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Binding of textile azo dyes by *Myrothecium verrucaria*

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SUMMARY

A strain of *Myrothecium verrucaria* that showed a high capacity for rapid decolorization of textile dye solutions was isolated from soil. As much as 70%, 86%, and 95% of Orange II, 10B (blue) and RS (red) dyes (color index no. 15510, 20470, 23635), respectively, were adsorbed from solutions of approximately 0.2 g dye per liter in 5 h by approximately 4.5 g dry weight of cells per liter of dye solution. Intact cells showed a higher adsorption capacity than disrupted cells for Orange II and RS but not for 10B. Dye bound to cells was recoverable by extraction with methanol and methanol-treated cells were able to be recycled, albeit with a slightly diminished dye-binding capacity. The Tween detergents were shown to reduce dye adsorption. Dyes strongly bound to the fungal biomass required sonication in dH_2O or in Triton X-100 or extraction with methanol for their removal. These results suggest that hydrophobic/hydrophilic interactions are important in dye binding.

INTRODUCTION

Azo dyes are employed extensively by the textile and dyestuff industries. Their presence in dye wastewaters creates a pollution problem. Since they are relatively recalcitrant to aerobic biological treatments [9], chemical and physical methods have been employed in their removal [3]. However, several publications have reported decolorization of dye solutions by bacteria; *Pseudomonas* sp. [13], *Bacillus cereus* [4,12] and *Streptomyces* spp. [7] and by the white rot fungus *Phanerochaetes chrysosporium* [1,2,7]. Biological decolorization has been attributed to biodegradation and to fungal adsorption [1,13]. Azoreductases [13] and lignin peroxidases [1,7] have been implicated in the biodegradation. Considerable decolorization (50–90%) of dye solutions by fungi occurs in the first 1–6 h and is probably primarily a result of adsorption to the fungal biomass [1]. Recently, our laboratory also reported novel fungal isolates which showed a good adsorption capacity toward textile dyes and dye wastewater [5]. On the other hand, biodegradation would be expected to occur over a longer time frame. The high level of removal of dye by adsorption to biomass in a short time period may in itself be the basis for a treatment process for removal of dye and for its recovery and reuse. This is very much like a classical chromatography method. Preliminary investigations employing wastewater from dye houses showed that addition of fungal biomass was as much as 100-fold more effective than activated sludge in removal of dye and that irrespective of the broad pH ranges of the different dye wastewaters the removal rate was high [5]. In this study, we examined further the adsorption (external binding) and absorption (internalization) of the three azo dyes: Orange II, 10B (blue) and RS (red) (Fig. 1) and, in particular, with a view to the mechanistics of their binding to *Myrothecium verrucaria*.

MATERIALS AND METHODS

Microorganisms and culture preparation. Soil and dye wastewaters were screened for fungi with decolorizing ability by plating on nutrient agar (Difco) and potato dextrose agar (Difco) supplemented with each of three dyes at 0.1 g/l: Orange II, RS (H/C, red), 10B (H/C, blue) (Fig. 1) obtained from Everlight Chemical Industrial Co., Taiwan. Antibiotics (penicillin G and kanamycin, 3 mg/ml) were added to the media to discourage bacterial growth. Colonies surrounded by decolorized zones were selected. Isolates were then tested in submerged culture. One spore-forming filamentous fungus was shown to consistently remove high levels of dye. Based on its growth and colony characteristics [8] and on comparison with two *M. verrucaria* strains (ATCC 9095 and 36315) this microorganism was identified as *M. verrucaria* and desig-

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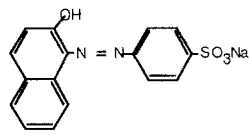
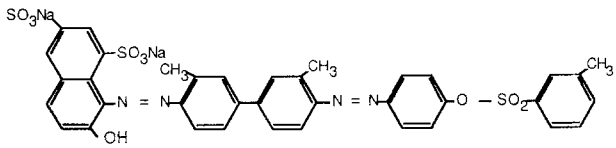
Orange- II

C. I. No. 15510
MW 350



RS(H/C)

C. I. No. 23635
MW 811



10B(H/C)

C. I. No. 20470
MW 616

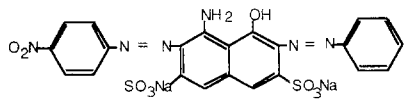


Fig. 1. Molecular structures of Orange II, RS (red) and 10B (blue) dyes.

nated as strain DCB D-1. Dye biodegradation was observed inconsistently and considered present when 'dyed' culture mycelia to which dye had been added became colorless, that is identical to cultures to which no dye had been added. An isolate of DCB D-1 which had lost its dye biodegradation properties was selected for this study into the mechanistics of dye removal. Thus biodegradation could not contribute to decolorization. *M. verrucaria* were first grown in potato dextrose broth at 28 °C in 500-ml Erlenmyer flasks on a shaker (70 mm throw diameter, 175 rpm) for 3 days before use.

Estimation of decolorization. Three-day old cultures were centrifuged (3000 rpm, 10 min), supernatants removed and an amount of the wet cell cake (1.5% dry weight) was added to an aqueous dye solution to give a wet weight to volume ratio of 1:3. Dye solutions were 0.25, 0.22 and 0.26 g/l for Orange II (Acid Orange; C. I. No. 15510), 10B (Naphthol Blue Black, Amido Black 10B, Acid Black I; C. I. No. 20470) and RS (Acid Red 114; C. I. No. 23635) [14] dyes, respectively. The degree of decolorization was measured as the change in the absorbance of the supernatant. Optical densities of properly diluted supernatants were measured in a Beckman DU-62 spectrophotometer. The dye in these samples is referred to as the non-absorbed dye (fraction 1). Measurements were made at the characteristic wavelengths for the dyes: RS, 510; 10B, 620 and Orange II, 485 nm. We did not observe significant pH shifts in the dye adsorption experiments. Furthermore, it was not a factor of concern over a pH range of 3–10 for all three dyes. High performance liquid chromatography (HPLC) data backed up the notion that the mass of dye removed is proportional to the reduction in absorbance of dye solution (data not shown). The HPLC method employed an ion-pair reverse-phase separation method on a C-18 column, PIC-A reagent mobile phase (1 ml/min) and 280 nm UV detection [11].

Cell fractionation. Mycelia collected by centrifugation were homogenized in dH_2O (1:2; cell mass:dH_2O) and sonicated (3–12 min, Heat Systems-Ultrasonic Inc., Model W-385). Disruption was verified by microscopic examination (Nikon, Optiphot). The samples were then centrifuged (10000 rpm, 15 min) and the absorbance of the supernatant measured. This fraction is referred to as the dH_2O-sonicated fraction (fraction 2). The disrupted pellet was then resuspended in 0.1% Triton X-100 (Sigma) (1:2; cell mass:dH_2O), resonicated, centrifuged and the absorbance measured as above. This fraction is referred to as the Triton X-100-sonicated fraction (fraction 3). The remaining pellet was then extracted with methanol at a cell wet weight to volume ratio of 1:8 and the absorbance of the resulting supernatant measured. This fraction is referred to as the methanol fraction (fraction 4). Dye recovery refers to the amount of dye recovered compared to the control dye solution incubated without cells, i.e., (sum of dye in all fractions $(1+2+3+4)$/dye in no-cell control) $\times$ 100. The amount of dye in fractions in g/l was determined from extinction coefficients, obtained from standard curves, for the dyes at the given wavelength.

RESULTS AND DISCUSSION

A strain of the *Myrothecium verrucaria* DCB D-1 was isolated and shown to decolorize dye-containing solutions by adsorption and biodegradation [5]. Preliminary laboratory results indicate that the biodegradation trait of this culture was not stable and a strain lacking this property was conveniently selected for studies on adsorption alone.

Dyes RS (red) and 10B (blue) were adsorbed rapidly and to a high degree by *M. verrucaria* while Orange II was adsorbed at a slower rate and to a lesser extent (Fig. 2). More than 50% of the dye RS was removed in the first few minutes of exposure. Equilibrium was obtained at about

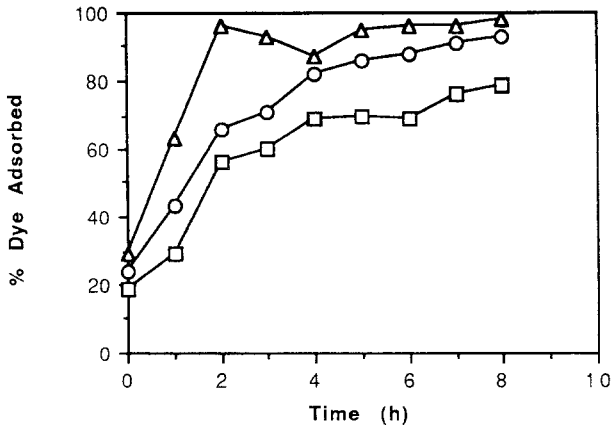


Fig. 2. Rate of adsorption of Orange II (□), 10B (○) and RS (△) dyes by *M. verrucaria*.

4–6 h. Some slight variation in the percentage adsorption of the different dyes was observed with different batches of cells. Results are averages of three determinations of a single experiment. One kg of wet mycelial cells was estimated to have a removal capacity of 4 g of RS dye molecules when saturated after repeated exposures to the dye solution [5].

Methanol extraction of dye-bound cells desorbed 39%, 35% and 53% of the Orange II, 10B and RS dyes, respectively. Visual examination of the extracted cell mass showed that dye was still present.

At this stage it was not clear whether the term adsorption represented simply external binding or external binding plus internalization. In order to examine further the internalization of dye in cells and to investigate the possible role of intracellular compartmentation of dye in decolorization, intact and disrupted (sonicated) cells were incubated for 1 or 24 h in the dye solutions (Fig. 3). After 1 h in the presence of either of the three dyes, the intact cells had adsorbed more or less the same amount of dye as the disrupted cells (Fig. 3a). After 24 h the dye adsorption increased for the intact cells (Fig. 3b). This increase was only slight for disrupted cells with the exception of 10B dye which came close to that of the intact cells. The increased binding with intact cells suggests that the integrity of the cell is important for the binding capacity and that some dye is internalized. In addition, autoclaved cells removed Orange II dye equally well if not to a better extent than intact resting cells (data not shown). Autoclaving of cells may have modified the permeability of the cell mass. The visual appearance of autoclaved cells or methanol-extracted non-autoclaved cells in the absence or presence of bound dye remained unchanged from that of untreated cells.

Methanol-treated cells retained, to a large extent, their capacity to adsorb dyes (Fig. 4). This effect was more

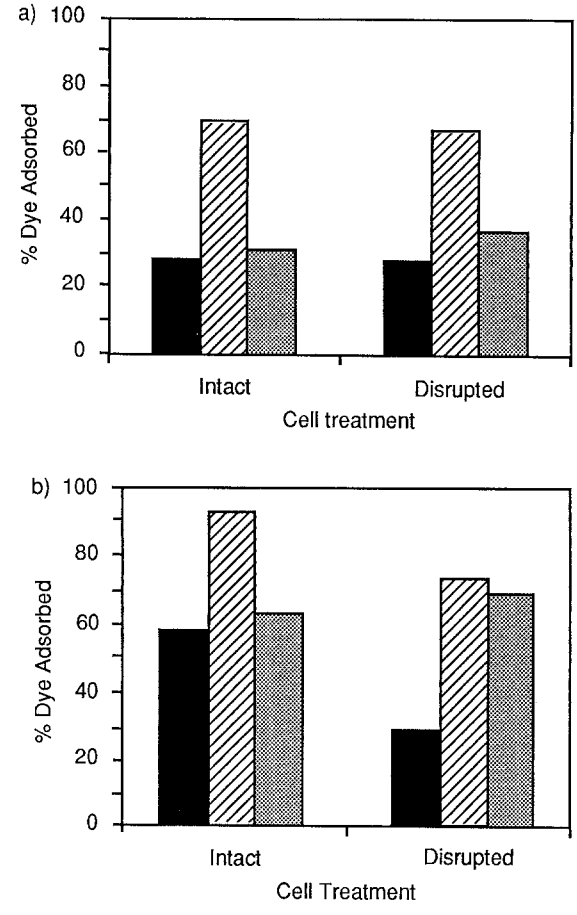


Fig. 3. Adsorption of Orange II (■), RS (▨) and 10B (▩) to intact and disrupted cells after (a) 1 h and (b) 24 h.

pronounced with RS than with Orange II and 10B dyes. Methanol may be expected to influence the hydrophobic/hydrophilic interactions between the dyes and the biomass. These latter results also demonstrate that methanol-

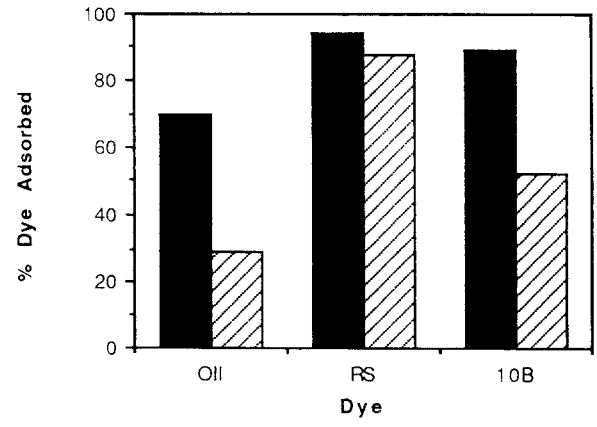


Fig. 4. Adsorption of Orange II (OII), RS and 10B for 1 h to cells pretreated in dH_2O (■) or methanol (▨).

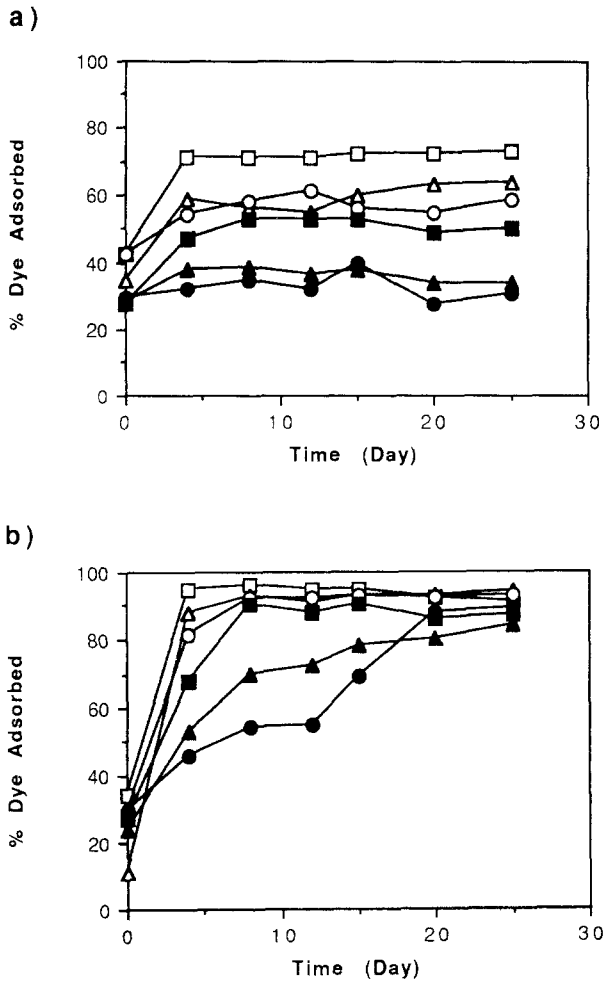


Fig. 5. Effect of Tween-20 on the adsorption of (a) Orange II and (b) 10B dyes: absence of Tween-20 (□), 0.1% (△), 0.25% (○), 0.5% (■), 1.0% (▲) and 2.0% (●) Tween-20. 0 day represents a 1.5 h exposure time.

extracted cells could be recycled for further dye removal, albeit at a slightly lower efficiency. More extensive rinsing of the cell pellet with dH_2O to remove traces of methanol may permit recovery of the total binding ability.

The mass to volume and the mass to charge ratios of each dye were held approximately constant for all experiments. However, the molar concentration of the Orange II dye was approximately 2-fold that of the other dyes due to its lower molecular weight. Thus a slight increase in the adsorption percentage would be expected at a concentration one-half that of the present Orange II concentration, that is at a molar concentration equivalent to the other dyes. The molecular weight, the degree of sulfonic acid substitution and the positioning of the azo linkage(s) and of the sulfonic acid functional group(s) of the dyes may influence the rate and level of adsorption [9].

The influences on dye binding by glucose, salts, pH, shaking and the dye concentration were examined previously [5] and were shown to affect only slightly the adsorption of dye. However, the presence of the non-ionic surfactant Tween-20 during dye removal resulted in a considerable reduction in the adsorption capacity of the cells (Fig. 5). Surfactants are occasionally employed during textile dying and thus may be present in dye wastewaters. Thus our results suggest that the presence of detergents in wastewaters may reduce the binding efficiency of the cells. In general the higher the concentration of Tween the lower the adsorption. The adsorption of Orange II (Fig. 5a) appeared to be affected to a greater extent than that of 10B (Fig. 5b). The effect of Tween diminished with time especially for 10B but not Orange II (Fig. 5b).

Both Tween-20 and -80 brought about a reduction in the degree of RS dye adsorption (Table 1). The Tween surfactants are non-ionic polyoxyethylene sorbitol esters. Tween-20 contains a monolaurate (C:12) moiety while Tween-80 contains a monooleate (C:18:1) moiety. Tween-80 appeared to prevent binding to a greater extent than

TABLE 1

Cellular localization of RS dye after incubation of *M. verrucaria* in the presence or absence of 1% Tween 20 or Tween 80

Fraction	Recovery (%)[a]					
	dH_2O control		Tween-20		Tween-80	
	30 min	8 days	30 min	8 days	30 min	8 days
(1) Non-adsorbed	48	1	80	56	70	66
(2) dH_2O-sonicated	30	15	17	23	18	21
(3) Triton X-100-sonicated	12	44	2	4	2	4
(4) Methanol extract	1	19	0	2	0	1

[a] Percentage of the control, i.e. dye solution in the absence of cells.

Tween-20; that is the amount of non-adsorbed dye was higher for Tween-80 than for Tween-20 but only after exposure to dye for more than 10 days (data not shown). Thus the type of fatty acid in the Tween structure influenced the degree of binding.

Surfactants effect the integrity and function of cell membranes by modifying hydrophobic-hydrophilic interactions. These results therefore suggest that the membrane of the cells as well as the cell wall may be involved in optimal binding. However, Tween detergents are also generally well tolerated by microorganisms and in fact may even be consumed as carbon sources [6]. Thus at the concentrations used they may bring about only minimal changes to the cell membrane. The observed effects may result rather from a modification of the hydrophobic/hydrophilic environment which influences dye binding than from a direct modification of the cell membrane structure. Other detergents, ionic in nature, may bring about different modifications in dye binding. However, non-detergent ions such as phosphate and changes in pH had little effect on dye binding [5]. Mechsner and Wuhrmann [4] demonstrated that treatment with toluene of *B. cereus* increased the passage of azo dye: possibly by modifying the lipid fraction of the cell membrane.

Since the largest proportion of recoverable dye was obtained with an initial H_2O-sonication, cells in the presence of Tween-20 or Tween-80 (Table 1) after 8 days appeared to bind less strongly the bound dye than cells in dH_2O. Cells in the absence of Tween required sonication in both dH_2O and Triton X-100 to desorb the bound-dye. This was also reflected in the total amount of recovered dye, that is the sum of all fractions, which was higher in the presence of Tween than in its absence (Table 2). Visual and microscopic examination of cultures exposed to dye for long time periods suggested that dye became more tightly bound to the biomass.

Bound Orange II dye was more easily desorbed than bound 10B dye (data not shown). The bound Orange II dye was desorbed after dH_2O- and Triton X-100 sonication and the resulting pellet was colorless. However, considerable color was desorbed in the methanol fraction from the 5-day-old 10B dye-treated cells. Orange II dye was recoverable either by sonication in dH_2O and Triton X-100 or by extraction with methanol. However, some color remained with cells treated with 10B or RS dyes after successive sonication and methanol extraction steps. After the methanol extraction step, at a cell mass to volume ratio of 1:8, the cell pellet remained slightly colored. The amount of dye recovered with the successive sonication-extraction protocol decreased with time of incubation and was lower for cells incubated in the absence of Tween (Table 1). This suggests again that dye is bound more strongly in the absence of Tween. The dH_2O-sonicated sample would be expected to contain the intracellular compartment and the Triton X-100-sonicated sample to contain the membrane fraction. These results also suggest internalization of dye. However, the possibility that externally bound dye was released by sonication could not be excluded.

TABLE 2

Total recovery of RS dye, after successive desorption treatments, from mycelia exposed in the presence or absence of 1% Tween-20 or -80

Exposure time (days)	Recovery[a] (%)		
	dH_2O control	Tween-20	Tween-80
0[b]	91	99	90
8	79	85	91
18	84	72	94
28	32	66	80
39	49	52	75

[a] Percentage of the control, i.e., dye solution in the absence of cells. Total recovery is the sum of the non-adsorbed dye (Fraction 1) and the dye released from cells in successive desorption fractions: dH_2O-sonicated (Fraction 2), Triton X-100-sonicated (Fraction 3) and methanol extracted fractions (Fraction 4) i.e. (sum of dye in all fractions $(1+2+3+4)$/dye in no-cell control) $\times$ 100.

[b] 0 days represents 30 min.

The results presented in this report suggest that a large proportion of dyes when exposed to *M. verrucaria* bound to the chitin of the fungal cell wall. Chitin, a polymer of glucose, has a similar structure to cellulose and thus dyes might be expected to bind to chitin in a similar manner to other biological polymers such as cotton. Dyes are also known to bind to proteinacious materials such as wool. Binding may also occur in a relatively specific manner, and this phenomenon has been exploited in the purification of certain enzymes [10]. Thus intracellular protein may also participate in the binding by fungal biomass. However, the presence of an intact membrane improved binding for most dyes. Thus the membrane and intracellular compartments may play a role in dye binding.

This study demonstrates that the fungal biomass of *M. verrucaria* may be employed in an efficient manner to remove and recycle textile dyes from aqueous solutions. The mechanism of binding of dyes appears to involve hydrophobic/hydrophilic interactions.

ACKNOWLEDGEMENTS

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