Bioremediation of direct dyes in simulated textile effluents by a paramorphogenic form of *Aspergillus oryzae*

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ABSTRACT

Azo dyes are extensively used for coloring textiles, paper, food, leather, drinks, pharmaceutical products, cosmetics and inks. The textile industry consumes the largest amount of azo dyes, and it is estimated that approximately 10–15% of dyes used for coloring textiles may be lost in waste streams. Almost all azo dyes are synthetic and resist biodegradation, however, they can readily be reduced by a number of chemical and biological reducing systems. Biological treatment has advantages over physical and chemical methods due to lower costs and minimal environmental effect. This research focuses on the utilization of *Aspergillus oryzae* to remove some types of azo dyes from aqueous solutions. The fungus, physically induced in its paramorphogenic form (called 'pellets'), was used in the dye biosorption studies with both non-autoclaved and autoclaved hyphae, at different pH values. The goals were the removal of dyes by biosorption and the decrease of their toxicity. The dyes used were Direct Red 23 and Direct Violet 51. Their spectral stability (325–700 nm) was analyzed at different pH values (2.50, 4.50 and 6.50). The best biosorptive pH value and the toxicity limit, (which is given by the lethal concentration (LC_{100}), were then determined. Each dye showed the same spectrum at different pH values. The best biosorptive pH was 2.50, for both non-autoclaved and autoclaved hyphae of *A. oryzae*. The toxicity level of the dyes was determined using the Trimmed Spearman–Karber Method, with *Daphnia similis* in all bioassays. The Direct Violet 51 (LC_{100} 400 mg · mL^{-1}) was found to be the most toxic dye, followed by the Direct Red 23 (LC_{100} 900 mg · mL^{-1}). The toxicity bioassays for each dye have shown that it is possible to decrease the toxicity level to zero by adding a small quantity of biomass from *A. oryzae* in its paramorphogenic form. The autoclaved biomass had a higher biosorptive capacity for the dye than the non-autoclaved biomass. The results show that bioremediation occurs with *A. oryzae* in its paramorphogenic form, and it can be used as a biosorptive substrate for treatment of industrial waste water containing azo dyes.

Key words | *Aspergillus oryzae*, azo dye, bioremediation, biosorption, *Daphnia similis*, paramorphogenic form, toxicity

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INTRODUCTION

Dye effluents are among the major pollutants released into the environment, mainly by textile and dyestuff industries. Azo dyes predominate and are the largest class of dye, with the greatest variety of colors. These dyes have become a great concern in effluent treatment due to their color and potential toxicity to animals and humans alike (Nam & Renganathan 2000; Abraham *et al.* 2003). The advantages of biological treatment over physicochemical methods are its low cost and minimal disturbance to the environment (Ashoka *et al.* 2002). There has been increasing importance placed on the control of water pollution in recent years. Although the release of dyes into the

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environment constitutes a small proportion of water pollution, dyes are visible in small quantities due to their bright colors. Stricter government legislation is holding textile industries to increasingly higher standards of treatment regarding waste effluents.

Currently, the removal of dyes from effluents is performed using physicochemical means. Such methods are often very costly and, although the dyes are removed, the accumulation of concentrated sludge and the emission of toxic substances create a disposal problem (Corso & Almeida 2009). Thus, there is a need to find alternative treatments that are effective in removing dyes from large volumes of effluents at a low cost, such as biological or combination systems. However, industrial discharge from textile and dyestuff industries (major producers of azo and sulfonated compounds) is generally resistant to biological treatment. Both anaerobic and aerobic methods are used for the bioremediation of azo dyes (O'Neill *et al.* 2000). A large number of microorganisms, including bacteria, yeast and fungi, produce different groups of enzymes. Hydrolytic enzymes (e.g., cellulase, xylanase, azoreductase, pectinase, etc.) are generally produced through fungal cultures, as such enzymes are used in nature by fungi for growth. *Trichoderma* spp. and *Aspergillus* spp. have been widely used for the production of these enzymes (Choy *et al.* 1999).

Among all the waste technologies investigated, bioremediation has emerged as the most desirable approach for treating many environmental pollutants. Bioremediation is a pollution control technology that uses biological systems to catalyze the degradation or transformation of various toxic chemicals into less harmful forms. The general approaches to bioremediation are to enhance natural biodegradation by native organisms (intrinsic bioremediation) and perform environmental modification by applying nutrients or aeration (biostimulation) or the introduction of microorganisms (bioaugmentation) (Jin *et al.* 2008). Bioremediation is similar to the use of plants for restoring contaminated sites (phytoremediation). The ability of microorganisms to transform a variety of chemicals has led to their use in bioremediation processes. A number of microorganisms have recently been studied to discover their degradation capacity in the remediation of pollutants (Kumar *et al.* 2011; Ogugbue & Sawidis 2011).

In the present study, normal and autoclaved hyphae of *Aspergillus oryzae* were used to determine their effect on the removal of the dyes Direct Red 23 and Direct Violet 51 from an aqueous solution and study the decrease in toxicity related to *Daphnia similis*.

MATERIALS AND METHODS

Dyes and chemicals

Direct azo dyes with formulations designated Direct Red 23 (DR-23) and Direct Violet 51 (DV-51) were obtained from Imperial Chemistry Industries, a dye manufacturing unit in Rio Claro, São Paulo, Brazil. All chemicals used were of analytical grade: DR-23 $\lambda_{max} = 505$ nm; DV-51 $\lambda_{max} = 550$ nm (Figures 1 and 2).

Microorganism and culture conditions

Aspergillus oryzae CCT 5321 was obtained from the Tropical Culture Collection – Andre Tosello Foundation (Campinas, São Paulo, Brazil). Pure culture was maintained on nutrient agar slants. The nutrient medium used for decolorization studies was composed of (g L^{-1}) glucose 5, peptone 5, beef extract 3 and agar 20. The methodology described by Head (1998) for producing *Aspergillus niger* pellets was followed to obtain the paramorphic forms of *A. oryzae* (Marcanti-Contato *et al.* 1997).

Dye toxicity test

For the toxicity test, dye concentrations ranged from 500 to 1,000 μg mL^{-1} for DR-23 and 125 to 500 μg mL^{-1} for DV-51, with pH 2.50, placed in contact with *D. similis* and cultivated based on the parameters established by Levine (1991)

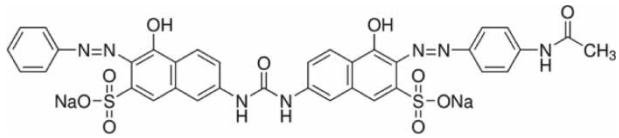

Figure 1 | Direct Red 23 chemical structure.

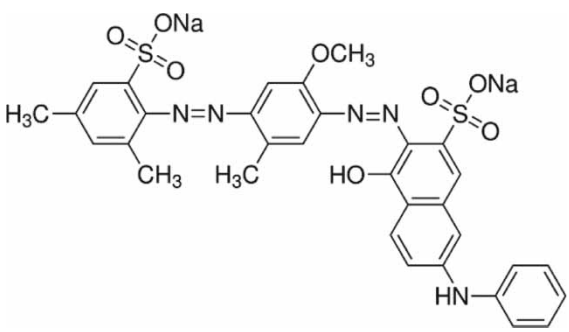

Figure 2 | Direct Violet 51 chemical structure.

to determine LC_{100} and LC_{50} values over a 48 h exposure time, using the Trimmed Spearman–Karber method (Hamilton *et al.* 1977).

Biosorption experiments

Aspergillus oryzae samples were divided into two groups – one non-autoclaved and the other autoclaved – and submitted to biosorption tests at concentrations of 0 to 15 mg (dry weight) for DR-23 and 0 to 6 mg for DV-51, at pH values of 2.50, 4.50 and 6.50. The dye was added to a concentration of 900 μg mL^{-1} for DR-23 and 400 μg mL^{-1} for DV-51. After 120 min, the supernatant was centrifuged at 6000 rpm and analyzed in a spectrophotometer. The amount of biomass needed for total dye removal was estimated from correlations of the data obtained in both experiments.

Toxicity test

Tests were performed to assess the toxicity of the dyes at a LC_{100} concentration limit and when in contact with different concentrations of non-autoclaved and autoclaved *A. oryzae* biomass. The toxicity of each dye at pH 2.50 was evaluated using the supernatant from the biosorption test with *D. similis* by the method mentioned above (Levine 1991).

RESULTS

Dye toxicity test

Dye toxicity tests were conducted for a period of 48 h. Increasing concentrations of the dye remained in contact with about 20 *D. similis* individuals for each concentration studied. Tables 1 and 2 demonstrate that the increase in dye concentration was accompanied by an increase in the mortality of individuals toward the limit of total mortality (LC_{100}).

Table 1 | Toxicity test for DR-23 with *D. similis* [7]

Dye concentration	μg mL^{-1}	500	600	700	800	900	1,000
D. similis	n°	20	20	20	20	20	20
Mortality rate	%	0	45	65	90	100	100

Estimated LC_{50}: 639.834 ± 33.646 μg mL^{-1}.
95% confidence interval.

Table 2 | Toxicity test for DV-51 with *D. similis* [7]

Dye concentration	μg mL^{-1}	125	150	200	250	300	400
D. similis	n^{o}	20	20	20	20	20	20
Mortality rate	%	0	5	40	65	90	100

Estimated LC_{50}: 219.297 ± 18.443 μg mL^{-1}.
95% confidence interval.

Biosorption experiments

The biosorption test with the dyes at an initial concentration of LC_{100} revealed that both the non-autoclaved and autoclaved *A. oryzae* biomass in pellet form (Marcanti-Contato *et al.* 1997) had a linear correlation of 0.9513 to 0.9643 and promoted a high degree of de-colorization in the solution (Figure 1). Adsorption techniques have recently gained favor due to their efficiency in the removal of pollutants that are too stable for conventional methods. Adsorption produces a product of high quality and is an economically feasible process (Schneider *et al.* 2004; Mitter *et al.* 2011) (Figure 3).

The estimate of *A. oryzae* pellets capable of totally removing the dyes from the solution was checked at different pH values. Table 3 demonstrates an enhanced interaction occurring at pH 2.50 and observed for both the autoclaved and non-autoclaved fungal biomass. Also at this pH value, the interactions were optimized and lower biomass concentrations were estimated for the thorough de-colorization of the aqueous solutions.

Monitoring of toxicity through de-colorization of direct dyes

Toxicity assays with *D. similis* were performed to determine the decrease in toxicity of reactive azo dyes treated with non-autoclaved and autoclaved *A. oryzae* biomass. The results of the toxicity tests are displayed in Tables 4 and 5, along with the LC_{100} and degree of toxicity decrease when the dyes were in contact with the biomass compared with control solutions without biomass.

In the interaction between the amount of biomass used and the decrease in toxicity of DR-23, the concentration of non-autoclaved biomass necessary to achieve LC_{50} 640 ± 34 μg mL^{-1} was 2.91 mg mL^{-1}, whereas the concentration of autoclaved biomass needed to reach the same limit was 2.08 mg mL^{-1} (Table 4). In the interaction between amount of biomass used and the decrease in toxicity of DV-51, the concentration of non-autoclaved biomass necessary to achieve LC_{50} 219 ± 18 μg mL^{-1}

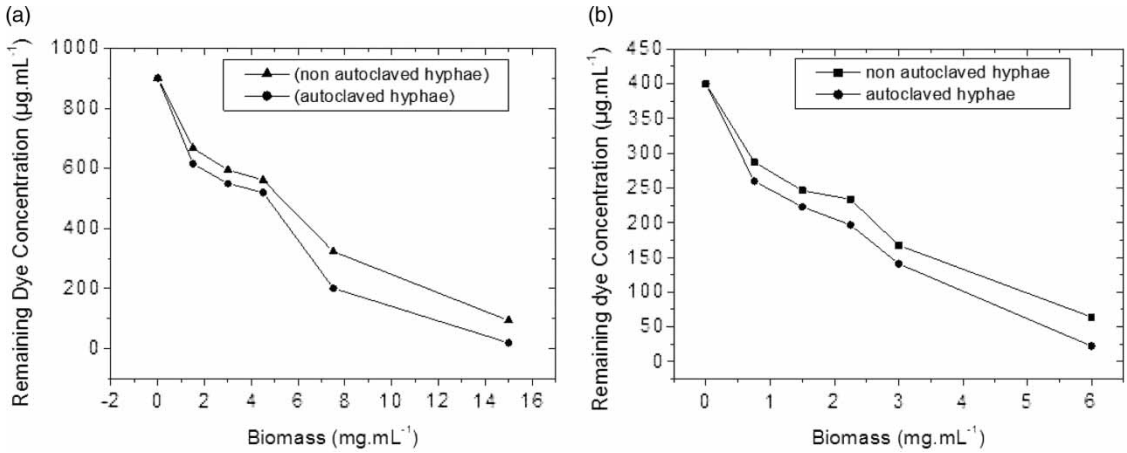

Figure 3 | (a) Toxicity removal from initial LC100 (900.00 mg mL^{-1}) biosorptive interaction between *A. oryzae* (autoclaved and non-autoclaved hyphae) and DR-23 at pH 2.50 after 120 min of exposure time; (b) Toxicity removal from initial LC100 (400.00 mg mL^{-1}) biosorptive interaction between *A. oryzae* (autoclaved and non-autoclaved hyphae) and DV-51 at pH 2.50 for 120 min of exposure time.

Table 3 | Total dye removal estimation from an initial 900 µg mL^{-1} concentration for DR-23 and 400 µg mL^{-1} for DV-51 using dry weight of *A. oryzae* (mg mL^{-1} of biomass) in paramorphic form at different pH values

pH	Non-autoclaved	Autoclaved
DR-23		
2.50	15.74	13.84
4.50	35.13	28.74
6.50	48.95	38.17
DV-51		
2.50	6.81	5.92
4.50	19.16	15.76
6.50	28.16	25.43

was 2.52 mg mL^{-1}, whereas the concentration of autoclaved biomass needed to reach the same limit was 2.03 mg mL^{-1} (Table 5).

DISCUSSION

Environmental toxicology is the study of the effects of toxic substances found in the environment (Duffus 1983). The control of these effects in the state of São Paulo (Brazil) is made explicit by the Brazilian environmental sanitation technology company, (Cetesb 1992), with norm *n*°L 5.017, which also indicates the most suitable statistical analyses for defining the degree of toxicity of a particular product.

The de-colorization of dyes occurs mainly as a result of two mechanisms: adsorption and biodegradation. The molecules that emerge during the biodegradation of dyes – denominated metabolites – are normally more toxic and carcinogenic than the molecules of the intact dye. The use of dyes that produce these byproducts has been drastically reduced in Europe through national regulations, but remains a problem in non-European countries (Schneider *et al.*

Table 4 | Bioassays for toxicity removal test of DR-23 in biosorption interaction with pellets of non-autoclaved and autoclaved *A. oryzae* after 120 min of exposure time at pH 2.50 and 48 h using *D. similis*

Initial dye concentration (µg mL^{-1})	(A) Non-autoclaved biomass (mg mL^{-1})	Remaining dye concentration (µg mL^{-1})	*D. similis* mortality rate (%)	(B) Autoclaved biomass (mg mL^{-1})	Remaining dye concentration (µg mL^{-1})	*D. similis* mortality rate (%)
900.00	0	900.00	100	0	900.00	100
900.00	1.50	667.38	55	1.50	615.29	40
900.00	3.00	594.53	35	3.00	549.38	20
900.00	4.50	560.96	25	4.50	519.56	10
900.00	7.50	322.97	0	7.50	200.68	0
900.00	15.00	93.34	0	15.00	19.05	0

(A) Calibration dye[ ] × biomass[ ]: dye concentration = 785.0052 + (−49.8683 × biomass); $R = 0.9643$; estimated biomass to achieve $LC_{50} = 2.91$ mg mL^{-1}; (B) Calibration dye[ ] × biomass[ ]: dye concentration = 753.0565 + (−54.42473 × biomass); $R = 0.9433$; estimated biomass concentration to achieve $LC_{50} = 2.08$ mg mL^{-1}.

Table 5 | Bioassays for toxicity removal test of DV-51 in biosorption interaction with pellets of non-autoclaved and autoclaved *A. oryzae* after 120 min of exposure time at pH 2.50 and 48 h using *D. similis*

Initial dye concentration ($\mu g\ mL^{-1}$)	(A) Non-autoclaved biomass ($mg\ mL^{-1}$)	Remaining dye concentration ($\mu g\ mL^{-1}$)	*D. similis* mortality rate (%)	(B) Autoclaved biomass ($mg\ mL^{-1}$)	Remaining dye concentration ($\mu g\ mL^{-1}$)	*D. similis* mortality rate (%)
400.00	0	400.00	100	0	400.00	100
400.00	0.75	287.28	75	0.75	259.68	65
400.00	1.50	246.24	60	1.50	222.63	50
400.00	2.25	233.53	55	2.25	196.73	40
400.00	3.00	167.21	20	3.00	140.53	5
400.00	6.00	64.08	0	6.00	22.23	0

(A) Calibration dye[ ] × biomass[ ]: dye concentration = 348.0491 + (−51.107 × biomass); $R = 0.9592$; estimated biomass to achieve $LC_{50} = 2.52\ mg\ mL^{-1}$; (B) Calibration dye[ ] × biomass[ ]: dye concentration = 333.7399 + (−56.34367 × biomass); $R = 0.9513$; estimated biomass concentration to achieve $LC_{50} = 2.03\ mg\ mL^{-1}$.

2004). Studies are needed to determine how much time is necessary for biodegradation to occur until there is a reduction in the toxicity of these compounds to the point at which they no longer have harmful effects, thereby promoting bioremediation. Biosorption involves a more rapid removal of large amounts of dye molecules that are still intact, which facilitates the removal of the source of toxicity. Lowest biomass concentration needed for total dye removal in biosorption tests was achieved at pH 2.50. This situation occurs because when the pH becomes more acidic there is an increase in positive ions (H^+) that attach to the cell wall, increasing its positive charge and thus favoring adsorption to sulfonic groups from both dye molecules.

A decrease in toxicity is the goal of all environmental studies (Vitor & Corso 2008). Toxicity reduction in azo dyes has been shown to occur under many conditions, particularly in association with microorganisms. Among such microorganisms, *A. oryzae* was chosen in the present study due to the fact that this fungus can be transformed into pellets of mycelial hyphae. This method was developed in our laboratory through physical induction, which was first successfully used with *A. niger* (Marcanti-Contato *et al.* 1997) and made it possible to apply mathematical calculations to a mycelial fungus system, which was unprecedented.

The present study evaluated the degree of dye toxicity and the possibility of dye removal from the environment through biosorption. The results demonstrated that Direct Red 23 had an LC_{100} of $900\ \mu g\ mL^{-1}$ and an LC_{50} of $640 \pm 34\ \mu g\ mL^{-1}$ (Table 1) and Direct Violet 51 had an LC_{100} of $400\ \mu g\ mL^{-1}$ and an LC_{50} of $219 \pm 18\ \mu g\ mL^{-1}$ (Table 2). Biosorption tests with Direct Red 23 and Direct Violet 51 at initial concentrations of LC_{100} demonstrated that both dyes were removed using autoclaved and non-autoclaved *A. oryzae* pellets. Moreover, autoclaved pellets proved more efficient than the living fungal biomass. This situation is because dye molecules are identified by living cells as strange, activating protection mechanisms from the cell and reducing its active adsorption sites. When autoclaved cells are used, adsorption becomes a physical–chemical system, as adsorption sites are active in their entirety, thereby increasing adsorption capacity. The dye removal profiles were traced from the toxicity limit (100% mortality) to the concentration estimated for LC_{50} (50% lethal) and a concentration at which the dye does not offer toxicity (LC_0).

CONCLUSION

In the present study, *A. oryzae* in pelletized living and dead biomass promoted the de-colorization and reduced the toxicity of direct textile dyes DR-23 and DV-51 within a short contact time. DR-23 completely immobilized all *D. similis* at $900\ \mu g\ mL^{-1}$ and LC_{50} for this dye was $640 \pm 34\ \mu g\ mL^{-1}$, whereas DV-51 completely immobilized all *D. similis* at $400\ \mu g\ mL^{-1}$ and LC_{50} for this dye was $219 \pm 18\ \mu g\ mL^{-1}$. Both autoclaved and non-autoclaved *A. oryzae* biomass promoted the removal and decreased the toxicity of these dyes in aqueous solutions. However, the autoclaved biomass demonstrated greater adsorption strength.

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