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Lignocellulolytic enzymes from *Fomes sclerodermeus* growing in solid-state fermentation

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Abstract

To investigate the growth and production of lignocellulosic enzymes from *Fomes sclerodermeus*, solid-state fermentation (SSF) was performed by using soy and wheat bran. Both media showed similar maximal values of dry weight loss of the substrate and biomass production, nevertheless enzyme production was markedly different according to the substrate used. Cellulases, xylanases and pectinases in soy bran medium reached the highest activity at 12 days of fermentation, while maximal manganese peroxidase (MnP) activity occurred at 15 days post-inoculation (14.5 U g^{-1}), laccase peaked (520 U g^{-1}) at the last sampling day (28). Using the medium with a mixture (1:1) of wheat and soy bran, the hydrolases reached the maximum value at 15 days being the amounts of pectinases produced 3-fold higher than those obtained using soy bran as substrate. However, the amounts of ligninases were lower, the values measured for MnP and laccase were 4.64 and 66.7 U g^{-1}, respectively. Therefore, the high enzyme production along with the very low cost of the substrate, showed the suitability of the system *F. sclerodermeus*-SSF for industrial purposes, being the enzyme production different for each enzymatic system according to substrate composition.

Keywords: *Fomes sclerodermeus*; Endoglucanase; Endoxylanase; Laccase; Polygalacturonase; Manganese peroxidase

1. Introduction

Enzyme production is a growing field of biotechnology. Most enzyme manufacturers produce enzyme using submerged fermentation (SmF) techniques. There is, however, a significant interest in using solid-state fermentation (SSF) techniques to produce a wide variety of compounds such as antibiotics (Adinarayana et al., 2003), organic acids (Kumar, Jain, Shanker, & Srivastaba, 2003) and a wide variety of enzymes (Pandey, Selavakumar, Soccol, & Nigam, 1999). In general, enzyme titers in SSF are higher than in SmF, when comparing the same strain and fermentation broth (Romero-Gómez, Augur, & Viniegra-González, 2000; Viniegra-Gonzáles et al., 2003).

The organisms able to degrade efficiently the major components of lignocellulosic materials, cellulose, hemicellulose and lignin, are white rot fungi. These fungi possess hydrolytic enzymes like cellulases, pectinases and xylanases, which typically are induced by their substrates. Lignin is a polymer of phenylpropane units connected by different C–C and C–O–C linkages. This molecule is degraded by a ligninase complex (LC) composed of at least three enzyme activities: lignin peroxidase (LiP) (Tien & Kirk, 1983), manganese dependent peroxidase (MnP) (Glenn & Gold, 1985) and laccase. LiP is a heme protein with high oxidation potential. This enzyme can oxidize phenolic and non-phenolic substrates (Banci, Ciofi-Baffoni, & Tien, 1999; Mester et al., 2001). MnP is considered unable to oxidize non-phenolic substrates, although its capacity to depolymerizate synthetic (Wariishi, Valli, & Gold, 1991) or natural (Hatakka, Lundell, & Hatakka, 2001) lignins *in vitro* has been shown. Laccase belongs to a family of multicopper oxidases, which has a wide range

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of reducing substrates like polyphenols and methoxy-substituted phenols (Thurston, 1994). The presence of appropriate primary substrates like ABTS [2,2'-azino-*bis* (3-ethylbenzthiazoline-6-sulfonic acid)] or 1-HBT (1-hydroxybenzotriazole) can act as cooxidants extending its range of substrates (Bourbonnais, Paice, Freiermuth, Bodie, & Borneman, 1997). Cellulose is a linear polymer of glucose units, which can be hydrolyzed by the action of endoglucanases, cellobiohydrolases and β-glucosidases. Hemicellulose is a heterogeneous, branched polymer. The backbone of the polymer is built up by sugar monomers like xylose, in this case xylanases are the enzymes involved in its degradation. Similar to cellulases the xylanases can act synergistically to achieve hydrolysis, predominant enzymes within this system are endo 1,4 β-xylanases which attack the polysaccharide backbone, and β-xylosidases, which hydrolyze short xylooligosaccharides to xylose (Coughland & Hazlewood, 1993). Pectin is a polymer built of polygalacturonic acid backbone with rhamnose interruption with short side chains of neutral sugar mainly of arabinose and galactose. This molecule can be hydrolyzed by the action of pectinases.

All of these enzymes are industrially important, therefore, organisms able to produce them are interesting in view of the potential importance in industrial processes like bioremediation, biobleaching of pulp paper, degradation and detoxification of recalcitrant substances or in the food industry, thus the efficient production of these enzymes in a low-cost medium is interesting for biotechnological applications.

Fomes sclerodermeus BAFC 2752 is a white rot basidiomycete isolated in Tucumán, Argentina. Previously, the production of both laccase and MnP by this fungus in defined liquid media was demonstrated (Papinutti & Forchiassin, 2000). The aim of this work was to study the growth and production of predominant lignocellulases by the white rot fungus *F. sclerodermeus* in natural low-cost media containing as substrates solid residues originating from two of the most important cultures worldwide, soy and wheat.

2. Materials and methods

2.1. Organism and culture conditions

Fomes sclerodermeus (Léveillé) Cooke BAFC 2752 (Cepario Micológico de la Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires), was maintained in MEA (malt extract 1.2%, glucose 1%, agar 2%) medium at 4 °C.

Soy bran (4–12 mesh) and wheat bran (8–20 mesh) purchased at a local market were used as fermentation substrates. Two different media were prepared, soy bran 4 g (SB) or soy bran 2 g plus wheat bran 2 g (SWB), distilled water 16 ml. The media were sterilized by autoclaving at 121 °C for 20 min. Erlenmeyer flasks were inoculated with 2 agar cubes (25 mm^2) cut out from the margin of a 10-d-old colony growing on MEA medium. Cultures were incubated at 28 °C and 90% humidity to avoid evaporation, and they lasted for 28 d in total.

Weight losses were determined by drying the content of each flask to constant weight at 80 °C. Crude extract was obtained by adding distilled water (5:1, w/w) stirring for 20 min, followed by filtration and centrifugation. All of the steps for crude extraction were performed at room temperature. The supernatant was stored at −20 °C until needed. For all experiments, measurements were carried out in triplicate parallel cultures. The values are reported as the mean with a standard deviation (SD) less than 10%.

2.2. Analysis of proteins, reducing sugars and chitin

Soluble proteins in the crude extract were determined by the Bradford method (Bradford, 1976) using BSA as the standard. The chitin content of dried samples and mycelium from malt extract liquid cultures was determined by measuring *N*-acetylglucosamine (NAGA) released from chitin after hydrolysis with 6N HCl (Plassard, Mousain, & Salsac, 1982). Reducing sugars of the crude extract were assayed by the method of Somogyi and Nelson (Nelson, 1944) using glucose as the standard.

2.3. Enzyme assays

Predominant enzymes of each group were measured. Cellulases and xylanases were incubated at 50 °C; polymetilgalacturonase (PMG), polygalacturonase (PG), MnP and laccases were assayed at 30 °C. Laccase: was determined at 420 nm ($\varepsilon_{420} = 36\ mM^{-1}\ cm^{-1}$) using 5 mM ABTS as substrate (Bourbonnais, Paice, Reid, Lanthier, & Yaguchi, 1995). Measurements were made in 0.1 M sodium acetate buffer, pH 3.6. Manganese peroxidase (MnP): was determined using phenol red as a substrate. The reaction product was measured at 610 nm ($\varepsilon_{610} = 22\ mM^{-1}\ cm^{-1}$) (Glenn & Gold, 1985). The reaction mixture contained 50 mM succinate buffer, pH 4.5, 0.01% phenol red. The addition of H_2O_2 (0.1 mM final concentration) initiated the reaction. Endoglucanase, endoxylanase, PMG and PG were determined measuring the reducing sugars produced after hydrolysis of the substrate by the Somogyi–Nelson method (Nelson, 1944). Measurements were made in 0.1 M sodium acetate buffer, pH 4.8, using the following substrates: carboxymethycellulose (CMC) 0.5% for endoglucanase; xylan from oat spelt 0.2% for endoxylanase; pectin from apple 0.1% for polymethylgalacturonase (PMG); polygalacturonic acid for polygalacturonase (PG). Enzyme activity has been expressed in International Units (U), as the amount of enzyme needed to release 1 μmol of product in 1 min. In terms of production, the activity was defined as U g^{-1} dry wheat/soy bran (U g^{-1}).

3. Results

In this study, the growth and lignocellulolytic enzyme production by *F. sclerodermeus* were characterized. The