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Degradation of natural lignins and lignocellulosic substrates by soil-inhabiting fungi imperfecti

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Abstract

The most powerful lignin-degraders among the 82 microbial strains isolated during a screening of ligninolytic microorganisms from forest soil were identified as *Penicillium chrysogenum*, *Fusarium oxysporum* and *Fusarium solani*. These fungi imperfecti mineralized 27.4%, 23.5% and 22.6% of ^{14}C-labelled milled wood lignin (MWL) from wheat straw after 28 days of incubation in liquid media. Degradation of MWL from pine by *P. chrysogenum* was 8% and 19% when it was evaluated by spectrophotometry and Klason lignin, respectively, but this substrate was hardly mineralized. All fungi were able to attack the hemicellulosic, cellulosic and also lignin fractions of wheat straw during solid-state fermentation, *F. solani* being capable of degrading about 25% of both carbohydrates and lignin. When the selected fungi were tested for dye decolourization, they all readily attacked the polymeric dye Remazol brilliant blue R (RBBR) and also poly R-478 to a minor extent.

Keywords: Soil-inhabiting fungi; Solid-state fermentation; Lignocellulosic substrate; Natural lignin

1. Introduction

Fungi are among the main primary consumers of decomposable materials in soil [1]. It is generally assumed that fungi imperfecti, the main fungal group in soil, are only cellulose-decomposers and that lignin decomposition is brought about mainly by white-rot basidiomycetes [1,2]. However, the role of the latter fungi in lignin bioconversion in soil is questionable, because soil environmental conditions are not favourable to their active metabolism. Particularly, soil pH is usually higher and the C/N ratio lower than reported for optimal ligninolysis with white-rot basidiomycetes [3]. In addition, degradation of synthetic and industrial lignins and other recalcitrant substrates by soil-inhabiting fungi imperfecti in pure culture has been reported by many authors [4–9], demonstrating their potential for degrading natural lignins in their ecological niche.

Environmental scientists have become increasingly interested in ligninolytic fungi for the clean-up of toxic organic chemicals, in soils and water. This is because their lignin degradation systems are not very substrate-specific and can oxidize a great variety of compounds including environmental pollutants [10]. The list of xenobiotic compounds degraded by the

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white-rot fungus *Phanerochaete chrysosporium* includes chlorinated compounds, polycyclic aromatic hydrocarbons (PAHs), dyes and others [11–15]. Soil inhabiting fungi can also degrade fungicides and biocides, PAHs, dyes and other xenobiotics like starch-plastic films [16–20]. These last findings suggest the ecological importance of non-basidiomycete soil fungi in the degradation of sediment-borne pollutants.

We have previously reported the isolation from forest soils of several fungi imperfecti exhibiting ligninolytic capacity [7–9]. In this work we present the results of our research into the ability of these fungi to degrade natural lignins and lignocellulose substrates in both liquid and solid-state culture, as well as their capacity to decolourize two polymeric anthrone-type dyes.

2. Materials and methods

2.1. Soil samples

Soil samples were taken from forest regions of Tenerife, where plant litter derives predominantly from *Pinus canariensis* (7 samples) or from trees and shrubs of an endemic thermophilic laurel forest (6 samples). These soils have been formed on volcanic materials and are classified as ferrallitic and fersiallitic soils. The samples were collected from horizon A where organic matter content varies from 2 to 18% and the C/N ratio between 8 and 17 [21].

2.2. Microbial populations

Counts of total aerobic bacteria and fungi were carried out using multiple C-N source agar supplemented with cycloheximide, and rose-bengal medium with chloramphenicol, respectively. To isolate actinomycetes, soil samples were thermically treated and inoculated on glucose-asparagine-casamino acids agar supplemented with cycloheximide, polymyxin and penicillin, and on arginine-glycerol salts agar with cycloheximide [9].

We also tested the microbial populations able to grow on mineral salts-agar media, supplemented with suitable antibiotics and containing purified Kraft lignin (PKL) as single carbon source [9].

2.3. Fungal strains

Penicillium chrysogenum (MUCL 31363) and *Fusarium oxysporum* (MUCL 30736) were isolated from pine forest soils and *Fusarium solani* (MUCL 35071) from those under laurel forest [9]. These fungi were selected on the basis of their relatively abundant growth on PKL-medium, their results from several biochemical tests and, especially, their ability to catabolize both lignin-related phenylpropanoid acids and lignin-model dehydrogenative polymerizates of coniferyl alcohol (DHPs) [9].

2.4. Substrates

Lignocellulose ^{14}C-labelled specifically in the lignin component from *Pinus canariensis* (^{14}C-lignin-lignocellulose, spec. act. 4000 dpm/mg) was prepared by feeding twigs L-^{14}C-phenylalanine through their cut stems as previously described [22]. Inactive and ^{14}C-labelled milled wood lignin (^{14}C-MWL, spec. act. 9015 dpm/mg) from pine were prepared as described before [23] from unlabelled and ^{14}C-lignin-labelled pine lignocellulose. Inactive MWL was purified to remove residual carbohydrates [23] whose content was reduced as far as 1.3%. ^{14}C-MWL from wheat straw (spec. act. 21 300 dpm/mg) was provided by J. Trojanowski (Forstbotanisches Institut der Universität Göttingen, Göttingen, Germany), and prepared from uniformly ^{14}C-labelled plant tissue.

Wheat straw for solid-substrate fermentation was thoroughly washed with boiling water, dried, and milled to 40 mesh particle size.

2.5. Media and culture conditions

Fungal strains were cultured as described before [9] in high N medium (25.6 mM N, L-asparagine + NH_4NO_3). D-Glucose was added as an easily metabolized carbon source at a final concentration of 55.5 mM for both *Fusarium* strains and of 5.5 mM for *P. chrysogenum* that is optimal for its ligninolytic activity [8].

For degradation studies, inactive MWL or radiolabelled substrates were added at 0.1% (w/v) or to give between $1–2 \times 10^5$ dpm per culture, respectively. Experimental cultures were inoculated with

one millilitre of spore suspensions as previously described [8] and incubated in 250-ml Erlenmeyer flasks containing 25 ml of medium with shaking (reciprocal 50 strokes per min) at 28°C for 28 days. Uninoculated controls were incubated and treated identically with inoculated cultures and both cultures and controls were performed in triplicate.

Solid-substrate fermentations were carried out in 500-ml Erlenmeyer flasks, containing 4 g dry, ground wheat straw and 25 ml medium. No glucose was added to the cultures for solid-substrate fermentation. A duplicate set of flasks was prepared for each study.

2.6. Radiorespirometric assay

$^{14}CO_2$ evolved from metabolized ^{14}C-labelled substrates was trapped with 10% NaOH as indicated by Haider and Trojanowski [24] and radioactivity was measured in a liquid scintillation counter (Optiphase III Kabi Pharmacia). Mineralization was expressed as the percentage of added radioactivity recovered as $^{14}CO_2$.

2.7. Analytical methods

For measurement of inactive MWL degradation, the lignin remaining after culture incubation was measured by spectrophotometry at 280 nm and by residual acid-insoluble Klason lignin and degradation was expressed as the relative decrease in lignin, with the amount in uninoculated flasks as 100% [8].

For analysis of wheat straw after solid-substrate fermentations, the contents of flasks were harvested by filtration through preweighed Whatman no. 1 filter paper. The ash content was determined by burning 400 mg of dry matter at 550°C for 3 h. Loss in dry matter was estimated by weighing the dried substrate (60°C, 72 h) at the beginning and the end of the fermentation. Acid detergent fibre (lignin + cellulose) and acid detergent lignin (Klason lignin) were determined as previously described [25]. This includes the insoluble fraction in acid detergent followed by 72% sulphuric acid treatment. The amounts of hemicellulose and cellulose were calculated as the difference in mass before and after acid detergent extraction and sulphuric digestion, respectively. Finally, the lignin content was corrected for acid-insoluble ash.

2.8. Dye decolourization

For dye decolourization studies, cultures were supplemented with 0.02% (w/v) Remazol brillant blue (RBBR) or poly R-478. RBBR and poly R decolourizations was measured according to Pasti and Crawford [26] as the decrease in the $A_{592/500}$ and $A_{520/350}$ ratios, respectively.

3. Results and discussion

During a screening of ligninolytic microorganisms from forest soils, 82 microbial strains were selected and preserved after several subcultures in solid media containing purified Kraft lignin (PKL) as sole carbon source [9]. Fungi comprised more than 50% of the selected microorganisms (44 strains), whereas they represented 26% of the strains isolated in PKL-media and less than 10% of those in nutritive media (Table 1).

Although microbial numbers varied considerably between the different soil samples in both rich- and PKL-media (Table 1), the number of strains able to

Table 1
Total microbial population from forest soil and potential ligninolytic microorganisms isolated using selective solid media containing purified Kraft lignin (PKL) as sole carbon source

	Pine forest	Laurel forest
cfu's/g soil in rich media [a]	1.7×10^5–9.1×10^6	6.7×10^5–1.7×10^7
% Fungi	8.7	9.6
% Bacteria	91.3	90.4
cfu's/g soil in PKL-media [a]	5.6×10^3–5.5×10^5	3.5×10^5–9.3×10^5
% Fungi	26.1	26.5
% Bacteria	73.9	73.5
Selected strains [b]	42	40
% Fungi [c]	52.4	55
% Bacteria [d]	47.6	45

[a] Population range in 7 (pine forest) and 6 (laurel forest) soil samples.
[b] Able to propagate on PKL-media.
[c] Identified strains: 12; Basidiomycete: 1; Fungi imperfecti: 11 (*Penicillium*: 6 strains; *Fusarium*: 2 strains; *Aspergillus*: 2 strains; *Pestalotia*: 1 strain).
[d] Actinomycetes: 15 strains; Gram-positive spore forming rods: 20 strains: Gram-positive cocci: 1 strain; Gram-negative rods: 2 strains.

grow in the latter was low, representing less than 15% of the cfu's isolated in nutritive media. This result shows the resistance of lignin to metabolization. No statistically significant difference was found between total populations from laurel and pine forest soils. However, laurel forest populations growing in PKL-media were significantly greater than the pine ones ($P < 0.05$).

Among the 82 selected strains, actinomycetes and Gram-positive spore-forming rods were the predominant bacteria and fungi imperfecti were the most abundant fungi identified, the genus *Penicillium* predominating (Table 1). From these strains, five fungi were selected according to several biochemical tests in relation to lignocarbohydrate metabolism and cultured in the presence of ^{14}C-labelled DHPs, *P. chrysogenum*, *F. oxysporum* and *F. solani* being the strains exhibiting the highest mineralization efficiencies [9]. Moreover, *P. chrysogenum* was capable of attacking industrial (Kraft and organosolv) lignins [8].

The ability of these *Penicillium* and *Fusarium* strains to degrade natural lignin and lignocarbohydrate substrates was tested in both liquid and solid-state cultures. As much as 27.4%, 23.5% and 22.6% of ^{14}C-MWL from wheat straw was mineralized by *P. chrysogenum*, *F. oxysporum* and *F. solani* in liquid culture after 28 days (Fig. 1). Moreover, more than 70% of the $^{14}CO_2$ was released by all fungi after only one week of incubation.

On the other hand, when ^{14}C-MWL and ^{14}C-lignin-lignocellulose from pine were assayed, mineralization rates were less than 3%. However, degradation of MWL from pine by *P. chrysogenum* was estimated to be 8% and 19% when it was evaluated by spectrophotometry and Klason lignin content, respectively. It should be borne in mind that $^{14}CO_2$ determination generally underestimates lignin degradation since it does not account for incorporation of ^{14}C into the cell wall and into other lignin-derived compounds.

It has been reported that grass lignins are more easily degraded than wood lignins by white-rot fungi and actinomycetes [27,28], which can be attributed to intrinsic differences between the two lignin types. Monocotyledon lignins possess some particular structural and chemical characteristics such as the presence of significant amounts of phenolic acids

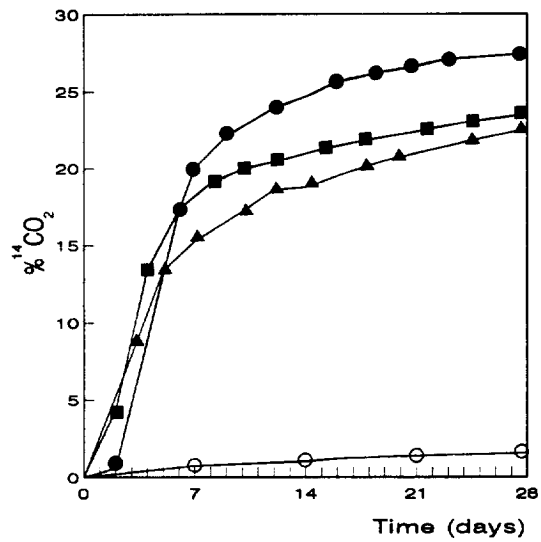

Fig. 1. Cumulative $^{14}CO_2$ released from ^{14}C-MWL from wheat straw (% initial) by *Penicillium chrysogenum* (●), *Fusarium oxysporum* (■), *Fusarium solani* (▲) and uninoculated control (○) in liquid media. Data represent the mean values for 3 replicate cultures, the standard errors being less than 4% of the mean.

[29] and their high solubility in sodium hydroxide [30], which may influence biodegradation patterns.

Solid-substrate fermentation is a more natural environment than liquid culture for lignin-degrading fungi. Therefore, we tested fungal abilities to degrade wheat straw under these conditions. Although growth was not quantified, visual inspection clearly indicated that *F. solani*, *F. oxysporum* and *P. chrysogenum* mycelia permeated the substrate when they were grown on wheat straw without any additional carbon source, causing losses of dry matter between 24.3% and 28.5% in a period of four weeks (Table 2). The tested fungi significantly degraded the hemicellulosic, cellulosic and lignin fractions of wheat straw compared with uninoculated controls (Table 2). *F. solani* degraded the carbohydrate fraction (hemicellulose + cellulose) simultaneously with lignin (27% and 25.1%, respectively). However, both *F. oxysporum* and *P. chrysogenum* consumed more polysaccharides (30.4% and 25%, respectively) than lignin (about 15%), resulting in a proportional increase in this component in the residual substrate.

The experimental data presented indicate that fungi imperfecti belonging to the common microbial

soil flora were capable of degrading natural lignins, even to the same extent as the carbohydrate fraction of lignocellulosic substrates. Although the degradation levels were lower than those described for white-rot fungi [28,31], such processes were carried out under high N concentration which increases lignin degradation in soil [32], but inhibits it in cultures of most white-rot fungi [3]. This suggests the significant role of such fungi in the bulk of carbon turnover from lignin and lignocarbohydrate plant residues in soil, where occurrence of the white-rot basidiomycetes is very sparse when compared with fungi imperfecti [2,33].

As is well known, ligninolytic systems are highly unspecific and the ligninolytic microorganisms can degrade many xenobiotics including polymeric dyes that have even been proposed as substrates for testing ligninolytic activity [11,26,34]

The polymeric dye RBBR, an anthracene derivative, is used as a starting material in dye production and represents an important class of often toxic and recalcitrant organopollutants. *P. chrysogenum*, *F. oxysporum* and *F. solani* readily decolourized RBBR (Fig. 2A), the decolourization by *F. solani* being greater than by *P. chrysogenum* and *F. oxysporum*. Dye decolourization by the two latter fungi was slower, beginning 7 days after inoculation, just as dye decolourization by *F. solani* was completed. All three fungi also decolourized poly R-478, but to a lesser extent (Fig. 2B). In addition to these results, we have previously reported the decolourization of another polymeric dye, poly B-411, by *P. chrysogenum* and *F. oxysporum* in liquid culture [7,8].

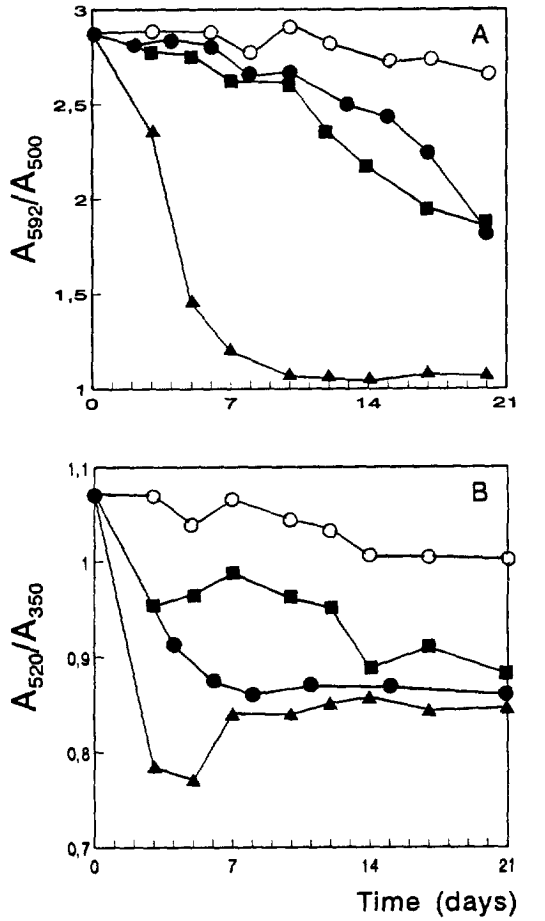

Fig. 2. Decolourization of RBBR (A) and poly R-478 (B) during 21 days by *Penicillium chrysogenum* (●), *Fusarium oxysporum* (■), *Fusarium solani* (▲) and uninoculated control (○) in 0.02% dye basal media. Data represent the mean values for duplicate cultures.

Table 2
Losses in total weight and structural components after solid-state fermentation of wheat straw by three selected soil-inhabiting fungi imperfecti

Fungi	Loss from uninoculated control [a] (%) [b]			
	Dry matter	Hemi-cellulose	Cellulose	Lignin
P. chrysogenum	22.6±0.1	22.9±0.6	26.3±0.3	15.4±4.6
F. oxysporum	26.8±2.5	31.7±0.5	29.6±3.6	15.4±3.5
F. solani	25.8±3.1	23.6±6.2	30.2±3	25.1±3.4

[a] Fibrous composition of wheat straw in control, hemicellulose: 31.5%; cellulose: 51.3%; lignin: 12.6%.
[b] Data are means±S.E. of duplicate cultures.

The clean-up of toxic organic chemicals in soils by indigenous microorganisms already adapted to the soil habitat is probably more economical than the introduction of foreign microorganisms. The results presented here indicate that the selected soil fungi possess an estimable decolourizing ability and further research into their potential to degrade other polyaromatic compounds and dyes could be interesting for bioremediation of contaminated soils.

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