3-1-1998

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PII: S0043-1354(97)00378-3

Wat. Res. Vol. 32, No. 5, pp. 1713–1717, 1998

Printed in Great Britain
0043-1354/98 $19.00 + 0.00

RESEARCH NOTE

BIODEGRADATION OF CONGO RED BY *PHANEROCHAETE CHRYSOSPORIUM*

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(First received March 1997; accepted in revised form March 1997)

Abstract—The azo dye Congo Red, was degraded extensively by the wood rotting basidiomycete, *Phanerochaete chrysosporium* in agitated liquid cultures and in solid malt agar cultures. Upon addition of Congo Red to agitated liquid cultures, the dye was adsorbed to the mycelial pellets in both ligninolytic and non-ligninolytic cultures followed by extensive degradation only in the ligninolytic cultures. This fungus, grown from conidiospores, readily degraded up to 718 μM (500 mg/l) Congo Red in 2.0% malt agar. Decolorization of Congo Red in malt agar plates was suppressed by the addition of supplemental nutrient nitrogen indicating that the lignin degrading system of *P. chrysosporium* may be important in the biodegradation of this dye. This is supported by the observation that Congo Red is a substrate for purified lignin peroxidase H8. These results are of interest because it had been previously reported that Congo Red was not a substrate for lignin peroxidase nor was it extensively degraded by this fungus. © 1998 Elsevier Science Ltd. All rights reserved

Key words—*Phanerochaete chrysosporium*, lignin peroxidase, Congo Red, azo dyes

INTRODUCTION

Azo dyes are extremely versatile colorants and are the largest group of synthetic colorants known (Anliker, 1979). As a consequence, they are also the most common group of synthetic colorants released into the environment. Although they do not generally display extreme acute toxicity, azo dyes are an environmental problem because of the large amounts that are released and their resistance to microbial degradation (Anliker, 1979). The long term deleterious health effects of exposure to azo dyes has not been completely assessed. When it does occur, biodegradation (particularly by anaerobic bacteria) can lead to the reduction of the azo bond producing mutagenic and/or carcinogenic compounds (Brown and Dietrich, 1983).

Phanerochaete chrysosporium has been shown to degrade a wide variety of environmentally hazardous compounds to carbon dioxide (Bumpus *et al.*, 1985). The list includes nitroaromatics, polycyclic aromatic hydrocarbons and chlorinated organics (Bumpus, 1989; Kennedy *et al.*, 1990; Valli *et al.*, 1992; Bumpus and Tatarko, 1994). The degradation by *Phanerochaete chrysosporium* of various classes of dyes, including azo dyes, has also been reported (Bumpus and Brock, 1988; Cripps *et al.*, 1990; Paszczynski and Crawford, 1991; Paszczynski *et al.*, 1991, 1992; Capalash and Sharma, 1992; Pasti-Grigsby *et al.*, 1992; Spadaro *et al.*, 1992; Bumpus, 1995).

Cripps *et al.* (1990) reported that Congo Red was resistant to decolorization by *P. chrysosporium* in nutrient nitrogen limited cultures and that substantial amounts of Congo Red remained tightly bound to the fungal mycelium after 12 days of incubation (Cripps *et al.*, 1990). Furthermore, of the dyes tested only Congo Red appeared not to be a substrate for lignin peroxidases. In contrast, a more recent report by Ollikka *et al.* (1993) showed that Congo Red is indeed a substrate for lignin peroxidases. It was reported that 54% decolorization of Congo Red occurred in the presence of a crude preparation of lignin peroxidase and hydrogen peroxide (Ollikka *et al.*, 1993). We have reexamined this apparent discrepancy.

MATERIALS AND METHODS

Chemicals

The disodium salt of Congo Red, 3,3′-[[1,1′ biphenyl]-4,4′diylbis-(azo)]bis[4-amino-1-naphthalenesulfonic acid]

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(87% purity) and 3,4 dimethoxy benzyl alcohol (veratryl alcohol) were purchased from Aldrich Chemical (Milwaukee, WI). Except for malt extract and agar (Difco Laboratories, Detroit, MI) all other chemicals were purchased from Fischer Scientific (Pittsburgh, PA).

Microorganism

P. chrysosporium BKM-F-1767 was obtained from the Forest Products Laboratory (U.S. Department of Agriculture, Madison, WI) and maintained on 2% malt agar slants.

Enzymes

Crude lignin peroxidases were obtained from the extracellular fluid of nutrient nitrogen-limited cultures of *P. chrysosporium* and concentrated in a Millipore Minitan Concentrator (Millipore, Bedford, MA). This concentrated extracellular material was dialyzed overnight against 5.0 mM sodium acetate buffer, pH 4.0, and stored frozen at −20°C until use. Purified lignin peroxidase H8 was a gift from S.D. Aust (Utah State University, Logan, UT).

Culture conditions

P. chrysosporium was grown in agitated liquid cultures as described by Tuisel *et al.* (1990). In some experiments the ammonium tartrate concentration in the medium was raised from 1.2 mM (nutrient nitrogen limited cultures) to 12 mM (nutrient nitrogen sufficient cultures). In other experiments, *P. chrysosporium* spores were suspended in distilled water, removed from the surface of stock malt agar slants by scrapping gently with an inoculating loop and used to inoculate (2 drops from a 2.0 ml serological pipet of spores in water having an $A_{650\ nm} = \sim 0.5$) the center of petri plates containing solid 2.0% malt agar (2% malt extract and 2% agar). In some experiments malt agar plates were supplemented with Congo Red and increased amounts of glucose and ammonium tartrate. Malt agar plates were incubated at 37°C in a humidified growth chamber to prevent desiccation of the agar.

Biodegradation

Agitated nutrient nitrogen limited and nutrient nitrogen sufficient liquid cultures were grown for six days at which point Congo Red in distilled water was added to give a final concentration of 71.8 μM (50 mg/l) or 718 μM (500 mg/l). To determine dye decolorization, samples were removed from the cultures at predetermined times, diluted with distilled water, centrifuged (16 000 × *g* for a minimum of 5 min), and the supernatant absorbance at 500 nm was determined using a Cary 3 UV visible spectrophotometer (Varian Instruments Group, Palo Alto, CA).

To determine dye decolorization in solid cultures, 2.0% malt agar plates were incubated for a predetermined time. The plates were then chilled and the surface mycelium was removed with tissue paper (Kimwipes, Kimberly-Clark, Roswell, GA). Initial experiments revealed that substantial amounts of Congo Red were adsorbed to the mycelium upon melting of the agar plates. If mycelium removal using tissue paper was omitted, decolorization of dye due to adsorption to mycelium would be mistaken for decolorization due to biodegradation and the degree of biodegradation would thus be overestimated. Considerable care was taken not to remove any of the agar while removing the mycelium. The entire agar plate was then melted and diluted with distilled water to a concentration appropriate for determination of absorbance at 500 nm (*i.e.*, $A_{500} = \sim 1$). Absorbance at 500 nm was determined in a thermostated (70°C) cell in the Cary 3 spectrophotometer. Decolorization was then calculated taking into consideration the volume of water used for dilution.

HPLC analysis

Analytical HPLC analysis was performed on a Waters 600E system equipped with a Waters 990 photodiode array detector (Millipore, Millford, MA). Chromatography of Congo Red was achieved using a gradient system (Waters gradient curve 6) from 100% water to 100% acetonitrile in 50 min followed by isocratic elution using 100% acetonitrile for 10 min. The flow rate was 1.0 ml/min. Separation was achieved using a Beckman Ultrasphere ODS column (4.6 × 250 mm) (Beckman Instruments, San Ramon, CA).

Enzyme assays

Enzyme activity (veratryl alcohol oxidase activity) was assayed as described by Tien and Kirk (1984). The oxidation of Congo Red was monitored at the wavelength maximum of the dye at each pH. The initial rate is expressed as the decrease in absorbance/minute. The enzyme mixture consisted of 0.5 μM lignin peroxidase H8 and 35.8 μM Congo Red in 50 mM sodium tartrate buffer at the indicated pH. Reactions were initiated by the addition of 250 mM hydrogen peroxide.

RESULTS

Upon addition of Congo Red to agitated nutrient nitrogen sufficient liquid cultures of *P. chrysosporium*, there was a rapid adsorption of the dye to the mycelial pellets in both live and autoclaved cultures (Fig. 1). The clearance from the medium of Congo Red (50 mg/l) was complete within five hours. Visual inspection of these cultures indicated that there was no apparent further decolorization. Virtually all of the Congo Red was tightly bound to the mycelial pellets. In some experiments, dyed pellets were used to inoculate malt agar plates. This resulted in rapid growth extending to the edge of the plate, indicating that the dye had little or no toxicity toward the fungus. In agitated nutrient

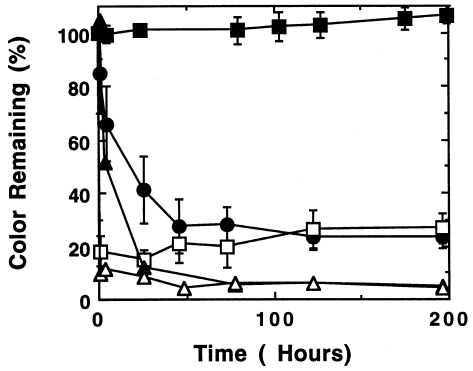

Fig. 1. Decolorization of Congo Red in agitated cultures of *P. chrysosporium*. Congo Red (500 mg/l) was added to six day old cultures of nutrient nitrogen limited (closed circles, $n = 6 \pm$ S.D.), autoclaved nutrient nitrogen limited cultures (closed triangles), nutrient nitrogen sufficient cultures (open squares, $n = 4 \pm$ S.D.), autoclaved nutrient nitrogen sufficient cultures (open triangles), and distilled water (closed squares, $n = 4 \pm$ S.D.). Decolorization was monitored spectrophotometrically at 500 nm. It should be noted that the symbols (open and closed triangles) for the final time points of the autoclaved controls overlap.

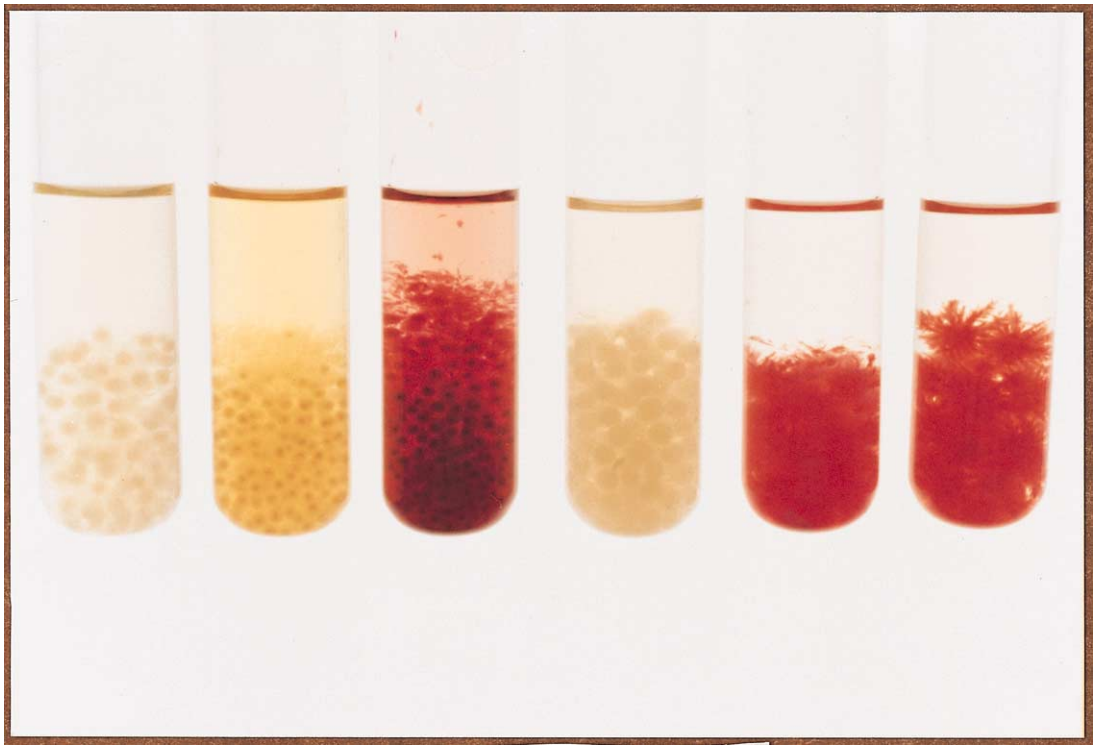

Fig. 2. Photograph of *P. chrysosporium* mycelial pellets obtained from agitated cultures after incubation for 200 h with and without Congo Red (50 mg/l). From left to right: nutrient nitrogen limited pellets with no addition, nutrient nitrogen limited pellets with Congo Red, autoclaved nutrient nitrogen limited pellets with Congo Red, nutrient nitrogen sufficient pellets with no addition, nutrient nitrogen sufficient pellets with Congo Red, autoclaved nutrient nitrogen sufficient pellets with Congo Red.

nitrogen limited liquid cultures, there was also extensive decolorization of the media due to adsorption, but the Congo Red in these cultures was substantially degraded as there was an extensive loss of all the red color and the gradual appearance of unidentified light yellow product(s). Autoclaved controls of both nutrient nitrogen limited and nutrient nitrogen sufficient cultures adsorbed Congo Red at a rate similar to the live cultures. A photograph illustrating the adsorption of Congo Red to nutrient nitrogen limited and nutrient nitrogen sufficient pellets is presented in Fig. 2.

P. chrysosporium also decolorized Congo Red in solid cultures (Fig. 3). These cultures were grown from conidiospores of the fungus and the dye did not appear to cause long term toxic effects. Cultures containing 500 mg/l of Congo Red did experience a short period of inactivity with regard to decolorization for approximately two days after which the maximum rate of decolorization of $31.2\% \pm 2.9\%$/day, $n = 3$, was reached between days 3 to 5. Cultures that contained 50 mg/l of Congo Red had a maximum rate of decolorization between days 2 to 5 of $26.7\% \pm 3.6\%$/day, $n = 3$. The surface mycelium did not adsorb Congo Red. Decolorization in the plates started at the surface and gradually spread downwards through the plate to the bottom.

Addition of supplemental nutrient nitrogen and organic carbon to the 2.0% malt agar plates suppressed decolorization of Congo Red only in plates containing high amounts of nutrient nitrogen (12 mM ammonium tartrate) (Table 1). The lignin degrading system, but not growth, is known to be suppressed by high amounts of nutrient nitrogen

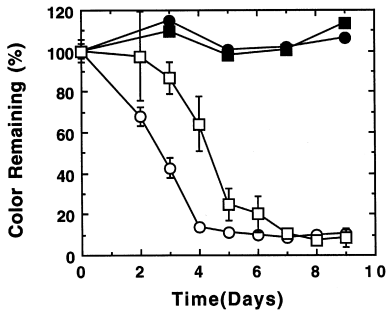

Fig. 3. Decolorization of Congo Red in malt agar by *P. chrysosporium*. *P. chrysosporium* was used to inoculate 2.0% malt extract agar plates (60×15 mm) containing 50 mg/l (open circles, $n = 3 \pm$ S.D.) or 500 mg/l (open squares, $n = 3 \pm$ S.D.) Congo Red. At predetermined times the mycelium was removed and the entire agar plate was melted, diluted with distilled water, and absorbance at 500 nm was determined in a thermostated (70°C) Cary 3 spectrophotometer. Controls consisted of uninoculated plates containing 50 mg/l (closed circles) or 500 mg/l (closed squares) Congo Red.

Table 1. Decolorization of Congo Red by *P. chrysosporium* grown on malt agar plates

Ammonium tartrate (g/l)	Glucose concentration (g/l)		
	0	1.0	10.0
	50 mg/l Congo Red		
0	87.8 ± 2.2	85.4 ± 3.6	88.2 ± 1.4
0.22	83.3 ± 4.2	85.6 ± 1.2	83.7 ± 3.4
2.2	40.0 ± 2.5	26.9 ± 4.3	21.3 ± 5.9
	500 mg/l Congo Red		
0	93.0 ± 4.4[a]	79.6 ± 1.9	77.9 ± 6.0
0.22	97.4 ± 1.0	85.9 ± 2.0 82.	77 ± 2.0
2.2	38.7 ± 15.0	31.4 ± 11.5	30.0 ± 21

[a]Numbers refer to the percent of Congo Red decolorized ± S.D. ($n = 4$) compared to uninoculated controls incubated under identical conditions. These sterile controls exhibited 21.8% ± 7.6 (50 mg/l control) and 11.5 ± 9.5 (500 mg/l control) decreases in absorbance over the incubation period.

(Kirk *et al.*, 1977). It should also be noted that in 2% malt agar, nutrient nitrogen is limited. Elemental analysis demonstrated that the carbon to nitrogen ratio in malt extract was approximately 50:1.

Lignin peroxidase H8 oxidized Congo Red. The effect of pH on decolorization of Congo Red by lignin peroxidase H8 is illustrated in Fig. 4. In addition to a decrease in absorbance, the absorbance maxima shifted to shorter wavelengths (i.e. hyposochromic shifts occurred) in all cases where oxidation was found to occur. There was no observed spectral change at pH 4.5 or in control experiments in which either lignin peroxidase H8 or hydrogen peroxide was omitted. Analysis of the enzymatic reaction by HPLC (data not shown) revealed that a number of oxidation products were formed upon incubation of Congo Red with lignin peroxidase H8 at pH 3.0. In agreement with the spectral assays no detectable oxidation occurred at pH 4.5 and no products could be detected by HPLC analysis.

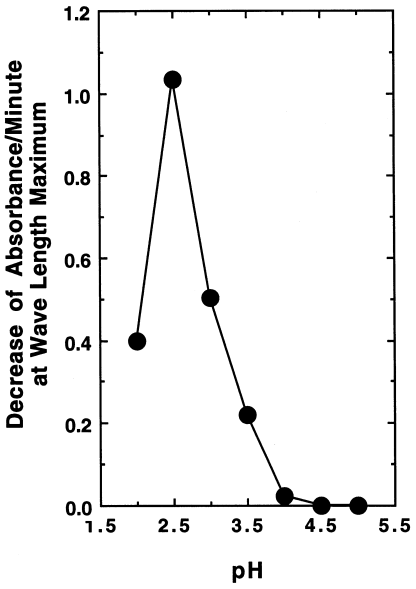

Fig. 4. pH profile of the oxidation of Congo Red by lignin peroxidase H8. The initial rate was calculated by measuring the decrease in absorbance of Congo Red at the wave length maximum for each pH. There was no observed oxidation at pH 4.5 or in controls.

DISCUSSION

P. chrysosporium has been shown to degrade a wide variety of compounds including an extensive number of azo dyes (Kirk *et al.*, 1977; Bumpus, 1989, 1995; Bumpus *et al.*, 1985; Bumpus and Brock, 1988; Cripps *et al.*, 1990; Kennedy *et al.*, 1990; Paszczynski and Crawford, 1991; Paszczynski *et al.*, 1991, 1992; Spadaro *et al.*, 1992; Pasti-Grigsby *et al.*, 1992; Capalash and Sharma, 1992; Ollikka *et al.*, 1993; Bumpus and Tatarko, 1994). Cripps *et al.* (1990) reported that the azo dye Congo Red was not readily degraded by *P. chrysosporium* nor did it appear to be a substrate for lignin peroxidases. However, Ollikka *et al.* (1993) reported that Congo Red was a substrate for lignin peroxidase. We confirmed the observation that Congo Red is indeed a substrate for lignin peroxidase and showed that it is also readily degraded in liquid and solid agar cultures.

Ollikka *et al.* (1993) studied lignin peroxidase mediated oxidation of Congo Red at pH 4.0. In the report by Cripps *et al.* (1990) the attempted enzymatic oxidation of Congo Red by crude lignin peroxidase was performed at pH 4.5. Although pH 4.5 is within the physiological pH range that is near optimum for growth and lignin degradation (Kirk *et al.*, 1977), it is outside the pH range for Congo Red oxidation by lignin peroxidase. The maximum rate of Congo Red oxidation occurred at pH 2.5, a value that agrees well with the published pH profile for veratryl alcohol (Tien and Kirk, 1984). Interestingly, the red to blue transition that is characteristic of Congo Red occurs between pH 5 and pH 3 suggesting that only the protonated form of the dye is oxidized.

Congo Red is readily degraded in nutrient nitrogen limited agitated cultures. The pH of these cultures at the time of addition of Congo Red was 4.28 ± 0.04 and may have been just acidic enough to allow oxidation of Congo Red by lignin peroxidase. Alternatively, the putative variations in the microenvironment of the fungal cultures may also have produced areas of sufficiently low pH for oxidation by lignin peroxidases to occur. The fact that there was no observable degradation in nutrient nitrogen sufficient cultures in which the lignin degrading system is repressed also indicates that the lignin degrading system has a critical role in the biodegradation of Congo Red.

Acknowledgement—This research was supported by NIEHS grant ESO 4492.

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