmol of DMP per minute at 30 °C and expressed as U ml⁻¹ (Moreira et al., 2004).

Endo-β-1,4-glucanase (EC 3.2.1.4) activity was determined by measuring the liberation of reducing sugar with the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959) using 0.5% carboxymethylcellulose (w/v) as substrate in 0.05 M sodium citrate buffer pH 5. Reactions were incubated at 50 °C for 30 min. Absorbance was measured at 540 nm in a Shimadzu UV3600 spectrophotometer. The carbohydrate fraction was extracted from the culture supernatant, and the amount of sugar liberated was calculated using a glucose standard curve. One endo-β-1,4-glucanase activity unit was defined as the amount of enzyme that releases 1 µmol of reducing sugar per minute at 50 °C.

Endoxylanase activity was determined indirectly through reducing sugars released by hydrolysis of soluble xylan beechwood (Sigma–Aldrich, USA) and subsequent detection by the 3,5-dinitrosalicylic acid method (DNS) previously described by Miller (1959).

The enzyme reaction was carried out at 50 °C for 60 min in sodium acetate buffer 0.05 M (pH 4.8) containing 1% beechwood soluble xylan (w/v). One endoxylanase unit was defined as the amount of enzyme that releases 1 µmol of reducing sugar per minute under assay conditions.

Enzyme activities were calculated on a chip weight basis and reported in U g⁻¹.

2.9. Analysis of wood composition

The chemical composition of non-treated and biotreated wood chips was determined according to the laboratory analytical procedure (LAP) and biomass analysis of the National Renewable Energy Laboratory (NREL). Sample preparation for compositional analysis was done according to NREL/TP-510-42620 (2008). Ethanol

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