Biodecolourization of azo dyes using *Phanerochaete chrysosporium*:
Effect of culture conditions and enzyme activities

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This study presents effect of culture conditions (temp. (28-39°C) & agitation speed (0-200 rpm)) on biodecolourization of azo dyes [Direct Red - 80 (DR-80) & Mordant Blue – 9 (MB-9)] over an initial concentration range of 10-100 mg/l using white rot fungus, *Phanerochaete chrysosporium* in batch shake flasks. Optimum values of temperature & agitation speed, respectively, for dye decolourization were found to be: DR-80, 39°C & 200 rpm; and MB-9, 37°C & 150 rpm. Both dyes were best removed at the least initial concentration (10 mg/l). Employing Arrhenius equation, activation energies for decolourization of dyes were estimated to be: DR-80, 32.67; and MB-9, 39.12 kJ/mol. Maximum enzyme activities of LiP and MnP were found to be more with DR-80 than with MB-9. Study proved very good potential of ligninase producing fungus in decolourization of industrial wastewaters containing synthetic dyes.

**Keywords:** Azo dyes, Dye decolourization, Lignin peroxidase, Manganese peroxidase, *Phanerochaete chrysosporium*

**Introduction**

Industries like textile dyeing, paper and plastic manufacture use different synthetic dyes and discharge 2,80,000 t of dye based wastewaters worldwide every year into the environment. Discharge of such coloured effluents into rivers and lakes reduces availability of oxygen and absorbs sunlight thus poses problems to photosynthetic plants and aquatic biota. Textile dyes and effluents have toxic effect on germination rates and biomass concentration of several plant species. Many dyes are reported to be carcinogenic. Dyes with azo-based chromophores are the largest group of synthetic dyes known and the most common group found in wastewaters. Azo dyes remain unutilized, found in textile effluents. Dye containing wastewaters can be treated by physical and/or chemical treatment processes (adsorption, coagulation-flocculation, oxidation and electrochemical methods). Although these methods are quite efficient in removing colour but suffer from secondary sludge generation problems, high cost, difficulty in adapting to a wide range of dye containing wastewaters etc. Biological methods are considered more sustainable and eco-friendly. White-rot fungus, *Phanerochaete chrysosporium*, has been intensively studied due to its ability to degrade polycyclic aromatic hydrocarbons (PAHs), dyes etc. This fungus secretes nonspecific oxidoreductases during secondary metabolism in response to nutrient limitation, and among these enzymes lignin peroxidase (LiP, EC 1.11.1.14) and manganese peroxidase (MnP, EC 1.11.1.13) are thought to play a crucial role in degradation of various xenobiotic compounds. In an earlier study, decolourization of two structurally different azo dyes [Direct Red-80 (DR-80) and Mordant Blue-9 (MB-9)] by *P. chrysosporium* was investigated both individually and in mixtures in batch shake flasks.

This study presents effects of culture conditions (temp. and agitation speed) and initial concentration (IC) of dye on decolourization of DR-80 and MB-9 by *P. chrysosporium*.

**Experimental Section**

**Dyes and Chemicals**

Azo dyes (DR-80 and MB-9) and veratryl alcohol (3, 4-dimethoxybenzyl alcohol, 96% pure) were purchased from Sigma Chemicals (St Louis, MO, USA). All other chemicals and solvents were of reagent grade and purchased from Merck® India Ltd.
Microorganism and Culture Conditions

White-rot fungus, *P. chrysosporium* (MTCC 787) used in decolourization experiments, was obtained from IMTECH, Chandigarh, India. Stock culture of organism was grown on potato dextrose agar (PDA) at 25°C and maintained at 4°C, and refreshed in every 30-40 days. For spore production, slants were incubated at 39°C for 2-6 days in medium22 (glucose, 10; malt extract, 10; peptone, 2; yeast extract, 2; asparagine, 1; KH₂PO₄, 2; MgSO₄×7H₂O, 1; thiamin-HCl, 1; and agar, 20 g/l). Media23 used for decolourization of dyes was optimized and composed of basal medium (KH₂PO₄, 20; MgSO₄, 5; and CaCl₂, 1 g/l), trace elements (MgSO₄, 3; MnSO₄, 0.5; NaCl, 1; FeSO₄·7H₂O, 0.1; CoCl₂, 0.1; ZnSO₄·7H₂O, 0.1; and CuSO₄, 0.1 g/l; AlK(SO₄)₂·12H₂O, 10; H₂BO₃, 10; and Na₂MoO₄·2H₂O, 10 mg/l; and nitrolotriacetate, 1.5 g/l) and other ingredients (glucose, 100 g/l; 2,2-dimethylsuccinate, 0.1 M (pH 4.2); thiamine, 100 mg/l (filter sterilized); veratryl alcohol, 4 mM (filter sterilized); and NH₄Cl, 4.68 g/l).

Dye Decolourization Experiments and Optimization of Process Variables

Dye decolourization experiments were performed as reported28 with some modifications. Erlenmeyer flasks (250 ml) containing decolourization media were inoculated with 10% (v/v) fungal spores (optical density ~ 1.0). Choosing an IC (10-100 mg/l) for both DR-80 and MB-9, dye decolourization by fungus was studied by incubating flasks at 180 rpm for 6 days at 39°C. Effect of agitation speed (0-200 rpm) and temperature (25-39°C) on dye decolourization was studied. In all experiments, initial pH 4.5 of media was set. Optimization of temperature and agitation speed on decolourization was performed for an IC (50 mg/l) for both dyes. Duplicate samples were taken during the experiments and analyzed for dye concentration and enzyme activities of LiP (lipase peroxidase) and MnP (manganese peroxidase).

Determination of LiP and MnP Activities and Residual Dye Concentrations

For enzyme assay, samples were centrifuged at 10,000 × g for 10 min at 4°C to remove fungal biomass and divided into two portions. One portion of supernatant containing enzymes was assayed for LiP activity at 310 nm using a UV-visible spectrophotometer (Cary 100, Varian, USA), which was based on the oxidation of veratryl alcohol to veratraldehyde29. Another portion of supernatant was assayed for MnP activity by monitoring the oxidation of 1 mM MnSO₄ in 50 mM sodium malonate buffer (pH 4.5) in the presence of 0.1 mM H₂O₂. One unit of enzyme is defined as the amount of enzyme that oxidized 1 mM of substrate per min and its activity is reported in U/l. Residual concentrations of DR-80 and MB-9 in the media were analyzed using same supernatant as above by measuring its absorbance at 528 nm (λ_max of DR-80) and 516 nm (λ_max of MB-9) respectively.

Results and Discussion

Effect of Dye Initial Concentration (IC) on Its Decolourization by Fungus

Decolourization (Fig. 1) of DR-80 and MB-9 by *P. chrysosporium* varied with the nature and IC of dyes. Low dye concentrations (10-45 mg/l) culture took less time to decolourize DR-80 than MB-9 under similar conditions and resulted in maximum (100%) decolourization efficiency. However, at high concentrations (> 75 mg/l), no significant difference was observed in efficiency and rate of dye decolourization. Difference in decolourization of dyes can be explained that mordant class of dyes, unlike direct class of dyes, form insoluble colour complexes with substrates due to the metal chelating group present within their structures, which, offers more resistance towards decolourization than direct dyes. Similar observations on differences in rate and efficiency of decolourization of different dyes due to microorganisms are reported28,31,32. Compared with low ICs, high concentrations of dyes exhibit inhibitory effect on fungus growth and therefore its efficiency to degrade the dyes28,31,33.

Maximum activities of LiP and MnP depended on the dyes (DR-80 or MB-9) and their ICs used in their respective media. For both dyes (IC, 10 mg/l), maximum activities of LiP and MnP, respectively were obtained as...
follows: DR-80, 118.6, 16.58 U/l; and MB-9, 115.48, 22.89 U/l. Overall, irrespective of dyes and its IC, LiP activity was more than MnP activity shown by fungus. Further, LiP and MnP activities were correlated with decolourization profiles of two dyes for an IC of 100 mg/l in their respective media (Fig. 2); rate of dye decolourization increased when enzyme activities were maximum. Sami & Radhouane\textsuperscript{34} also reported similar values of enzyme activities for olive mill wastewater decolourization.

**Optimization of Process Parameters**

DR-80 decolourization increased with the raise in temperature, and maximum decolourization was observed at 39°C (Fig. 3a). In case of MB-9, similar profile was observed and maximum decolourization obtained was at 37°C (Fig. 3b). Dye removal at elevated temperatures could be attributed to higher respiration and substrate metabolism rate by fungus, which however was inhibited at very high temperature\textsuperscript{35}. Activation energies ($E_a$) determined using Arrhenius equation for decolourization of both DR-80 and MB-9 were calculated to be 32.67 kJ/mol and 39.12 kJ/mol, respectively. Lower value of $E_a$ in case of DR-80 suggests that its decolourization is more easily favoured than decolourization of MB-9 by fungus\textsuperscript{36}.

At low agitation speed (50 rpm), decolourization efficiencies were obtained as: DR-80, 74%; and MB-9, 38%. Further increase in agitation speed resulted in higher decolourization efficiencies of the dyes. In case of DR-80, the highest (~99%) decolourization was obtained at 200 rpm. On the other hand, an agitation speed of 150 rpm was optimum for maximum decolourization (~93%) of MB-9. Yesilada et al\textsuperscript{37} also reported an optimum agitation speed of 150 rpm for maximum decolourization of a basic azo dye, Astrazon Red FBL, using *Fumalia trogii* in batch shake flasks. Effect of agitation speed on secondary metabolism, stability and activities of lignolytic enzymes secreted by *P. chrysosporium* has been reported\textsuperscript{38,39}. Thus, present study suggests that optimum conditions of both temperature and agitation are required for maximum stability and activity of enzymes responsible for decolourization of dyes. Further, compared with reported
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