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Laccase activities of *Penicillium chrysogenum* in relation to lignin degradation

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Abstract An extracellular laccase capable of oxidizing ABTS (the diammonium salt of 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) was detected in ligninolytic cultures of *Penicillium chrysogenum*. By contrast, no lignin peroxidase, manganese-dependent peroxidase or aryl-alcohol oxidase was detected at any time during culturing. Both ABTS laccase activity and mineralization of dehydrogenative polymerizate of coniferyl alcohol were regulated by the C/N ratio in the medium and partially inhibited in the presence of thioglycolic acid, suggesting that both events are associated. In the presence of several known laccase inducers neither ABTS laccase activity nor mineralization rates were enhanced. However, a new laccase was detected in *P. chrysogenum*, able to oxidize 2,6-dimethoxyphenol but not involved in lignin mineralization. Studies with the known ligninolytic basidiomycete *Trametes villosa* suggest that lignin degradation by this fungus also involves the action of laccase.

Introduction

Laccases (*p*-diphenol oxidase, EC 1.10.3.2) and peroxidases are two groups of enzymes involved in lignin degradation by fungi. However, some ligninolytic fungi from various ecological niches have very different enzyme activity patterns. Lignin peroxidase was first detected with *Phanerochaete chrysosporium* (Tien and Kirk 1983) and then with other white-rot fungi. However, laccases are not produced by *Phanerochaete chrysosporium*, but have been demonstrated for most other ligninolytic white-rot fungi, including *Trametes*, *Pleurotus* and *Phlebia* spp (Hüttermann et al. 1989). In *Dichomitus squalens*, extracellular manganese peroxidase and laccase were the only activities detected (Perie and Gold 1991). Its laccase is believed to be capable of demethoxylating lignin and its manganese peroxidase should be an important ligninolytic enzyme.

In spite of recent progress, more information is still needed, especially to reveal the ligninolytic systems in the different groups of fungi. White-rot fungi are responsible for lignin degradation in wood. However, most C turnover from plant-residue lignin in soil cannot be attributed only to white-rot fungi. Degradation of lignin by soil-inhabiting fungi imperfecti has been reported by many authors (Norris 1980; Milstein et al. 1984; Kadam and Drew 1986; Carnicero et al. 1992; Rodríguez et al. 1994; Falcón et al. 1995), but the responsible systems have been studied insufficiently.

The purpose of the present work was to characterize the ligninolytic activities of a *Penicillium chrysogenum* strain isolated from soil on the island of Tenerife (Falcón et al. 1995). For comparison, a known ligninolytic fungus from the Basidiomycetes class – *Trametes villosa* – was also included in the study.

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Materials and methods

Microorganisms

Penicillium chrysogenum (MUCL 31363) was isolated through screening for ligninolytic microorganisms from pine forest soil in Tenerife, Canary Islands (Rodríguez et al. 1994; Falcón et al. 1995). *Trametes villosa* (*Polyporus pinsitus*) was obtained from the CBS-collection, Baarn, The Netherlands, strain no. 678.70.

Media and culture conditions

P. chrysogenum was cultured as described earlier (Rodríguez et al. 1994) in media with a low glucose concentration (1 $g l^{-1}$). Nitrogen at concentrations of 51.2, 25.6 and 0.16 mM was added as a combination of L-asparagine and NH_4NO_3 to give the following C/N ratios: 0.65, 1.3 and 200 respectively. *T. villosa* was grown in the culture medium according to Haider and Trojanowski (1975) with glucose (5 $g l^{-1}$) as carbon source and L-asparagine (0.6 $g l^{-1}$) as nitrogen source to give a C/N ratio of 8.3 (optimal for this species).

Unlabelled dehydrogenerative polymerizate of coniferyl alcohol (DHP) was at 80 μg ml^{-1} concentration in the cultures for enzyme assays. Specifically β-^{14}C-labelled DHP was added to give 40 nCi/culture for degradation studies. Both DHP were prepared following the method of Haider and Trojanowski (1975).

Cultures of *P. chrysogenum* were occasionally supplemented with Kraft pine lignin polymer (0.1 $g l^{-1}$) purified as described earlier (Rodríguez et al. 1994), Tween 80 (0.2 $g l^{-1}$), thioglycolate (2 mM), xylidine (0.05 mM) or benzyl or veratryl alcohol (0.1 mM), to test the effect of these compounds on fungal activities and lignin degradation (mineralization). Cultures of *T. villosa* were occasionally supplemented with organosolv lignin (1 $g l^{-1}$) or xylidine (0.05–0.5 nM) to stimulate the enzymatic activities.

Experimental cultures were grown in 250-ml conical flasks containing 25 ml medium with shaking (50 strokes/min) at 28°C.

Enzyme assays

Laccase, lignin peroxidase, manganese-dependent peroxidase and aryl-alcohol oxidase were periodically assayed in cultures of *P. chrysogenum* and *T. villosa* throughout the incubation period. Cells were harvested by centrifuging and extracellular fluids were occasionally concentrated tenfold by ultrafiltration using a filter with a 10-kDa-molecular-mass cut-off. Time-course results represent the average values from two individual flasks.

Laccase activity was routinely determined by monitoring the oxidation of the diammonium salt of 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) or 2,6-dimethoxyphenol (DMP) at 420 nm (Bourbonais and Paice 1990) and 468 nm (Paszczynski et al. 1985) respectively. The reaction mixture (1 ml) contained 2 mM ABTS in a mixture of 100 mM sodium lactate/sodium succinate pH 5 containing bovine albumin (1 mg ml^{-1}) or 2 mM 2,6-dimethoxyphenol in 200 mM sodium tartrate, pH 5, and 250–500 μl culture fluid.

Occasionally laccase activity in cultures of *T. villosa* was estimated using tetramethylbenzidine (Trojanowski and Hüttermann 1990).

Lignin peroxidase was assayed by monitoring the oxidation of veratryl alcohol at 310 nm (Tien and Kirk 1984). The reaction mixture (1 ml) contained 2 mM substrate in 100 mM sodium tartrate pH 3.5 and 250–500 μl culture fluid. The reaction was initiated with the addition of 0.05–0.1 mM H_2O_2.

Manganese-dependent peroxidase was assayed using phenol red as substrate (Johansson and Nyman 1987). For a total volume of 1 ml, the reaction mixture contained 50 μg phenol red, 0.1 mM H_2O_2, 0.1 mM $MnSO_4$, 100 mM sodium succinate pH 5, 100 mM lactate, 1 mg bovine serum albumin and 250–500 μl culture fluid.

Aryl-alcohol oxidase was assayed by monitoring the oxidation of veratryl alcohol in the absence of H_2O_2.

Extracellular protein was measured by the method of Bradford (1976) using bovine serum albumin as standard.

Lignin degradation

Lignin mineralization was monitored using the radiorespirometric method (Haider and Trojanowski 1975). $^{14}CO_2$ released from metabolized β-^{14}C-labelled DHP was trapped in 10% (w/v) NaOH and the radioactivity measured in a liquid scintillation counter (Optiphase III, Kabi Pharmacia). All experiments were done in triplicate and degradation of lignin was expressed as the percentage of added [^{14}C]DHP radioactivity recovered as $^{14}CO_2$.

Results and discussion

In previous reports we have described the isolation of several soil fungi, capable of affecting the polymeric structure of lignin in pure culture (Rodríguez et al. 1994; Falcón et al. 1995). In particular, a strain of *P. chrysogenum* was able to degrade and transform some synthetic and industrial lignins. Moreover, lignin degradation by *P. chrysogenum* is affected by culture conditions (Rodríguez et al. 1994). In this work we confirmed this earlier finding by using ^{14}C-labelled synthetic lignin (DHP). *P. chrysogenum* mineralized 7.9% of the [β-^{14}C]DHP when grown in medium having an optimal C/N ratio of 1.3 (Fig. 1A). The highest $^{14}CO_2$ yield evolved per milligram of mycelium (6%) was also obtained in the culture having a C/N ratio of 1.3. By contrast, optimal rate of lignin degradation by *T. villosa* requires a C/N ratio of 8.3 (data not shown).

In this work we studied the production of extracellular ligninolytic activities. In the ligninolytic cultures of *P. chrysogenum* no lignin peroxidase, manganese peroxidase or aryl-alcohol oxidase could be detected even after the culture filtrates had been concentrated tenfold. At the same time, a laccase active was detected on ABTS but not on 2,6-dimethoxyphenol. This activity could be totally inhibited with a Cu-chelating agent, 0.02 mM thioglycolic acid.

The time courses for extracellular ABTS laccase activity as a function of the different C/N ratios are shown in Fig. 1B. The highest ABTS laccase activity was observed in the culture having a C/N ratio of 1.3 but the activity followed the same time course in all media assayed. It rose to a peak after 5–7 days of fungal growth and then declined rapidly. Therefore, the optimal C/N ratio for DHP mineralization was also the optimum for ABTS laccase production. In addition, more than 75% of the $^{14}CO_2$ evolved by *P. chrysogenum* was released between days 3 and 14, correlating with the expression of ABTS laccase. These results suggest that ABTS laccase activity is involved in the degradation of lignin by *P. chrysogenum*.

When 2 mM thioglycolic acid was added to the cultures of *P. chrysogenum* after 4 days of fungal growth, the ABTS laccase activity began to decrease until it was undetectable on day 9 (Fig. 2A). However, after this decline it rose to a second peak on day 11. The drastic decrease in ABTS laccase activity observed between days 7 and 9 in the presence of thioglycolic acid correlates with a decrease of up to 65% on day 8 in the DHP mineralization rates (Fig. 2B).

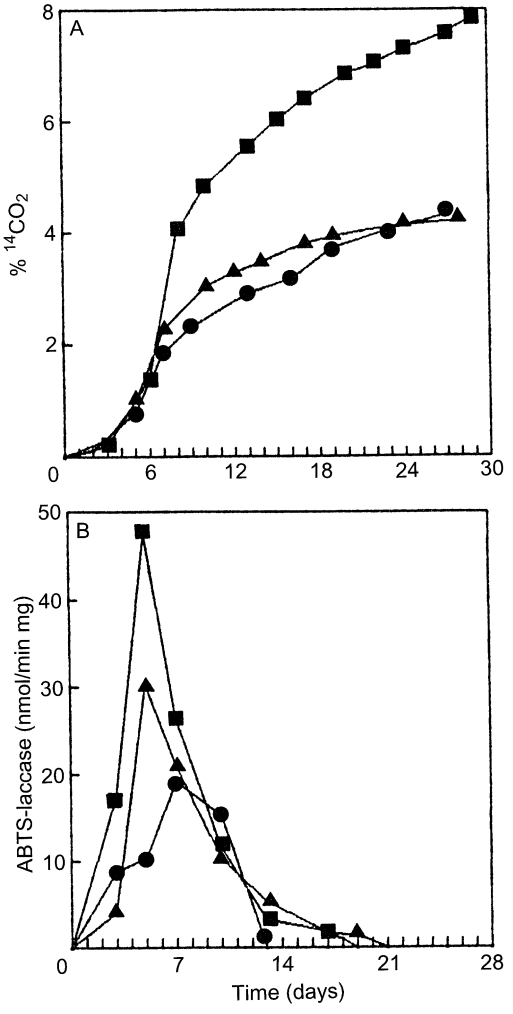

Fig. 1A, B Cumulative $^{14}CO_2$ released from β-^{14}C(side-chain)-labelled dehydrogenative polymerizate of coniferyl alcohol (% initial) (**A**), and laccase activity on the diammonium salt of 2,2′-azinobis-3-ethylbenzothiazoline (**B**), in cultures of *P. chrysogenum* grown in liquid media having a C/N ratio of 1.3 (■), 0.65 (▲) or 200 (●). Degradation experiments were performed in triplicate and the standard error was less than 10% of the mean. Activity values are the averages from two individual cultures

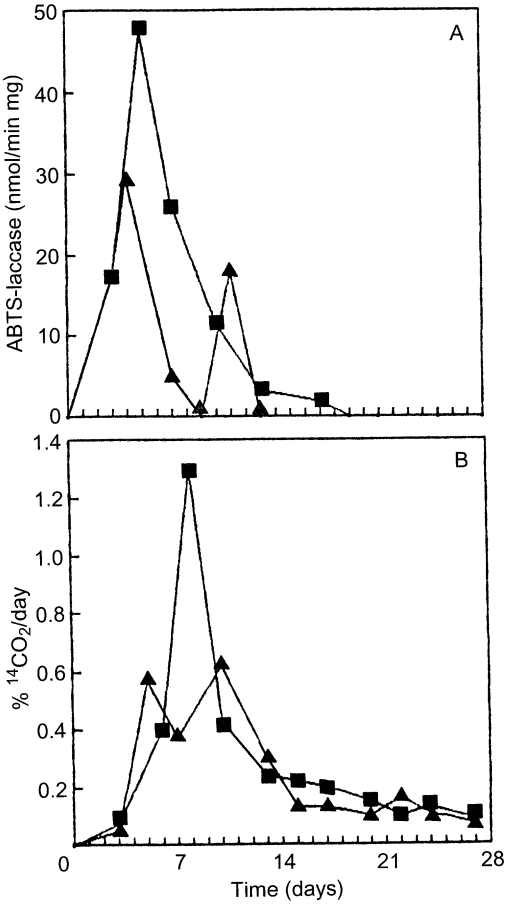

Fig. 2A, B Effect of thioglycolic acid on the ABTS laccase activity (**A**), and on the $^{14}CO_2$ released per day (**B**). *P. chrysogenum* was cultured in the medium with a C/N ratio of 1.3 with (▲) or without (■) addition of 2 mM thioglycolic acid 4 days after inoculation. Measurement details as in Fig. 1

It has been shown that addition of detergents (Rüttimann et al. 1992), aromatic compounds such as lignin (Rogalski et al. 1991) or xylidine (Fahraeus and Reinhammer 1962; Trojanowski and Hüttermann 1990) to fungal cultures may increase the production of laccase. Unexpectedly, when Tween 80, Kraft lignin, xylidine or benzyl or veratryl alcohol was added to cultures of *P. chrysogenum* a significant decrease in ABTS laccase activity was observed (Fig. 3). However, in the presence of these compounds, a second laccase, active only on 2,6-dimethoxyphenol but not on ABTS, was detected. This laccase activity on 2,6-dimethoxyphenol did not correlate over time with that on ABTS-laccase (Fig. 3). The effect of some of these 2,6-dimethoxyphenol laccase inducers is shown in Fig. 4. In the presence of Tween 80 or Kraft lignin a decrease in DHP mineralization of up to 44% was observed. This suggested that 2,6-dimethoxyphenol laccase activity is not involved in lignin degradation by *P. chrysogenum.*

Our studies with a known ligninolytic basidiomycete *T. villosa* suggest that lignin degradation by this fungus also involves the action of laccase. After 28 days, *T. villosa* mineralized 13.9% of the labelled DHP. We found high laccase levels in the cultures of *T. villosa*, when induced with xylidine or by organosolv lignin. However, only very low levels of lignin peroxidase activity were found in the cultures of this basidiomycete and the level of lignin peroxidase could not be induced by lignin or xylidine (Table 1). Although it has been reported that *T. villosa* produces manganese peroxidase (De Jong et al. 1992), extracellular Mn-dependent peroxidase activity was not detected in this study despite routine assays. The low Mn(II) concentration of the medium (1.8 ppm) may explain the absence of Mn-dependent peroxidase since expression of the genes for this enzyme is regulated in white-rot fungi directly by the Mn(II) concentration (Bonnarme and Jeffries 1990).

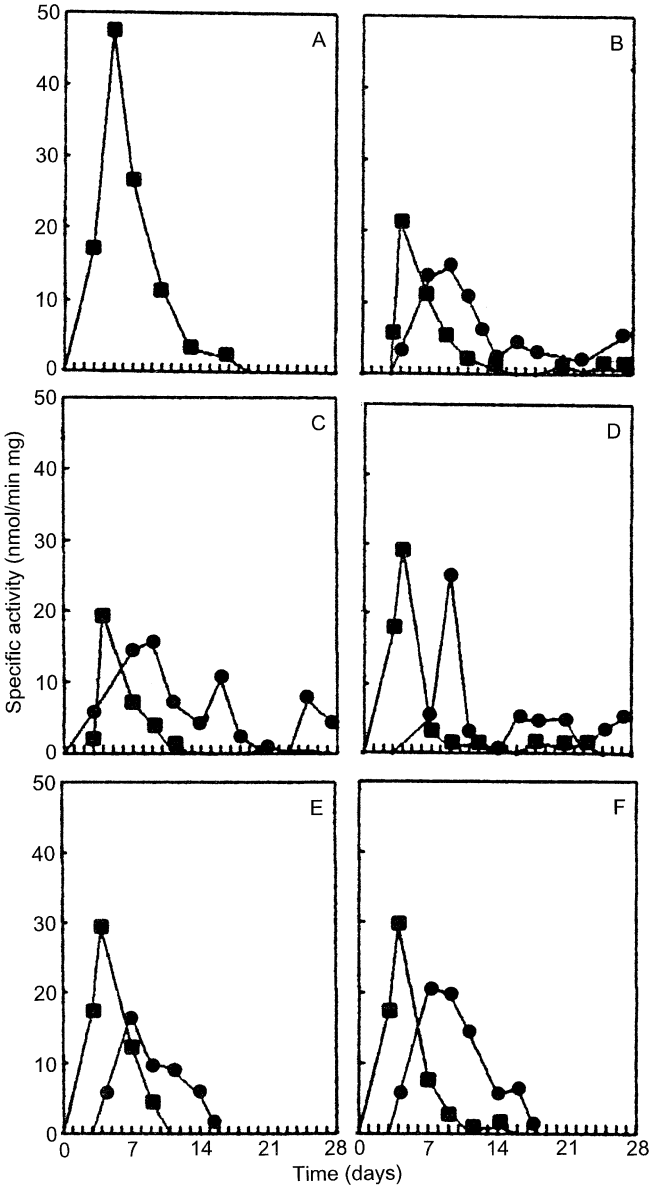

Fig. 3A–F ABTS (■) and 2,6-dimethoxyphenol (●) laccase activities in *P. chrysogenum* cultures without inducers (**A**), and in the presence of 0.1 $g l^{-1}$ Kraft lignin (**B**), 0.2 $g l^{-1}$ Tween 80 (**C**), 0.05 mM xylidine (**D**), 0.1 mM benzyl alcohol (**E**) or veratryl alcohol (**F**). Activity values are the average from two individual cultures

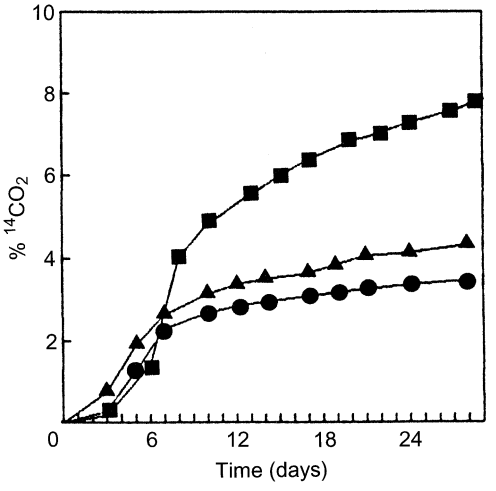

Fig. 4 Effect of 2,6-dimethoxyphenol laccase inducers on lignin mineralization. Cumulative $^{14}CO_2$ released in *P. chrysogenum* cultures without inducers (■) and in the presence of 0.1 $g l^{-1}$ Kraft lignin (▲) and 0.2 $g l^{-1}$ Tween 80 (●). Experiments were performed in triplicate, and the standard error was less than 10% of the mean

Table 1 Production of ligninolytic enzymes in induced and non-induced cultures of *T. villosa*. One unit of laccase activity is the change of absorbance A_{645} min^{-1} (ml medium filtrate)$^{-1}$ using 0.01% tetramethylbenzidine. One unit of lignin peroxidase activity is the increase in absorbance A_{310} min^{-1} (ml medium filtrate)$^{-1}$ using veratryl alcohol and H_2O_2

Inductor added	Enzyme activity	
	$10^2 \times$ Laccase (U)	Lignin peroxidase (U)
None	14.0	21
Organosolv lignin + xylidine	176.0	132
Xylidine	165	30

Laccase from *T. villosa* can be detected by using ABTS or 2,6-dimethoxyphenol, although it is three times more active on 2,6-dimethoxyphenol than on ABTS. Laccase activities, obtained by using both substrates, correlate over time, giving the same time course. Laccase was first detectable in *T. villosa* cultures on day 3, reached the first maximum on day 7, and persisted throughout the incubation period (Fig. 5A). When thioglycolic acid was added, the *T. villosa* laccase reached its maximum 3 days later, being initially strongly repressed, up to 90% on day 7. The maximal mineralization rate per day decreased by 25% in the presence of thioglycolic acid and was delayed by 3 days, correlating with the delay in laccase expression (Fig. 5B).

Results presented in this paper show that both *P. chrysogenum* and *T. villosa* seem to possess ligninolytic systems involving laccase activities.

The capability of laccases to oxidize phenolic OH groups and give phenoxy radicals is well known. Some laccases are also able to produce phenoxy radicals in lignin polymers, which can undergo several secondary reactions, e.g. depolymerization by splitting of *β-O*-ether bonds (Morohoshi and Haraguchi 1987).

Therefore, lignin can be degraded by different fungi imperfecti (e.g. *P. chrysogenum*) without peroxidases being involved. However, this conclusion cannot be extended to eliminate the role of peroxidase in lignin degradation by typical white-rot fungi (e.g. *Trametes* spp.).

We are presently carrying out studies on other lignin-degrading soil fungi to determine the components of their ligninolytic system. So far we have not detected

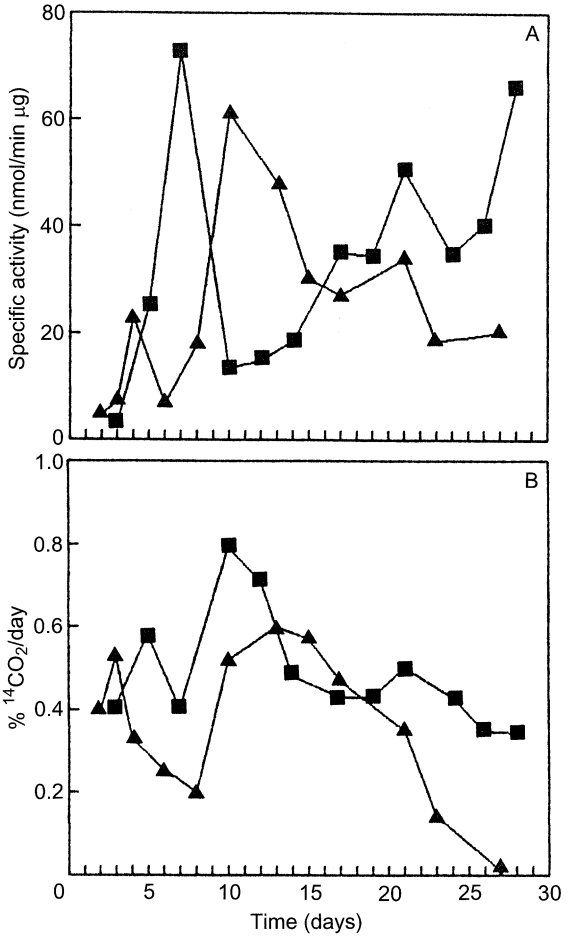

Fig. 5A, B Laccase activity, 2,6-dimethoxyphenol as substrate (**A**), and $^{14}CO_2$ released per day (**B**), in cultures of *T. villosa* in the absence (■) or presence (▲) of 2 mM thioglycolic acid added after 2 days of fungal growth. Measurement details as in Fig. 1

lignin peroxidase production by any fungus under study. By contrast, laccase activities and occasionally aryl-alcohol oxidases were found to be present.

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