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Simultaneous biodegradation of *p*-cresol and phenol by the basidiomycete *Phanerochaete chrysosporium*

Christian Kennes and Juan M. Lema

Department of Chemical Engineering, University of Santiago de Compostela, E-15706 Santiago de Compostela, Spain

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SUMMARY

The fungus *Phanerochaete chrysoporium* BKM-F-1767 was able to degrade high concentrations of *p*-cresol (up to 150 mg L^{-1}) provided that glucose was added as a carbon and energy source and conditions favourable to ligninolytic enzyme activities were used, i.e. a nitrogen-limited medium. The fungus also simultaneously degraded *p*-cresol (50 mg L^{-1}) and phenol (50 mg L^{-1}) in a mixture at similar rates. Kinetics of *p*-cresol biodegradation were almost identical whether the compound was tested individually or in a mixture with phenol.

INTRODUCTION

White-rot fungi such as *Phanerochaete chrysoporium* are highly efficient at degrading lignin and chlorolignin structures in the presence of a primary carbon and energy source such as glucose [15]. The production of ligninolytic enzymes occurs under nutrient-limited conditions [14].

Structures similar to several toxic aromatic compounds can be found in the lignin polymer and *P. chrysosporium* can also degrade molecules such as chlorophenols and polynuclear aromatic hydrocarbons [1,4,8,11,18,19,24]. Ligninolytic enzymes are often involved in the process [3,22]. The formation of carbon dioxide as an end product has been observed in many cases, both for lignin and for toxic compounds. Nevertheless, other products, sometimes unidentified, are also formed. The biodegradation mechanism and the enzyme complex involved are not fully understood.

Phenol and related compounds such as cresols are frequently encountered environmental contaminants. They appear in soil and groundwater as well as in wastewaters mainly originating from petroleum-related industries. They are also components of creosote. Their biodegradation by aerobic and anaerobic bacteria has been shown [2,5,7,10,16]. Yeasts and other eucaryotes also metabolize cresols and use them as a carbon source [12,21].

The purposes of the present work were to test the ability of the white-rot fungus *P. chrysosporium* to degrade *p*-cresol and phenol, to compare the fate of the compounds when used individually or in a mixture, and to determine the influence of environmental factors on the biodegradation.

Correspondence to: C. Kennes, Dept of Chemical Engineering, University of Santiago de Compostela, E-15706 Santiago de Compostela, Spain.

MATERIALS AND METHODS

Microorganism

P. chrysosporium BKM-F-1767 (ATCC 24725) was obtained from the Spanish Type Culture Collection, Valencia, Spain. It was maintained on glucose-malt extract agar by growing the fungus at 37 °C and storing the plates at 4 °C until needed.

Culture conditions

For biodegradation studies, *P. chrysoporium* was grown in 30-ml amber screw cap flasks containing 5 ml of a previously-described medium [25] (pH 4.5) that had been autoclaved and then supplemented with 2 mg L^{-1} of a filter-sterilized thiamine solution. An aqueous solution of *p*-cresol or phenol was added at the desired concentration. One 4-mm diameter agar plug of the mycelium pregrown on plates was used as inoculum. After inoculation, the flasks were maintained in the dark and incubated statically at 37 °C.

Triplicate samples were analyzed daily by sacrificing three vials. As controls, cultures of the fungus grown for one week on glucose were autoclaved before addition of *p*-cresol or phenol and the flasks were incubated. Sterile media were also used as controls. Fungal biomass dry weight was measured after holding the filtered culture medium overnight at 90 °C.

Analytical methods

Both *p*-cresol and phenol as well as their biodegradation products were analyzed by HPLC. A Hewlett–Packard HP 1090 chromatograph, series II, equipped with a diode array detector and connected to a HP Vectra 486/33U computer (HP, Barcelona, Spain) was used for the analysis. A Supelcosil LC 8 column (Teknokroma, Barcelona, Spain) allowed separation of the desired compounds using a

methanol:water gradient (30:70 to 50:50) with a flow rate of 1.5 ml min^{-1}. Absorption was measured at 280 nm.

Enzyme assays

Lignin peroxidase (LiP) and manganese peroxidase (MnP) activities were determined in the extracellular medium as described previously [20,25]. Laccase was estimated by oxidation of 2,6-dimethylphenol [20].

RESULTS

Biodegradation of p-*cresol*

Two sets of triplicate flasks were inoculated with *P. chrysoporium* under nitrogen-limiting conditions in the presence of 50 or 150 mg L^{-1} *p*-cresol, corresponding respectively to 0.46 and 1.38 mM. One week before starting the biodegradation experiment, flasks with cells pregrown for 8 days on glucose were autoclaved prior to the addition of *p*-cresol. They were used as controls as were sterile media. The daily biomass increase estimated by means of dry weight determinations gave evidence that *p*-cresol did not prevent growth (Fig. 1). Biodegradation of *p*-cresol started at the end of the fifth day of incubation irrespective of its initial concentration. Although difficult to estimate, by comparing the curves in Fig. 2, the rate of biodegradation seems to be slightly higher in the presence of 150 mg L^{-1} than with 50 mg L^{-1}. The final dry weight obtained after complete disappearance of the phenolic compound was similar to the dry weight obtained with glucose alone, suggesting that *p*-cresol did not sustain fungal growth (Fig. 1). Towards the end of *p*-cresol disappearance a new peak appeared on the HPLC chromatogram with characteristic optimal absorbance peaks around 230 nm (major absorbance) and 280 nm (minor absorbance) over the UV-visible range. The absorption spectrum was similar to the spectrum obtained with pure veratryl alcohol. After an increase in absorbance intensity of that intermediate product (measured at 280 nm), the HPLC-peak area slowly decreased as the culture was incubated further at 37 °C. This compound had

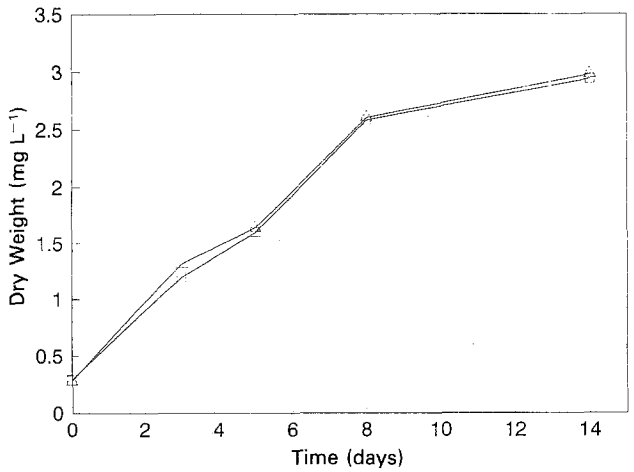

Fig. 1. Dry weight increase in the presence (□) or absence (△) of *p*-cresol, in the presence of glucose in the culture medium.

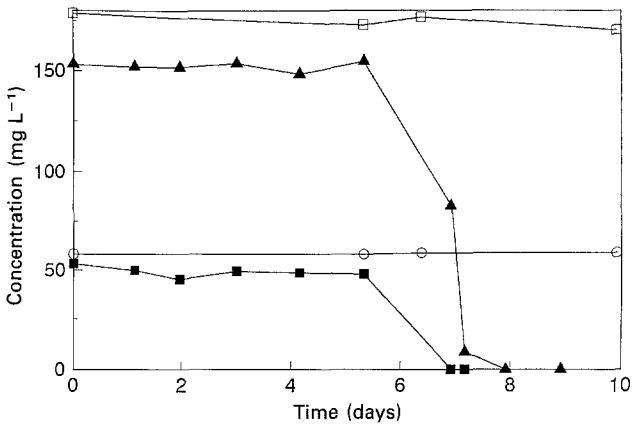

Fig. 2. Biodegradation of 50 mg L^{-1} (■) and 150 mg L^{-1} (▲) *p*-cresol by *P. chrysoporium*, under N-limiting conditions, and corresponding controls (□,○).

not completely disappeared by the end of the experiment. No other aromatic intermediate compound was detected, measuring absorbances at 230 nm, 280 nm and 250 nm (typical of quinones such as *p*-benzoquinone). This suggests that a significant degradation was attained. *p*-Cresol remained present in the controls (Fig. 2), even after 20 days incubation (data not shown). The fact that biodegradation occurred under N-limiting conditions and the fact that it started only after several days incubation suggested a typical involvement of a ligninolytic enzyme system.

Parameters influencing biodegradation of p-*cresol*

In order to determine whether the fungus could use *p*-cresol as its sole source of carbon and energy, experiments were started both under N-limiting and under N-sufficient conditions using the same controls as above. One series of triplicate flasks contained *p*-cresol as the sole possible carbon/energy source. In the other series of flasks, glucose was added. Biodegradation of *p*-cresol (150 mg L^{-1}) did not occur in the absence of glucose (Fig. 3) even after 20 days

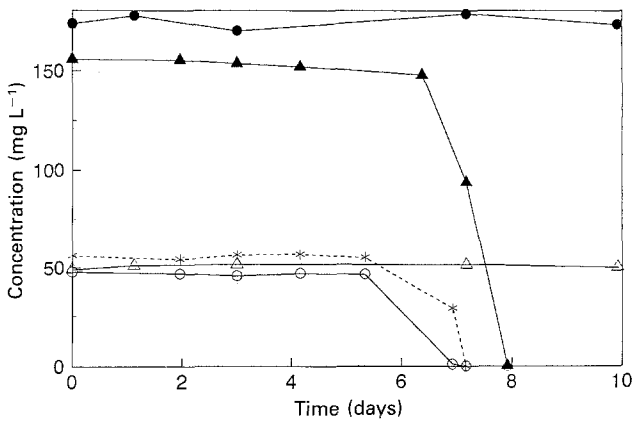

Fig. 3. Biodegradation of *p*-cresol under N-limiting conditions in the presence (▲) or absence (●) of glucose and under N-sufficient conditions (△) in the presence of glucose. Simultaneous biodegradation of *p*-cresol (○) and phenol (*) is also represented.

(data not shown) whereas the expected removal was observed in its presence under N-limiting conditions. In vials where the fungus was pregrown on glucose and where *p*-cresol was later added as the sole carbon source, a gradual decrease in dry weight was observed a few days after adding the compound. Under N-sufficient conditions, biodegradation was inhibited even in the presence of glucose and using only 50 mg L^{-1} *p*-cresol to avoid possible inhibition effects due to high concentrations of the toxic compound.

Simultaneous biodegradation of p-*cresol and phenol*

The biodegradation of *p*-cresol (50 mg L^{-1}) followed a similar pattern when 50 mg L^{-1} phenol was added to the culture medium as when testing *p*-cresol as a single substrate. Degradation of both compounds started after 5–6 days incubation, showing that *P. chrysoporium* is also able to degrade phenol under N-limiting conditions. Its biodegradation starts as soon as the environmental conditions are such that the ligninolytic enzyme system is switched on.

Role of LiP and MnP in p-*cresol and phenol degradation*

The presence of LiP and MnP in the culture medium was checked under various experimental conditions. When the culture medium was incubated in the presence of *p*-cresol alone (50 or 150 mg L^{-1}), phenol alone (50 mg L^{-1}) or with a mixture of both aromatic compounds (50 mg L^{-1} each), MnP activity was clearly detected as well as low LiP activity. Laccase, which was also tested for, was not present, as might have been expected with *P. chrysosporium*. Biodegradation of the phenols started as soon as ligninolytic enzyme activity was detected in the medium.

DISCUSSION

The ability of the white-rot fungus *P. chrysoporium* BKM-F-1767 to degrade lignin has led several groups to study the behaviour of the organism in the presence of related compounds sometimes found as part of the lignin structure. Toxic compounds such as polynuclear aromatic hydrocarbons [8], pentachlorophenol [1,18], pesticides [17] and azo dyes [9] are only a few examples. In many—though not all—cases, biodegradation is partly due to the presence of an active and widely studied ligninolytic enzyme system [6].

In the present work, we demonstrated the ability of the white-rot fungus to degrade *p*-cresol, individually or in mixture with phenol, under environmental conditions favourable to the production of ligninolytic enzymes (N-limiting conditions). After a lag period of about five days, the compounds were rapidly degraded in a few hours with no significant differences in the biodegradation rates for *p*-cresol and phenol. Similar *p*-cresol biodegradation kinetics were observed whether the compounds were tested individually or in a mixture with phenol, suggesting that the presence of phenol (50 mg L^{-1}) did not inhibit biodegradation of *p*-cresol (50 mg L^{-1}). MnP and LiP activities were detected in the culture medium as soon as biodegradation of the organic compounds started.

These phenolic compounds could not be used as growth substrates by the white-rot fungus and an additional carbon source, i.e. glucose, is required for growth. Complete mineralization of organic pollutants, including highly toxic polychlorinated phenols, has been observed in a few cases with aerobic and strict anaerobic bacteria [13,23,26]. However, in the case of *P. chrysosporium* BKM-F-1767, recalcitrant organic compounds are often not fully mineralized and, together with carbon dioxide, other products are formed. In the case of *p*-cresol, an intermediate product absorbing at 280 nm and around 230 nm was detected. It showed the same UV-visible absorbance spectrum as pure veratryl alcohol. The peak area, measured at 280 nm, increased during breakdown of *p*-cresol and only partly dissappeared afterwards, proving the intermediate product (veratryl alcohol) accumulated to some extent. Molecules such as *p*-benzoquinone and other related quinones which strongly absorb around 250 nm were not detected, suggesting that aromatic intermediates were not present and a significant detoxification was attained. For phenol as well as for *p*-cresol, at all initial concentrations tested (up to 150 mg L^{-1}), the phenolic compounds were completely removed. This was true with individual compounds as well as with a mixture of both compounds (50 mg L^{-1} each).

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