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Short contribution

Comparison of abilities of white-rot fungi to mineralize selected xenobiotic compounds

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Summary. The abilities of the white-rot fungi *Chrysosporium lignorum, Trametes versicolor, Phanerochaete chrysosporium* and *Stereum hirsutum* to mineralize 3,4-dichloroaniline, dieldrin and phenanthrene were investigated. *S. hirsutum* did not mineralize any of the test compounds but the other strains partly mineralized them all to varying degrees. The relative degradation rates per unit biomass were *T. versicolor* > *C. lignorum* > *P. chrysosporium*. Evidence was obtained for the production of water-soluble metabolic intermediates but no attempt was made to characterize these. It was found that mineral salts-glucose medium supplemented with trace mineral nutrients, vitamins and 1.5 mM 3,4-dimethoxybenzyl alcohol (veratryl alcohol) resulted in the highest mineralization rate. At no time in these experiments was there detectable extracellular ligninase (lignin peroxidase) activity.

Introduction

The white-rot fungi are capable of degrading lignin present in woody plant tissue. Some of these organisms have been shown to produce one or more extracellular peroxidase enzyme systems termed ligninases or lignin peroxidases, which are involved in complex radical-mediated attack upon lignin (Hammel et al. 1986; Bumpus and Aust 1987a). It has been shown that some white-rot fungi can also cleave a variety of organic chemicals, including polycyclic aromatic hydrocarbons (Bumpus 1989; Haemmerli et al. 1986; Sanglard et al. 1986), DDT (Köhler et al. 1988; Bumpus and Aust 1987b), polychlorinated biphenyls (Eaton 1985), hexachlorocyclohexane (Bumpus et al. 1985), dichloroaniline (Hallinger et al. 1988) and pentachlorophenol (Mileski et al. 1988). Most of these studies have employed strains of *Phanerochaete chrysosporium,* although degadation by *Funalia gallica, Coriolus vesicolor, Poria cinerescens,* and *Pleurotus ostreatus* has been reported (Eaton 1985; Hallinger et al. 1988). Whilst the observed degradation has generally been attributed to ligninase-mediated reactions, it should be noted that there is some evidence that ligninase is not associated with DDT degradation in cultures of *P. chrysosporium* (Köhler et al. 1988).

The observed degradation of compounds that may be present in the environment has led to the suggestion that white-rot fungi could be used in treatment processes for waste-water streams (Chang et al. 1985) and for the clean-up of contaminated soil and ground water (Morgan and Watkinson 1989). Whilst there is clear potential for the biotechnological application of white-rot fungi for waste treatment, most of the data available have been obtained using strains of *Phanerochaete chrysosporium.* The aim of this research was to investigate the degradation of a number of xenobiotic compounds by a wider selection of ligninolytic fungi.

Materials and methods

Organisms. The following strains were used: *P. chrysosporium* ATCC 24725 and ATCC 20696; *Chrysosporium lignorum* CL1; *Trametes versicolor* PV1; *Stereum hirsutum* SH1. Strains CL1 and PV1 were supplied by Mr. Jon Wright, formerly of Portsmouth Polytechnic, UK. Strain SH1 was kindly provided by Dr. Alan Rayner, University of Bath, UK. Strains were maintained on slopes of malt extract agar stored under mineral oil at 4° C. Working cultures were grown on malt extract agar plates at 30° C.

Preparation of inocula. All strains except *T. versicolor* sporulated on agar media. Spore suspensions were prepared by pipetting sterile mycological peptone containing 0.5% (v/v) Tween 80 onto the surface of agar cultures and gently dislodging the spores by means of a sterile glass spreader. Mycelial debris was removed from the suspensions by means of filtration through pads of sterile glass wool and suspensions were stored in darkness at 4° C. Mycelial inocula were also employed. These were grown in 500-ml portions of malt extract broth at 30° C with rotary agitation at 200 rpm for 3-5 days. The cultures were harvested by centrifugation at 3500*g* and 20° C for 10 min, washed twice in sterile culture medium (see below) and then resuspended in fresh medium.

Determination of xenobiotic mineralization. Mineralization was determined by measuring the release of $^{14}CO_2$ from radiolabelled

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phenanthrene, 3,4-dichloroaniline and dieldrin. Unlabelled phenanthrene (98%+) and 3,4-dichloroaniline (98%) were obtained from Aldrich (Gillingham, Dorset, UK). Unlabelled analytical standard dieldrin was kindly provided by colleagues in the Analytical and Formulation Chemistry Department at the Sittingbourne Research Centre. [9-^{14}C]Phenanthrene, [U-^{14}C]dieldrin and [U-^{14}C]3,4-dichloroaniline (all 99%+ radiochemical purity) were obtained from Amersham (Aylesbury, Bucks., UK). Solutions were prepared by dissolving unlabelled and radiolabelled compounds in the appropriate proportions in acetone to give a specific activity of 1 $\mu Ci \cdot mg^{-1}$. Suitable volumes of the xenobiotic solutions were added to sterile conical flasks to provide 1 mg compound and the solvent allowed to evaporate prior to inoculation.

Two culture media were employed. W-Medium was prepared according to Eaton (1985). Modified Kirk's medium (based on Kirk et al. 1978) contained ($g \cdot l^{-1}$): KH_2PO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.05; $CaCl_2$, 0.01; glucose, 10.0; ammonium tartrate, 0.22; 2,2-dimethylsuccinate, 2.9. The pH of the medium was 4.5. After autoclaving, the medium was supplemented with 1 $ml \cdot l^{-1}$ mineral salts supplement, 0.5 $ml \cdot l^{-1}$ vitamin supplement (Kirk et al. 1978) and 1.5 mM 3,4-dimethoxybenzyl alcohol (veratryl alcohol; Aldrich). Into the conical flasks were placed 100-ml portions of the relevant culture media plus inoculum. For mycelial inoculation the pellet from the malt extract starter culture was resuspended in a suitable volume of sterile medium and this was dispensed into the culture vessels. For spore inoculation, 1 ml of a spore suspension was added to each flask. Haemacytometer counts demonstrated that spore inoculation per flask was between 8×10^5 and 2×10^6. Concurrent killed control cultures (autoclaved inoculum) were prepared. Flasks were stoppered with sterile rubber Subaseals and incubated statically at 30° C. At weekly intervals the quantity of $^{14}CO_2$ released was determined. The headspaces of the flasks were sparged for 30 min with CO_2-free air and the effluent gas was passed through 8-ml portions of Carbosorb (Canberra Packard, Pangbourne, Berks., UK) to trap any CO_2 present. To the Carbosorb was added 12 ml Permafluor V scintillation fluid (Canberra Packard) and the radioactivity determined in an LKB Rackbeta liquid scintillation counter.

At the end of the experimental period the quantity of radiolabel present in water-soluble compounds was determined. Cultures were filtered through columns of glass wool and the filter washed with three 100-ml portions of distilled water. The collected filtrate and washings were pooled and 0.1-ml samples were added to 4 ml Optiphase Safe scintillation fluid (Pharmacia-LKB, Milton Keynes, UK). Radioactivity was determined using an LKB Rackbeta liquid scintillation counter.

Assay techniques. Ligninase activity was determined according to the method of Tien and Kirk (1984). Extracellular protein was determined by means of the Biorad protein assay reagent (Biorad, Watford, UK). The standard employed was bovine serum albumin. Glucose in the culture supernatant was determined by means of the Sigma (Poole, Dorset, UK) assay kit 16-UV. Biomass was determined by means of dry weight. Cultures were filtered through pre-weighed oven-dried discs of filter paper, rinsed thoroughly with distilled water and dried at 105° C until the residue weight was constant.

Results and discussion

Although measurements of mineralization underestimate overall biodegradation rates since they do not take into account the proportion of parent compound that is not converted to CO_2, the determination of CO_2 release provides a simple and unequivocal measure of xenobiotic breakdown. The use of W-medium demonstrated that *C. lignorum*, *T. vesicolor* and *P. chrysosporium* strains ATCC 24725 and ATCC 20696 were capable of mineralizing at least one of the compounds tested. In contrast, the *S. hirsutum* strain did not produce significant quantities of CO_2 from any of the substrates. The CO_2 evolution from the parent compounds was observed to occur at a linear rate during the 70-day incubation period. The mineralization rates obtained per unit biomass are given in Table 1. It has previously been demonstrated that the composition of the medium may have a significant impact upon both ligninase enzyme production and rates of biodegradation by white-rot fungi (Tonon and Odier 1988; Leisola et al. 1985).

It was found in this study that a medium modified from that of Kirk et al. (1978) gave linear mineralization rates over the 70-day test period that were between two and ten times greater than those obtained using W-medium (Table 1). Mineralization was also observed to continue at a linear rate throughout the 70-day incubation period. This medium is supplemented with trace mineral salts, vitamins and 3,4-dimethoxybenzyl alcohol (veratryl alcohol) and the cause of the enhanced mineralization was not determined. However, it is known that veratryl alcohol and mineral nutrients enhance ligninase activity in laboratory cultures of white-rot fungi (Asther et al. 1988; Leisola et al. 1985; Tonon and Odier 1988).

It is difficult to compare the mineralization rates obtained in this study with those reported elsewhere since few papers provide data concerning the biomass

Table 1. Observed rates of xenobiotic mineralization by strains of white-rot fungi grown in W-medium (Eaton 1985) or a glucose-mineral salts-veratryl alcohol medium modified from that of Kirk et al. (1978)

Compound	Mineralization rate ($\mu g \cdot g$ dry weight$^{-1} \cdot day^{-1}$)							
	Chrysosporium lignorum		*Trametes versicolor*		*Phanerochaete chrysosporium* ATCC 24725		*Phanerochaete chrysosporium* ATCC 20696	
	W-Medium	Kirk medium	W-Medium	Kirk medium	W-Medium	Kirk medium	W-Medium	Kirk medium
3,4-Dichloroaniline	2.8	5.0	6.0	47.7	0.7	6.9	0.5	ND
Dieldrin	0.2	0.6	0.2	2.8	0.1	1.6	0.0	ND
Phenanthrene	0.7	ND	1.0	ND	0.2	ND	0.1	ND

Incubation was static at 30° C with an initial substrate concentration of 10 $\mu g \cdot ml^{-1}$. Data are the means of duplicate vessels. ND, not determined

Table 2. Partial mass balance of ^{14}C distribution from 70-day-old cultures of white-rot fungi cultivated in W-Medium (Eaton 1985) demonstrating conversion of radiolabelled substrates to CO_2 and water-soluble intermediates

Fungus	Substrate	% Radiolabel present in fraction			
		CO_2		Water-soluble	
		Killed control	Viable	Killed control	Viable
C. lignorum CL1	3,4-Dichloroaniline	0.7	6.0	23.0	87.0
	Dieldrin	0.2	1.5	9.5	13.0
	Phenanthrene	1.1	4.5	11.0	57.0
P. chrysosporium ATCC 24725	3,4-Dichloroaniline	1.2	3.5	32.0	88.0
	Dieldrin	0.1	0.8	4.0	6.0
	Phenanthrene	1.1	1.3	10.0	28.0

Data (means of duplicate vessels) are presented for corresponding viable and killed control cultures and are expressed as a percentage of the recovered radiolabel

concentration present. However, from the data of Sanglard et al. (1986) it can be calculated that the mineralization of benzo(a)pyrene by *P. chrysosporium* ATCC 24725 occurred at a rate of approximately 12.6 $\mu g \cdot g$ dry weight$^{-1} \cdot$day^{-1}. The data of Bumpus and Aust (1987b) permit calculation of the mineralization rate of a number of chlorinated pesticides by *P. chrysosporium* strains ATCC 24725 and ME466, such as DDT and methoxychlor, which were mineralized at rates up to 1.4 $\mu g \cdot g$ dry weight$^{-1} \cdot$day^{-1}. Thus, it can be concluded that the rates obtained in this study using the medium modified from that of Kirk et al. (1978) are comparable to those previously reported for similar xenobiotic compounds.

This study was not intended to investigate in detail the production of intermediates during the degradation process but a simple mass balance was performed on 70-day-old cultures of *P. chrysosporium* ATCC 24725 and *C. lignorum* in order to ascertain whether there was production of water-soluble materials during the degradation process. The results are illustrated in Table 2 and it can be seen that there was an increase in radioactivity in the aqueous phase of the active cultures. This effect was particularly marked for 3,4-dichloroaniline and phenanthrene but less apparent for dieldrin and the significance of the data for dieldrin requires further study. The data suggest that oxygenated intermediate compounds of greater solubility than the parent compound were produced by fungal activity (Mileski et al. 1988; Hallinger et al. 1988). These data serve to illustrate that a variety of metabolic products may be produced by the action of white-rot fungi on xenobiotics. Should these products be non-toxic they may prove to be acceptable end-points for a biological treatment process or may serve as substrates for other microorganisms in the environment. However, there is a risk that the metabolites may represent a metabolic dead-end, be of elevated toxicity or be of enhanced mobility in the environment. These potential problems must be considered prior to application of white-rot fungi for environmental clean-up and therefore the nature of metabolites produced from xenobiotics by white-rot fungi requires further study.

It has normally been found that the degradation of xenobiotics by white-rot fungi is associated with the production of extracellular ligninases. Indeed, attack upon xenobiotics mediated by cell-free enzyme preparations has been reported (Sanglard et al. 1986; Haemmerli et al. 1986; Bumpus and Aust 1987a; Aitken et al. 1989). However, in this study no ligninase activity was detected at any time in any of the degradative cultures. Since enzyme activity had been detected in other experiments employing the same strains, attempts were made to monitor general fungal activity to determine whether the test xenobiotics were inhibiting fungal metabolism. Measurements of glucose utilization, total extracellular protein production and biomass production indicated that there were no differences between fungal cultures grown in the presence and absence of the xenobiotics. Blondeau (1989) has described slow attack upon humic materials by *P. chrysosporium* DSM 1556 in the absence of detectable ligninase activity and Köhler et al. (1988) have elegantly demonstrated that DDT degradation by *P. chrysosporium* ATCC 24725 occurred independently of ligninase production. Furthermore, no attack upon DDT could be obtained using a partially purified ligninase preparation. It was concluded that the degradation of DDT was caused by an alternative enzyme system to ligninase and as such may have been responsible for the mineralization observed in the absence of ligninase activity. Alternatively, the ligninase produced under the conditions employed in this study may have been cell-associated or present in a form that was inactive in the standard enzyme assay (Tien and Kirk 1984). Further research is necessary to investigate this phenomenon.

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