Conditions for selective degradation of lignin by the fungus *Ganoderma australis*

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Summary. The white-rot fungus *Ganoderma australis* selectively degrades lignin in the ecosystem “palo podrido”. Using conditions that simulate those of “palo podrido” in the laboratory, it was found that low nitrogen content and low O_2_ tension stimulate the production of manganese peroxidase and lignin degradation, and depress cellulose degradation and cellulase production. The inverse is found at high nitrogen concentration and high O_2_ tension. This agrees with previous results indicating that low O_2_ tension and low nitrogen stimulate selective lignin degradation by this fungus.

Introduction

The ecosystem called “palo podrido” (in English, “rotten wood”), mentioned by Philippi (1893), and first described in detail by Knoche et al. (1929), occurs in the rain forests of southern Chile. It is characterized by a selective degradation of lignin by fungi and bacteria in native trees (e.g. *Nothofagus dombeyi*). Since the cellulose is conserved, “palo podrido” can be used as feed for cattle. The principal agent responsible for lignin degradation in this system was identified as the fungus *Ganoderma applanatum* (Dill and Kraepelin 1986). However, Martinez et al. (1991), based on fungal fatty acid composition, propose that the fungus found in “palo podrido” is a strain of *G. australis*.

Several groups have worked in the identification of the microorganisms present in “palo podrido” (Zadrazil et al. 1982; Dill and Kraepelin 1986; Dill and Kraepelin 1988; González et al. 1986) and in analysing its degradation products (Dill and Kraepelin 1986, 1988; González et al. 1986; Agosín et al. 1990). Recently, Dill and Kraepelin (1988) have formulated the hypothesis that selective degradation of lignin occurs under conditions of high humidity, low nitrogen content and low O_2_ tension. The purpose of this work was to analyse the effect of nitrogen content and O_2_ supply on the degradation of wood components by *G. australis*, and correlate them with the production of certain lignocellulolytic enzymes by the fungus.

Materials and methods

*Fungal strains.* *G. australis* was isolated from a sample of “palo podrido”. The fungus was grown for about 15 days in 1.5% agar plates containing 0.1% yeast extract and 2% malt extract at 22 °C, and kept for longer periods at 4°C in the same medium.

*Culture conditions.* Solid fermentation cultures were performed in 250-ml erlenmeyer flasks containing *N. dombeyi* wood chips (about 0.4 × 0.7 cm in size) and a humidity of 40%. This humidity was attained by adding a basal medium (sterile) containing the salts and vitamins described by Kirk et al. (1978). In order to establish the amount of medium required, the humidity present in the original wood was estimated by means of its dry weight. This humidity was chosen so that the wood chips would be slightly wet, to mimic the conditions of the Chilean rain forests. Since *G. australis* is a non-sporulating fungus, the cultures were inoculated each with ten 0.4 × 0.4 cm fragments of agar-free mycelium. The erlenmeyer flasks were kept tightly capped with rubber stoppers, and were incubated for 65 days at 22°C.

Aerobic conditions were kept in some flasks, subjecting them to aeration every 3 days by blowing humid sterile air for 2 min. Low O_2_ tension was achieved in other flasks by blowing for 3 min with sterile N_2_ at the beginning of the experiment, and keeping the flasks sealed for the duration of the incubation. Cultures were performed in triplicate.

The effect of nitrogen was studied as follows: certain flasks (low nitrogen) contained no added nitrogen (beyond that present in the wood), while others (high nitrogen) were supplemented with arginine (15 mM nitrogen) added dissolved in the basal medium.

*Enzyme recovery from the cultures.* At the end of 65 days, the chips were washed in order to extract enzymes produced by the fungus: 50 mM sodium acetate buffer pH 4.8 was added to the flasks (7% of the total flask volume); the flasks were shaken in a rotary shaker for 15 min at 100 rpm and 22°C, and the supernatants were recovered by centrifugation at 1100g for 15 min and passed through a glass filter.

*Wood extraction.* Some experiments were performed with intact chips and others with chips subjected to the following extraction procedure (Crawford 1978): (i) 48 h extraction in a Soxhlet with a 1:1 toluene-absolute ethanol mixture; (ii) three washes with the...
same solvent mixture; (iii) three ethanol washes; and (iv) air drying at room temperature.

Determination of enzyme activities. Endoglucanase activity was tested using 0.5% carboxymethylcellulose (medium viscosity) (Sigma, St. Louis, Mo., USA) as substrate incubated for 20 min at 50°C in 50 mM sodium acetate buffer, pH 4.8, the reducing sugars formed being determined by the Somogyi-Nelson method (Nelson 1944) (glucose was used as standard). Xylanase was measured using 0.5% oat spelt xylan (Sigma) as substrate in 50 mM sodium acetate buffer, pH 5.5, incubating the mixture for 10 min at 50°C and determining the reducing sugars as above (xylose was used as standard). One unit of activity was defined as the amount of enzyme producing 1 µmol reducing sugar per minute per millilitre of assay under the conditions used.

Manganese (Mn) peroxidase was estimated according to the method of Paszczynski et al. (1985) (reaction mixture: 0.1 mM vanillylacetone (Sigma), 0.1 mM MnSO4, 0.05 mM H2O2, 100 mM sodium tartrate buffer, pH 4.8) at 37°C. Activity was detected by recording the disappearance of the substrate at 336 nm, and was expressed as change in absorbance per minute per millilitre of assay. Table 1 shows that the activity measured is dependent on the presence of H2O2 and can be suppressed by complexing the Mn2+ with EDTA; if additional Mn2+ is added together with EDTA, activity is recovered.

Protein and dry weight determination. Protein was assayed by the method of Lowry as modified by Tan et al. (1984). Dry weight of the wood was estimated by incubating the chips at 105°C until they attained constant weight.

Determination of lignocellulose components in wood. The method of Effland (1977) was used for estimation of the lignin, cellulose and hemicellulose of wood. The results are expressed as the percentage dry weight of the wood.

Results and discussion

The fungus grew very slowly in the solid fermentation flasks containing unextracted wood, and enzyme activities were practically undetectable in the wood washings. Therefore, all the data from solid fermentation cultures discussed below correspond to fungi grown in extracted wood.

Table 2 shows the endoglucanase, Mn peroxidase and xylanase activities detected in cultures with and without added nitrogen and at high and low O2 tensions. The data indicate that high nitrogen content and high O2 tension stimulate the production of endoglucanase, while Mn-peroxidase and xylanase activities are higher at low nitrogen and O2. Fungal growth, estimated by visual inspection, (data not shown) is higher in the flasks with high nitrogen and O2, corresponding to the conditions of higher cellulase production and degradation of the primary substrate, cellulose. This is confirmed by the loss of dry weight, as shown in Table 3, which is highest for the samples taken from flasks containing high nitrogen and/or high O2.

Table 3 shows also the cellulose, hemicellulose and lignin content of the wood samples used for the same experiment, expressed as a percentage of the dry weight. Greatest losses in cellulose content were found in the samples from cultures grown at high nitrogen and O2, while lignin losses were highest under low nitrogen and microaerobic conditions. These findings correlate well with the endoglucanase and Mn-peroxidase levels found. No significant difference, however, was observed for the hemicellulose content.

Our results show that G. australis is capable of producing cellulases under certain culture conditions, as would be expected from a white-rot fungus. The production of these cellulases is supposedly inhibited or repressed under the conditions found in "palo podrido".

Our findings agree with the hypothesis put forth by Dill and Krapelin (1988) that lignin degradation is stimulated under microaerobiosis and low nitrogen content in the medium. Under those conditions production of cellulases is low while the levels of xylanase and Mn-peroxidase were the highest found. Lignin degradation was also maximal. These results do not agree with the conditions found to be optimal for lignin degradation by the white-rot fungus Phanerochaete chrysosporium. This fungus shows highest lignin degradation under 100% oxygen (Kirk et al. 1978). Reid and Seifert (1982), using several white-rot fungi, have shown that O2 stimulates not only the degradation of lignin but also that of the wood polysaccharides; therefore, selectivity of degredation would not be enhanced under these conditions. Different regulatory mechanisms may be responsible for the wood degradation processes in G. australis as compared to the other white-rot fungi. These mechanisms are as yet unknown.

It has been found that P. chrysosporium grows optimally at 38°C, while it is more active in lignin degradation at 28°C (Drew and Kadam 1979). Hence, it will be of interest to study the effect of temperature on fungal growth and lignin degradation by G. australis, and to determine any possible correlation between these variables.