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Degradation of Azo dyes by saprobic microfungi from mangrove and terrestrial forests in India

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ABSTRACT

Azo dyes are extensively used in textile industry to dye the fabrics. The unbound dye enters into the environment through textile industry effluents and contaminates the surrounding water bodies. Azo dyes are known to be cytotoxic and genotoxic to living beings. High amount of energy is required to break the stable N=N- bond of azo dyes. Microbial degradation of azo dyes by bacteria and fungi have been extensively studied. The present study was carried out employing 12 fungal strains, each of 6 from terrestrial and mangrove forests for decolorization of azo dyes. Laccase and peroxidase assays were performed to identify the positive strains. *Diaporthe* sp. of terrestrial forests and *Trimmatostroma* sp. of marine origin were positive for laccase assay while none of the other strains were positive for peroxidase test. Azo dye degradation studies were performed with positive Laccase fungal strains. Three azo dyes namely, Congo red, Crystal violet and Remazol brilliant blue were chosen for degradation studies. Degradation studies were performed with individual dyes at 12.5 and 25 µg/mL concentrations. The 25 µg/mLconcentration of Azo dyes was used to study the pH optima at 5,6,7,8 range by keeping temperature constant at 28°C and by incubating the treatments between 1 and 7 days to select the optimum conditions for Azo dye degradation by the selected fungi. *Diaporthe* sp. could degrade the azo dyes at pH 5 and/or 6 whereas *Trimmatostroma* sp.at pH 7 and/or 8 to the maximum extent at the end of the 7th day.

Keywords: Congo red, Crystal violet,*Diaporthe* sp, Laccase, Remazol Brilliant blue, *Trimmatostroma* sp,.

INTRODUCTION

Azo dyes, have an azo bond ($R_1N = NR_2$), where $R_1$and R_2 are aromatic groups, which can be substituted by sulphonated groups. R_1 and R_2 represent the biggest and most versatile groups and constitute about one-half of all the dyes produced (Mendez-paz *et al.*, 2005). Based on the solubility, azo dyes are divided into water soluble and fat soluble dyes. Water soluble azo dyes are used in textile industry to dye natural and synthetic fabrics whereas, fat soluble dyes are used for printing. Azo dyes have lot of applications in various industries like textile, food, cosmetics and paper printing. Azo dyes do not dye the fabric 100%, the binding efficiency of a dye varies from 50% to 98%. The unbound azo dyes are released as textile industry effluents and contaminate the surrounding surface and ground waters (Ganesh *et al*.,1994). The textile effluents consist 10-15 % of unbound dye and additives that are used in the coloring processes (Wang *et al.*, 2002). From water bodies, azo dyes enter into the food chain and show adverse effects on all the trophic levels. Azo dyes are highly cytotoxic and genotoxic and the aromatic amines (anilines) produced by the dyes act as carcinogens and/or mutagens (Martins *et al.*, 2001). Azo and nitro compounds have been reported to be reduced in sediments of aquatic bodies giving rise to potentially carcinogenic amines (Chen, 2006).

Bacterial and fungal mediated azo dye degradation has been extensively studied. Azo reductases, peroxidases and laccases are involved in degradation of the azo dyes. Bacteria produce all the three types of enzymes whereas fungi produce peroxidases and laccases(Saratale *et al*., 2011; Solis *et al.*, 2012). Laccase is a copper containing polyphenol oxidase. It is produced by some of the plants, fungi, bacteria and insects (Mayer and Staples, 2002). Laccases are produced byAscomycetous, Deuteromycetous and Basidiomycetous fungal species. Among these Basdiomycetes are the best producers of laccase (Shekher *et al.*, 2011).The most widely explored fungi with regard to dye degradation are the ligninolytic fungi (Bumpus,1995; 2003).Laccases catalyze the monoelectronic oxidation of a broad spectrum of substrates, for example, ortho- and para-diphenols, polyphenols, aminophenols, and aromatic or aliphatic amines, coupled with a full, four-electron reduction of O_2 to H_2O. Laccases are ecofriendly biocatalysts and have large applications in bleaching, deliginification of pulp, food improvement and decolorization of textile effluent (Kunamneni *et al*., 2008). In the present study we examined the dye decolorization potential of saprobic microfungi isolated from terrestrial and mangrove forest environments in India and the results are discussed.

MATERIALS AND METHODS

Fungal strains: Twelve microfungal cultures, of which six (*Trichoderma* sp., *Botryophaeria corticola, Diaporthe* sp., *Cytospora* sp., *Saccarodoella minuta,* Unidentified Coelomycetous fungus) were isolated from woody litter collected from Andaman forests and six marine fungal cultures (*Phaeoseptum* sp., *Trematosphaeria* sp., *Ellisembia* sp., *Trimmatostroma* sp., *Marasmiellus* sp., *Neptunella longirostris*) isolated from woody litter collected from Muthupet mangroves, were used in the present study.

Laccase assay: A basal medium composed of inorganic salts was prepared **[**KH2P04 (0.5 g/L), ammonium tartarate (0.5 g/L), MgS04.7H20 (0.1g/L), CaCl2.2H2O (0.01g/L), yeast extract (0.001g/L), CuS04.5H2O (0.001g/L), Fe(SO4)2 (0.001g/L) and MnSO4 (0.001g/L)**]**. This medium was supplemented with 1g/Lof 2, 2'-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid (ABTS), pH was adjusted to 5.5 and 16 g of agar per liter. Ten mL of glucose (20% w/v) sterilized separately was added per liter medium. Then medium was poured into Petri dishes and inoculated with 6 mm of fungal discs. The plates were incubated at 28°C for 5 days (Reanprayoon and Pathomsiriwong, 2012.)

Peroxidase assay: Azure B (0.01 %) was added to the above mentioned Basal medium with 10 mL of glucose (20 % w/v).and the medium was poured onto Petridishes. Sixmm fungal disc was inoculated on Petri dishes and allowed for

incubation at 28°C in the dark for 5 days (Reanprayoon and Pathomsiriwong,2012). This assay was performed to select laccase positive stains.

Degradation of azo dyes: Initial degradation studies of Azo dyes by different fungal strains were performed with three dyes namely Remazol Brilliant Blue, Crystal violet and Congo red. The fungal strains *Diaporthe* sp. and *Trimatostroma* sp. were selected as both these strains were positive for laccase activity. Fungal strains were inoculated into 100 ml of Boyd &Kohlmeyer broth (Yeast extract 1g, Peptone 2g, Dextrose 10g, Distilled water 1000 mL) amended with different concentration of azo dyes ranging from 10, 25, 50 and 100 µg/mL and the pH was adjusted to 5.5. Then cultures were incubated at 28°C for 7 days. Then the fungal mycelium was filtered through a muslin cloth and the filtrate was centrifuged at 3000 rpm for 15 minutes. The supernatant was collected and then the absorbance of the samples was taken at λ max of the respective azo dye with the help of a US-VIS Spectrophotometer by scanning at a range of 300-700 nm.Uninoculated Boyd & Kohlemeyer broth with above mentioned dye concentrations was used as control (Congo red, Remazol brilliant blue and Crystal violet λ max values are 497, 592 and 588 nm, respectively)

% of Dye Degradation = (A_b-A_a/A_b) X100

A_b = Absorbance before degradation A_a = Absorbance after degradation.

Parameteroptimization in azo dye degradation: To select the optimum parameters for azo dye degradation, pH and incubation periods were taken as variables whereas temperature (28°C) was kept as constant. In this study fungal strains were inoculated on Boyd & Kohlmeyer broth that contains 25µg/mLof azo dyes and the pH range of the medium was set to 5, 6, 7 or 8. The cultures were incubated at 28°C and for every 24h absorbance values were measured to determine the percentage of Azo dye degradation. This process was done till the end of the day 7.For all the above mentioned methods where ever the marine fungal spp. are tested, the 1L of media was prepared with 500 mL of distilled water and 500 mL of sea water.

RESULTS

Laccase and Peroxidase assays: The enzymes Laccases and Peroxidases have been implicated in the degradation of Azo dyes. To check whether either of the enzymes or only one of the enzymes was involved in the degradation of Azo dyes, enzyme assays were carried out by screening Laccase and Peroxidase activity. ABTS was used as a substrate for laccase assay. *Diaporthe* sp. of terrestrial origin and *Trimatostroma* sp. of marine origin were positive for laccase assay (**Table 1**). After incubation period the plates had turned into greenish blue color (**Figures 1A, 1B**). In the medium ABTS was a colorless compound. The formation of greenish blue color was due to the oxidation of ABTS by extracellular enzyme laccase produced by the fungal sp. Peroxidase assay was done with Azure B as a substrate.None of the fungi of terrestrial or marine origin showed positive reaction to peroxidase assay. This was confirmed by no clearance of blue color of the medium.

Table 1: Screening of Laccase activity by marine and terrestrial fungi

S. No.	Name of the Fungus	Host name	Site of collection	Fungal origin	Laccase assay
1.	*Diaporthe* sp.	*Gliricidia sepium*	Adazig, Middle Andaman	Terrestrial	+
2.	*Lasiodiploidia crassispora*	*Pterocarpus dalbergioides*	Wright mayo, South Andaman	Terrestrial	-
3.	*Trichoderma peltatum*	*Pterocarpus dalbergioides*	Near mohan Nagar, N orth Andaman	Terrestrial	-
4.	*Saccarodoella minuta*	*Gliricidia sepium*	Adazig, Middle Andaman	Terrestrial	-
5.	*Cytospora* sp 1	*Gliricidia sepium*	Adazig, Middle Andaman	Terrestrial	-
6.	*Cytospora* sp. 2	*Parishia insignis*	Shoal bay 14, South Andaman	Terrestrial	-
7.	*Trimmatostroma* sp1	*Avicennia marina*	Muthupet mangroves, Tamil Nadu	Marine	+
8.	*Trimmatostroma* sp.2	*Avicennia marina*	Muthupet mangroves, Tamil Nadu	Marine	-
9.	*Ellisembia* sp.	*Avicennia marina*	Muthupet mangroves, Tamil Nadu	Marine	-
10.	*Phaeoseptum* sp.	*Avicennia marina*	Muthupet mangroves, Tamil Nadu	Marine	-
11.	*Marasmiellius* sp.	*Avicennia marina*	Muthupet mangroves, Tamil Nadu	Marine	-
12.	*Neptunella longirostris*	*Avicennia marina*	Muthupet mangroves, Tamil Nadu	Marine	-

+ indicates presence of laccase activity; - indicates lack of laccase activity

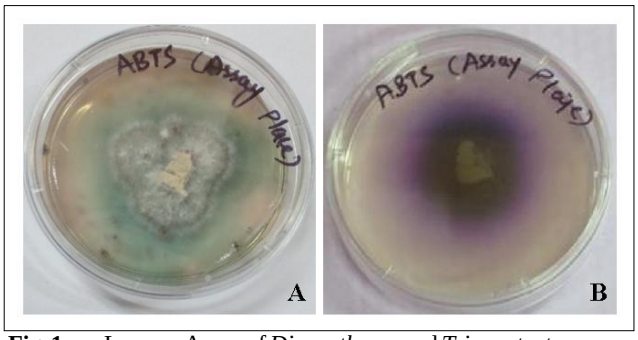

Fig. 1: Laccase Assay of *Diaporthe* sp. and *Trimmatostroma* sp. against ABTS substrate.

Degradation of Azo dyes: Azo dye degradation studies were performed with three azo dyes, namely Congo Red, Crystal violet and Remazol brilliant blue. The results show that both the fungal species i.e. *Diaporthe* sp. and *Trimmatostroma* sp. could degrade 12.5 and 25.0 µg/mL concentrations of all the three dyes (**Table 2, Figures 2A, 2B, 2C, 3A, 3B, 3C**) and both the strains were not able to grow on 50, 75 and 100 µg/mL concentrations of Azo dyes. Both *Diaporthe* sp. and *Trimmatostroma* sp. were laccase positive (**Figures 2A, 2B**) and were negative for peroxidase assays.

In **Figs. 1A** and **1B** oxidation of ABTS by *Diaporthe* sp. and *Trimmatostroma* sp. respectively has been demonstrated. ABTS was used as a substrate for laccase assay. After incubation period the plates had turned into greenish blue

Table2: Degradation of Azo dyes by *Diaporthe* sp. and *Trimmatostroma* sp

Name of the fungus	Percentage of azo dye degradation					
	Congo Red		Crystal Violet		Remazol Brilliant Blue	
	12.5 µg/mL	25.0 µg/mL	12.5 µg/mL	25.0 µg/mL	12.5 µg/mL	25.0 µg/mL
Diaporthe sp.	92	89	95	92	95	91
Trimmatostroma sp.	93.21	42	94	55	89	48

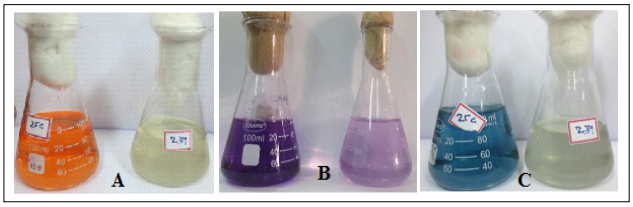

Fig. 2: **Degradation of Azo dyes by *Diaporthe* sp.:** A) Congo red, B) Crystal violet and C) Remazolbrilliant blue (25µg/mL). The two flasks in each panel represent the dyes on 1st day and after degradation at the end of the 7thday.

Fig.3: **Degradation of Azo dyes by *Trimmatostroma* sp.** A) Congo red, B) Crystal violet and C) Remazol brilliant blue (25µg/mL). The two flasks in each panel represent the dyes on 1st day and after degradation at the end of the 7th day.

color. In the medium ABTS was a colorless compound. The formation of greenish blue color was due to the oxidation of ABTS by extracellular enzyme laccase produced by the fungal species.

Optimization of parameters for azo dye degradation: Two parameters, namely, pH and incubation period were taken as variables and temperature (28°C) was set as constant to study the degradation of azo dyes by both the fungal species, *viz*. *Diaporthe* sp. And *Trimmatostroma* sp. Degradation studies were carried out at different pH readings: 5, 6, 7and 8 and incubation periods were from 1 to 7 days. In all the cases the percentage of dye degradation had increased from day 1 to day 7. Degradation of Congo red by *Diaporthe* sp. at the end of the 7th day of incubation period was observed as 96, 72, 52 and 29 % at pH 5, 6, 7 and 8, respectively. In the case of Crystal violet degradation of this dye by *Diaporthe* sp. at the end of 7thday was observed as 80, 97, 46 and 24 % at pH 5, 6, 7 and 8, respectively. Remazol brilliant blue degradation by *Diaporthe* sp. at the end of the 7th day of incubation period observed as 98, 98, 73 and 22% at pH 5, 6, 7 and 8, respectively. The results also show that less percentage of degradation at pH 7 and 8 was observed when compared to at pH 5 and 6 **(Table 3)**. *Diaporthe* sp could degrade Congo red at pH 5, Crystal violet at pH 5 and Remazol brilliant blue at pH 5 and/or pH 6 to the maximum extent**.**

Table 3: Degradation of Azo dyes by *Diaporthe* sp.

Incubation period (Days)	% Degradation of Congo Red				% Degradation of Crystal Violet				% Degradation of Remazol Brilliant Blue R			
	pH 5.0	pH 6.0	pH 7.0	pH 8.0	pH 5.0	pH 6.0	pH 7.0	pH 8.0	pH 5.0	pH 6.0	pH 7.0	pH 8.0
1	0	0	0	2	0	0	0	5	0	0	2	5
2	2	4	12	14	5	10	13	14	1	6	19	24
3	5	7	27	29	14	15	19	24	2	14	38	44
4	8	10	30	43	24	26	32	42	7	22	58	69
5	12	14	49	60	32	38	52	69	14	28	70	79
6	24	25	58	72	44	58	78	82	18	39	82	88
7	26	35	74	79	56	76	84	93	24	47	93	93

Trimmatostroma sp. was able to degrade Congo red at the end of 7th day to 26, 35, 74 and 79% at pH 5, 6, 7 and 8, respectively. Degradation of Crystal violet by *Trimmatostroma* sp. observed at pH 5, 6, 7 and 8 was 56, 76, 84 and 93 %, respectively. In the case of Remazol brilliant blue the degradation at the end of the 7th day observed was 24, 47, 93 and 93% at pH 5, 6, 7 and 8,respectively. Congo red and Crystal violet were degraded to maximum percentage at pH 8 whereas for Remazol brilliant blue it was observed at pH 7 and/or 8 **(Table 4)**.Less percentage of azo dye degradation was observed at pH 5 and 6 by *Trimmatostroma* sp. when compared to pH 7 and 8.

Table 4: Degradation of azo dyes by *Trimmatostromata* sp.

Incubation period (Days)	% Degradation of Congo Red				% Degradation of Crystal Violet				% Degradation of Remazol Brilliant Blue R			
	pH 5.0	pH 6.0	pH 7.0	pH 8.0	pH 5.0	pH 6.0	pH 7.0	pH 8.0	pH 5.0	pH 6.0	pH 7.0	pH 8.0
1	0	0	0	0	0	0	0	0	0	0	0	0
2	13	12	4	2	8	13	0	0	22	19	6	1
3	29	23	7	3	17	29	3	2	43	40	14	2
4	43	30	12	8	33	43	5	5	60	58	27	4
5	62	47	27	13	49	58	13	9	72	70	32	13
6	87	59	42	21	67	81	29	15	87	81	49	17
7	96	72	52	29	80	97	46	24	98	98	73	22

It can be inferred that pH5 and 6 are more favorable for the growth of *Diaporthe* sp. as well as the production of laccase, whereas for *Trimmatostroma* sp.pH 7 and 8 were more favorable for its growth and laccase production. This result is in consistency with the habitats from where these two fungi were isolated. While *Diaporthe* sp., isolated from terrestrial environment has shown a pH preference of 5 and 6, *Trimmatostroma* sp., being isolated from marine environment, has shown maximum degradation at pH 7 and 8. One more factor that could influence the degradationof azo dyes is their structural complexity and stability at different pH values.

DISCUSSION

In the present study initially 12 different fungal species, 6 from terrestrial forests of Andaman Islands and 6 marine fungi from Muthupet mangroves on the southeast coast of India were screened for laccase activity. This initial study led us to short list only two strains, one each from terrestrial and mangrove habitats, as they have shown a positive reaction to the laccase assay (**Table 1**). Degradation of azo dyes by Basidiomycetes has been extensively studied. The present study was carried out with wood degrading microfungi. The degradation of azo dyes from the fungal samples of unexplored areas in terms of diversity was performed for the first time. Twig samples normally do not support the large fruit bodies of the basidiomycetes and hence most of the twigs are normally degraded by ascomycetes and their anamorphs. Lignin degrading enzymes: peroxidases and laccases are responsible for the degradation of azo dyes.

Our enzymatic studies have shown that all of the fungal species tested were negative for peroxidase activity while *Diaporthe* sp. of terrestrial origin and *Trimmatostroma* sp. of marine origin were positive for laccase assay. Thus the azo dye degradation studies were carried out with these positive laccase strains. Broad spectrum substrate specificity of laccase was responsible for the degradation of different kinds of Azo dyes. Laccases act on azo dyes through nonspecific free radical mechanism that forms non phenolic compounds and avoids the formation of toxic aromatic amines (Chivukula and Ranganathan, 1995). The results of the present study have revealed that *Diaporthe* sp. and *Trimmatostroma* sp. were able to degrade azo dyes extensively. Our Initial azo dye degradation studies revealed that both the fungal species could degrade Congo red, Crystal violet and Remazol Brilliant Blue (**Table 2, Figs. 2A, 2B, 3A-C**). The later experiments showed that the extent of degradation of azo dyes by the fungal species was increasing

from the day 1 to day 7 of incubation periods (**Tables 3, 4**). Degradation of azo dyes recorded at pH 5, 6 and pH 7, 8 was maximum for *Diaporthe* sp. and *Trimmatostorma* sp., respectively at the end of day 7 (**Tables 2-4**). The extent of azo dye degradation depends on the structural complexity of the dyes and the physiochemical factors of the medium such as composition, pH, incubation period that promote the maximum growth of the fungal species and the production of extracellular laccase and also the specificity constant of the laccase on azo dyes.

Diaporthe sp. of terrestrial origin has shown azo dyes degrading ability to be maximum at pH 5 and 6. Whereas degradation of azo dyes by marine fungus *Trimmatostroma* sp. was found to be highest at pH 7 and 8. This result is not surprising as the former strain is isolated from a terrestrial environment and hence has shown the pH optima to be at 5 and/or 6. Whereas the latter fungus being of marine origin, has shown the pH optima to be at 7 and/or 8. *Diaporthe* sp. has been recorded on an unidentified twig from South Andaman Island in the present study. This ascomycete has deeply seated ascomata inside the host tissues and produces allantoid ascospores. Though basidiomycetes are the predominant fungi in lignocellulose degradation and production of ligninolytic enzymes including laccases, the ascomycetes have also been reported to be having the capability to produce laccases both from terrestrial and marine environments (Lyons *et al*., 2003; Castilho *et al*., 2009; D'Souza *et al*. 1996; Raghukumar, 2002; Raghukumar *et al.*, 1994, 1996). This is encouraging to undertake similar studies with microfungi (ascomycetes and their anamorphic fungi) colonizing woody substrata from terrestrial and marine environments.

Although our azo dye degradation studies were performed with fungi of novel source, more or less similar results were reported with other fungi. White rot fungi such as *Poria* sp., *Ganoderma* sp. and *Trametes* sp. When inoculated on to Carbon limited media with 50 µm Congo red, at the end of the 6^{th} day the degradation of Congo red by these species was observed as 93%, 77% and 89%, respectively (Selvam *et al.*, 2012). Two isolates of *Aspergillus niger* A1 and P1 degraded Crystal Violet (10 PPM) to 80.9 and 75.9 %, respectively in Czapek dox (pH 5.5) broth after 10 days of incubation period (Ali *et al.*, 2016).Though the present study serves as a preliminary report on the capabilities of the two fungal strains, namely, *Diaporthe* sp. and *Trimmatostroma* sp. in terms of producing laccase and in azo dye degradation, further studies are needed to establish their efficacy in field applications.

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