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Decolourization of azo dyes and a dye industry effluent by a white rot fungus *Thelephora* sp.

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Abstract

A white rot fungus *Thelephora* sp. was used for decolourization of azo dyes such as orange G (50 μM), congo red (50 μM), and amido black 10B (25 μM). Decolourization using the fungus was 33.3%, 97.1% and 98.8% for orange G, congo red and amido black 10B, respectively. An enzymatic dye decolourization study showed that a maximum of 19% orange G was removed by laccase at 15 U/ml whereas lignin peroxidase (LiP) and manganese dependent peroxidase (MnP) at the same concentration decolourized 13.5% and 10.8%, orange G, respectively. A maximum decolourization of 12.0% and 15.0% for congo red and amido black 10B, respectively, was recorded by laccase. A dye industry effluent was treated by the fungus in batch and continuous modes. A maximum decolourization of 61% was achieved on the third day in the batch mode and a maximum decolourization of 50% was obtained by the seventh day in the continuous mode. These results suggest that the batch mode of treatment using *Thelephora* sp. may be more effective than the continuous mode for colour removal from dye industry effluents.

Keywords: Azo dyes; Decolourization; Dye industry effluent; Laccase; Lignin peroxidase (LiP); Manganese dependent peroxidase (MnP); *Thelephora* sp.

1. Introduction

Synthetic dyes are extensively used for textile dyeing, paper printing, colour photography and as additives in petroleum products. During the dyeing process, 10–15% of dye is discharged in the effluent. Approximately a half of all known dyes are azo dyes, making them the largest group of synthetic colourants. Azo dyes and their pigments are versatile and the most common synthetic colourants released into the environment. They are also typically amenable to structural modification and representative azo dyes can be made to bind most synthetic and natural textile fibers. Approximately 10,000 different dyes and pigments are used for industries and over 7×10^5 tons of these dyes are annually produced worldwide (Zollinger, 1987).

Several amino-substituted azo dyes including 4-phenylazoaniline and *N*-methyl- and *N*,*N*-dimethyl-4-phenylazo anilines are mutagenic as well as carcinogenic. In mammals, azo dyes are reduced to aryl amines by cytochrome p450 and a flavin-dependent cytosolic reductase (Fujita and Peisach, 1977). Microbial decolourization has been proposed as a less expensive and less environmentaly intrusive alternative. Various bacteria and fungi have decolourizing abilities and an extensive review of microbiological decolourization is available (Glenn and Gold, 1983). At present decolourization of various kinds of azo dyes by various fungi has been reported (Bumpus et al., 1988; Cripps et al., 1990; Rafii et al., 1990; Paszezynski et al., 1991). Colleen et al. (1990) demonstrated the degradation of azo and heterocyclic dyes of orange II, tropeolin O, congo red and azure B by *Phanerochaete chrysosporium*. The degradation of azo, anthraquinone, heterocyclic, triphenylmethane and polymeric dyes by *P. chrysosporium* has been intensively studied (Bumpus et al., 1988; Cripps et al., 1990; Kling and Neto, 1991; Ollikka et al., 1993).

Adsorption of dyes to the microbial cell surface is the primary mechanism of decolourization (Knapp et al.,

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1995). Wang and Yu (1998) reported the adsorption of acid green 27, acid violet 7 and indigo carmine dyes on living and dead mycelia of *Trametes versicolor*. Also, enzymes such as lignin peroxidase (LiP), manganese dependent peroxidase (MnP) and laccase, all of which are involved in lignin degradation, have been reported to decolorize dyes (Vyas and Molitoris, 1995). Dyes with different structures are decolourized at different intrinsic enzymatic rates and high dye concentration results in the slower decolourization rate. Kim et al. (1996) demonstrated the presence of H_2O_2-dependent enzyme activity decolourizing remazol brilliant blue R in the culture filtrate of *Pleurotus ostreatus* in a chemically defined medium. Young and Yu (1997) suggested the binding of dyes to the fungal hyphae, physical adsorption and enzymatic degradation by extracellular and intracellular enzymes as major mechanisms for the colour removal. As a result of the studies on the physical sorption and the enzymatic degradation of the adsorbed dye molecules, the enzymatic degradation of adsorbed dyes has been regarded as a major mechanism in the regeneration of dye adsorption capacity of the mycelium.

Kim and Shoda (1999) have purified and characterized the novel peroxidase (Dyp) that is responsible for the dye-decolourizing acitivty of *Geotrichum candidum* Dec 1. Nine of the 21 types of dyes were decolourized by Dec 1 and in particular anthroquinone dyes were highly decolourised. Swamy and Ramsay (1999) reported that in the white rot fungus, *Trametes versicolor*, lignin peroxidase (LiP) was not detected during decolourization of the azo dye of amaranth, while laccase and manganese peroxidase (MnP) were detected in the decolourizing cultures. A white rot fungus, *Thelephora* sp., was isolated from the Western Ghats of South India and characterized for its ligninolytic enzymes (Selvam, 2000), but no studies have been conducted yet on the biotechnological applications of this fungus to decolourization of azo dyes. In the present study, the feasibility of using *Thelephora* sp. for azo dye decolourization and colour removal from a dye industry effluent was examined.

2. Methods

2.1. Microorganism and media

Thelephora sp. was isolated from stumps of a burnt tree in the Western Ghats region of Tamilnadu, India. The fungus was identified based on the keys provided previously (Bakshi, 1971; Gilbertson and Ryvarden, 1986). Fungal growth was cut out, sterilized with 1% mercuric chloride solution, repeatedly washed with sterile distilled water as described previously (Watling, 1971) and inoculated on 2% malt agar medium. The fungal growth on a plate was sub-cultured for 6 days at 37 °C and maintained on malt agar slants. Then, the spores were harvested without disturbing the mycelial growth using a camel hair brush and filter-sterilized. The spore concentration was adjusted to 10^5 spores/ml and used as an inoculum for further studies. Growth kinetics, the ligninolytic enzyme production, and dye decolourization studies were carried out in a C-limited medium (M14) of Janshekar and Fiechter (1988), to which spores in the one-tenth volume of the medium were inoculated.

2.2. Ligninolytic enzyme production

Enzymes, lignin peroxidase (LiP), manganese-dependent peroxidase (MnP) and laccase were purified from the culture filtrate by acetone precipitation (66% v/v) and Sephadex G-100 column chromatography. The LiP activity was determined using a spectrophotometer according to the method of Linko (1988). One unit of enzyme activity was defined as the amount of enzyme oxidizing one μmole of veratryl alcohol in 1 min. MnP was assayed using a spectrophotometer by the method of Kuwahara et al. (1984). One unit of enzyme activity was expressed as the amount of enzyme capable of increasing one optical density (OD) at 610 nm in a min. The laccase activity was determined spectrophotometrically as described by Evans (1985) with guaiacol as a substrate. In this case, one unit of the enzyme activity was expressed as the amount of enzyme able to increase one OD at 440 nm.

2.3. Decolourization of azo dyes

The ability of the fungus to degrade the azo dyes, orange G, congo red, and amido black 10B was studied in C-limited medium. Fungal spore (10^5 spores/ml) was inoculated and incubated at 30 °C for 6 days in an orbital shaker. After 6 days, a dye (orange G-50 μM/congo red-50 μM/amido black 10B-25 μM) was added. Samples were withdrawn at regular time intervals and filtered, and the OD of the clear filtrate was measured at 479, 497 and 618 nm, respectively, for orange G, congo red and amido black 10B. For enzyme treatment, the reaction mixture containing 90 ml of dye at various concentrations was incubated with various concentrations (5, 10 and 15 U/ml as a final concentration) of LiP, MnP (in sodium succinate buffer, 20 mM; pH 4.5) or laccase (in phosphate buffer, 0.1 M; pH 7). At regular time intervals, the samples were withdrawn and the OD was measured.

2.4. Decolourization of textile dye effluent

To analyse the efficiency of treatment of a dye industry effluent, two modes of treatment were adopted,

since different modes can show different efficiencies in the treatment. The ability of the fungus to remove colour from a dyeing industry effluent was assayed in the modified C-limited medium (Janshekar and Fiechter, 1988). The medium contained the textile dye effluent instead of distilled water in equal volume. The pH of the solution was adjusted to 4.5. To the effluent-amended medium (950 ml), 50 ml of spore suspension (10^5 spore/ml) was inoculated and maintained at 39 °C. Samples were withdrawn at regular time intervals and analysed for colour removal. The intensity of effluent colour was measured at 490 nm. For enzyme treatment, the reaction mixture containing 90 ml of the effluent, various concentrations (5, 10 and 15 U/ml as a final concentration) of LiP, MnP (in sodium succinate buffer, 20 mM; pH 4.5) and laccase (in phosphate buffer, 0.1 M; pH 7.0) were added. The reaction mixture was incubated at 37 °C for 1 h. After 1 h, the colour intensity of the effluent was measured. Boiled enzyme was used as a control.

3. Results and discussion

3.1. Decolourization of azo dyes by mycelia

The growth and decolourization efficiency of the test fungus is shown in Table 1. It was observed that *Thelephora* sp. was able to decolourize only 33.3% of orange G within 9 days, whereas congo red was removed upto 97.1% within 8 h and amido black 10B up to 98.8% in 24 h. White rot fungi are the only microorganisms known to be capable of complete mineralization of lignocellulosic polymers. Cripps et al. (1990) reported that *P. chrysosporium* could remove 87–93% of orange 11, tropeolin O and congo red within five days. Spadaro et al. (1992) established that *P. chrysosporium* was capable of mineralizing a variety of toxic azo dyes and the mineralization of aromatic rings of azo dyes was dependent on the nature of ring substituents. Heinfling et al. (1997) reported that *Bjerkandera adusta* and *T. versicolor* removed 95% of HRB 8 dyes within four days. When compared with these results, the newly isolated *Thelephora* sp. has a superior potential to decolourize some azo dyes such as congo red or amido black 10B.

Table 1
Decolourization of azo dyes by *Thelephora* sp. in liquid medium

Incubation period	Mycelial growth (mg/l)	Percent colour removal (mg/g)	Dye removal/mg mycelial
[*] Orange G (50 μM)			
3	80 ± 2.1	7.7 ± 0.7	1.0
6	82 ± 3.4	17.5 ± 0.8	2.3
9	84 ± 4.1	33.3 ± 0.6	5.5
[**] Congo red (50 μM)			
2	70 ± 3.4	75.1 ± 0.7	18.8
4	70 ± 4.3	76.9 ± 0.5	19.2
6	73 ± 5.1	94.2 ± 0.3	23.2
8	73 ± 4.3	97.1 ± 0.6	23.9
[**] Amido black 10B (25 μM)			
4	70 ± 4.6	52.4 ± 0.8	5.8
8	71 ± 4.1	86.6 ± 0.9	9.4
12	71 ± 2.6	87.8 ± 0.6	9.5
16	70 ± 3.4	90.2 ± 0.9	9.9
20	70 ± 5.1	93.9 ± 0.3	10.3
24	69 ± 3.5	98.8 ± 0.4	11.0

Values are mean of three replicates and ± standard deviation.
[*] Incubation period in days.
[**] Incubation period in hours.

3.2. Treatment of azo dyes by enzymes

Ligninolytic enzymes of *Thelephora* sp. were able to decolourize to some extent all of the three azo dyes examined (Fig. 1). A maximum of 19% orange G was removed by laccase (15 U/ml) whereas LiP and MnP

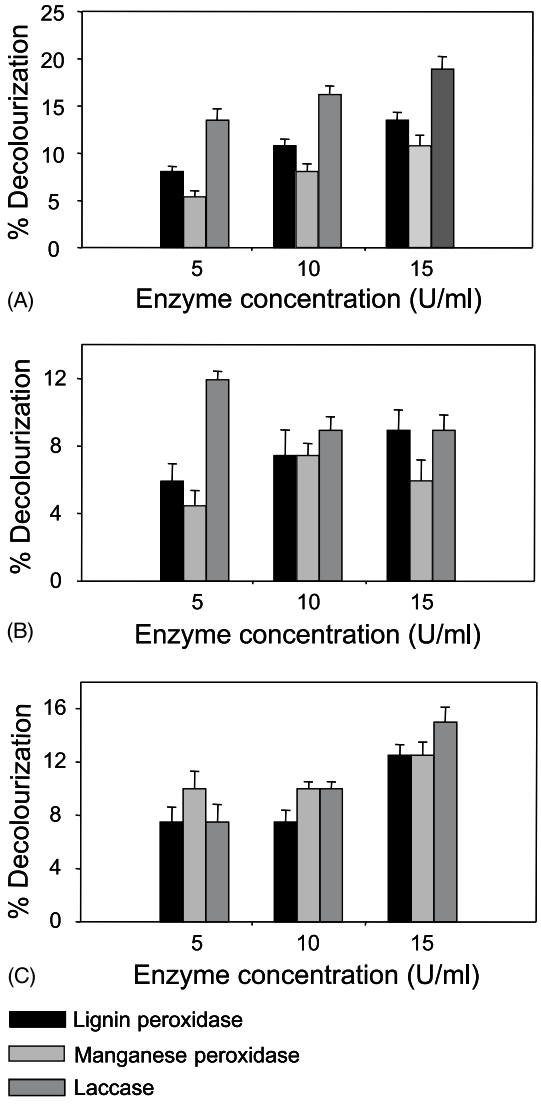

Fig. 1. Decolourization of azo dyes by purified ligninolytic enzymes of *Thelephora* sp.

decolourized 13.51% and 10.8%, respectively (Fig. 1(A)). A maximum decolourization of 12.0% and 15.0 % for congo red and amido black 10B was recorded by laccase enzyme at 5 and 15 U/ml than at other concentration of enzymes (Fig. 1(B) and (C)). Laccase showed the highest degradation of the azo dyes. Ollikka et al. (1993) showed 54% decolourization of congo red in the presence of crude preparation of lignin peroxidase and hydrogen peroxidase as these enzymes utilize congo red as a substrate. Vyas and Molitoris (1995) reported that lignin peroxidase (LiP), manganese-dependent peroxidase (MnP) and laccase, all of which are involved in lignin degradation, participate in the decolourization of the dyes.

3.3. Treatment of a dye industry effluent in two modes

In the study on the treatment of a dye industry effluent, mycelia of *Thelephora* sp. removed 61% of colour in a batch mode from the effluent on the third day of incubation whereas in a continuous mode only a maximum of 50% on the seventh day (Fig. 2). The enzyme laccase at 15 U/ml concentration removed 15% of colour (Fig. 3). Sayadi and Ellouz (1993) reported a positive role for veratryl alcohol with lignin peroxidase in decolourization of olive mill waste water by a *P. chrysosporium* culture. Wang and Yu (1998) reported that adsorption and degradation of dye molecules on living fungal hyphae might provide a mechanism for a feasible application of white rot fungi in continuous treatment of an industrial effluent. Rodriguez et al. (1999) reported that several industrial dyes were decolourized biocatalytically by extra cellular enzymes. Both mycelium and purified enzymes of *Thelephora* sp. were able to decolourize the azo dyes. Mycelia of the test fungus effectively removed 61% of the dyes, which was obtained under conditions not optimized for azo dye degradation and colour removal. Therefore, there is a potential application in the biological treatment of industrial effluents contaminated with azo dyes.

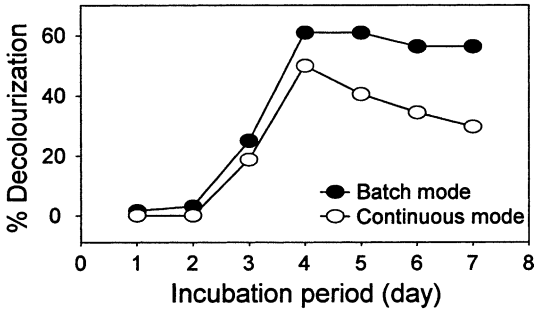

Fig. 2. Decolourization of dye industry effluent by culture of *Thelephora* sp.

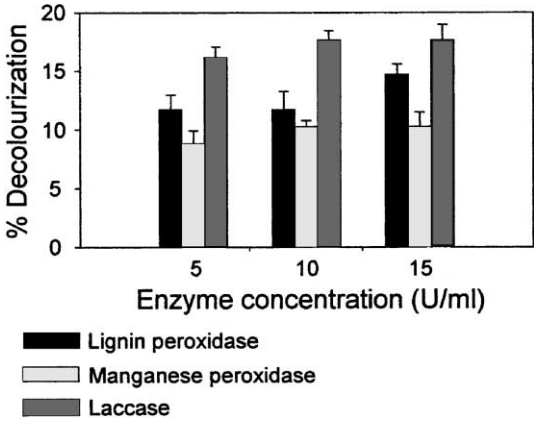

Fig. 3. Decolourization of dye industry effluent by purified ligninolytic enzymes of *Thelephora* sp.

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