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Bioremediation of Methyl Orange, a synthetic textile azo dye by a halotolerant bacterial strain

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KEYWORDS

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A B S T R A C T

Increasing industrialization and urbanization results in the discharge of waste to the environment, which in turn creates more pollution. The discharge of toxic effluents from various textile industries adversely affects the water resources, soil fertility, aquatic organisms and ecosystem integrity. Bioremediation of textile dyes has been of considerable interest since it is inexpensive, eco-friendly and produces a less amount of sludge. In the present study, effluent samples were collected from various textile and dyeing industries located in and around Arni, Tiruvannamalai District, Tamilnadu, India and were exploited for the screening and isolation of bacterial strains that were capable of decolorizing the textile dye, Methyl Orange. Physico-chemical properties of the effluent samples were analyzed. Five bacterial strains, TVU-M1 to TVU-M5 capable of decolorizing Methyl Orange were screened and isolated from various effluent samples. Out of which, TVU-M4 isolate *(Bacillus* sp. Strain TVU-M4) exhibited maximum decolorization efficiency of 88.24 % within 32 h of incubation. HPLC chromatogram and FTIR spectrum of 24 h extracted metabolites showed significant change in the positions of peaks, when compared to control dye spectrum, indicating the biodegradation of Methyl Orange.

Introduction

Ever since the beginning of mankind, people have been using colorants for painting and dyeing their surroundings, their skins and their clothes. The first evidence of the use of colorant materials by man goes as far as 15000-9000 BC, in the walls of the Altamira cave in Spain. The pioneering synthesis of mauveine by W. H. Perkins started the era of synthetic dyes, with chemical and physical properties better suited to contemporary demands, better level of quality and more reproducible techniques of application (Clark *et al.*, 1993; Vijayanand and

Hemapriya, 2013). Now there are more than 1, 00,000 commercially available dyes whilst over 7 x 10^5 metric tons of dyestuffs are produced annually (Wong and Yu, 1999). Dyes are used in textile industry, leather tanning industry, paper production, food technology, agricultural research, light-harvesting arrays, photo electrochemical cells, hair coloring and cosmetics. Moreover these compounds have been employed for the control of the efficacy of sewage and wastewater treatment, for the determination of specific surface area of activated sludge and for ground water tracing (Forgacs *et al.,* 2004; Hemapriya and Vijayanand, 2013).

Wet processing in textile industry generates large amounts of a wastewater whose pollution load arises not only from the removal of impurities from the raw materials themselves but also from the residual chemical reagents used for processing. The extreme diversity of raw materials and production schemes employed poses problems in assessing effluent characteristics and subsequently defining pollution control technologies (Correia *et al.,* 1994). During textile processing, inefficiencies in dyeing result in large amounts of dyestuff being directly lost to the wastewater, which ultimately finds its way into the environment. The amount of dye lost is dependent upon the dye application class, varying from only 2% loss when using basic dyes to a 50% loss when certain reactive dyes are used (O'Neill *et al.,* 1999; McMullan *et al.,* 2001; Pearce *et al.*, 2003). Color present in the dye effluent gives a straightforward indication of water being polluted (Nigam *et al*., 1996). In addition to being aesthetically displeasing, it interferes with the penetration of sunlight, affects photosynthesis and algal based biological treatment systems like stabilization ponds and aerated lagoons (Strickland and Perkins, 1995).

Dyes, by decreasing light absorption, may significantly affect photosynthetic activity of aquatic life and may be toxic due to the presence of aromatics or heavy metals (Saratale *et al*., 2006; Hemapriya *et al*., 2010; Vijayanand and Hemapriya, 2013). Extensive work has been carried out on the pollution problems associated with the discharge of textile effluent. Any change in the water quality causes several psychological and biochemical disturbances in fish (Arunachalam *et al*., 1980). Toxic compounds from dye effluent get into aquatic organisms, pass through the food chain and ultimately reach humans, leading to various physiological disorders like hypertension, sporadic fever, renal damage, cramps, etc., Bioaccumulation of toxicants depends on the availability and persistence of the contaminants in water, food and physico-chemical properties of the toxicants (Puvaneswari *et al*., 2006).

Traditional wastewater treatment technologies have proven to be markedly ineffective for handling wastewater of synthetic textile dyes because of the chemical stability of these pollutants. A wide range of methods has been developed for the removal of synthetic dyes from waters and wastewaters to decrease their impact on the environment. According to Kalyani *et al*. (2009), Bioremediation of textile effluents has been of considerable significance since it is inexpensive, eco-friendly and produces a less amount of sludge. The effectiveness of microbial decolorization depends on the adaptability and the activity of selected microorganisms including bacteria, actinomycetes, fungi, yeasts and algae capable of degrading azo dyes (Chen *et al*., 2003; Daneshwar *et al*., 2007). Considering the advantages and

potential applications of bioremediation processes in wastewater treatment, the present investigation targets on the bioremediation of Methyl Orange, a synthetic textile azo dye by a moderately halo-tolerant bacterial strain.

Methods

Sampling Site, Sample Collection and Physico-chemical analysis

The sampling site was the textile industries and dyeing units located in and around Arani, Thiruvannamalai District. Tamil Nadu, India. The effluent samples from both textile industries and dyeing units were characterized by its dark color and extreme turbidity. Samples were collected at the surface and at various depths and were placed in sterile polythene bags to prevent direct contact with air and transported to the laboratory in an ice box for further analysis. Physico-chemical properties of the effluent samples were analyzed (APHA, 1980).

Synthetic Azo Dye Used

The commonly used textile azo dye, Methyl Orange used in this study was procured from a local textile dyeing unit. Stock solution was prepared by dissolving 1 g of Methyl Orange in 100 ml distilled water. The dye solution was sterilized by membrane filtration (Millipore Millex ® - GS, 0.22 Mm filter unit), since azo dyes may be unstable to moist-heat sterilization. All the chemicals used in this study were of the highest purity available and of an analytical grade.

Toxicity studies with *Channa channastriata*

Channa channastriata used in this study was transferred and incubated in a container containing 1 L of water with 100 ppm of Methyl Orange and the control was setup by transferring and incubating another fish of same species, size and age in a container containing 1 L of water (without any dye products). Optimum conditions such as PH 7.0, proper aeration and room temperature was provided for 48 h. Following incubation, the fishes were killed (Fig.2) and the histopathology studies were carried out with the gills and skin of fish samples (control and test).

Isolation and Screening of Bacterial Strains Decolorizing Methyl Orange

The effluent samples were serially diluted and incubated over basal nutrient agar medium containing 50 ppm of Methyl Orange at 37°C for 5 days. Colonies surrounded by halo (decolorized) zones were picked and streaked on nutrient agar plates containing azo dyes (Wong and Yuen, 1996). Different colonies of dye decolorizing bacteria were picked and re-streaked several times to obtain pure cultures. Decolorization extent of the isolates were determined by measuring the absorbance of the culture supernatant at 470 nm using UV-visible spectrophotometer (Hitachi U 2800), according to Hemapriya *et al*. (2010).

HPLC Analysis of Decolorized Sample

10 ml of decolorized samples were taken after 24 h of incubation, centrifuged at 12,000 g for 30 min, and filtered through 0.45 µm membrane filter (Millipore). The filtrates were then extracted with diethyl ether and flash evaporated in rotary vacuum evaporator in temperature controlled water bath (50°C) and residues were dissolved in 2 ml of HPLC grade methanol and used for analysis. These extracted samples were analyzed by HPLC having a mobile phase of

50:49.6:0.4% (methanol: water: disodium hydrogen phosphate).

FTIR Analysis of Decolorized Samples

The biodegraded azo dye samples were characterized by FTIR spectroscopy (Perkin-Elmer, Spectrum one). The analysis results were compared with the control dye. The FTIR analysis was done in the mid IR region (400-4000 cm^{-1}) with 16 scan speed. The samples were mixed with spectroscopically pure KBr in the ratio (5:95). The pellets were fixed in sample holder and then analyzed (Saratale *et al*., 2009).

Phytotoxicity Studies

Phytotoxicity tests were performed in order to assess the toxicity of the untreated and treated dye samples. The ethyl acetate extracted products of degraded azo dyes were dried and dissolved in 5 ml sterile distilled water to make a final concentration of 100 ppm. The Phytotoxicity tests were carried out on *Sorghum vulgare* Pers. (monocot) (Parshetti *et al*., 2006). 10 healthy plant seeds were treated separately with 5 ml of control dye and degraded products respectively/per day. Control sets were carried out using distilled water at the same time. Germination percentage as well as the length of plumule and radical was recorded after 7 days (Saratale *et al*., 2009).

Results and Discussion

Environmental pollution has been recognized as one of the major problems of the modern world. The increasing demand for water and the dwindling supply has made the treatment and reuse of industrial effluents as an attractive option. Textile effluents are of global concern because they color the drains and ultimately the receiving water bodies. The textile industries are multi-chemical utilizing concerns, of which various dyes are of importance. During the dyeing process substantial amount of dyes and other chemicals are lost in the wastewater (Vaidya and Datye, 1982). Moreover, these azo dyes and their intermediate aromatic amines are either toxic or mutagenic or carcinogenic, posing a potential health hazard to human kind (Carliell *et al*., 1995). So purification of textile effluents has become a matter of great concern (Fang *et al*., 2004). One promising strategy is the use of microbial strains that possess the ability to decolorize and mineralize synthetic dyes (Robinson *et al*., 2001).

Physico-Chemical Analysis of Effluent Samples

The average temperature at the sampling sites was around 35°C at day time. The physico-chemical characteristics of the effluent samples were shown in the Table.1. The pH values of the effluent samples were found to be alkaline. Total solids of S_2 and S_3 samples were found to be lower than the S_1 sample. The highest TSS content was encountered in S_3 sample. TDS content was almost same in both S_1 and S_3 samples. BOD value of S_1 sample was found to be higher than the S_2 and S_3 samples. However, the COD value was maximum in case of S_2 sample. The effluent samples collected from S_1, S_2 and S_3 sites were found to be dark blue, blackish blue and dark brown respectively.

Dye Stuff Used

The dye stuff used in this study was Methyl Orange with molecular formula of $C_{14}H_{14}N_3S_4O_3NaS$. The absorption maximum of this dye was 470 nm. The structure of Methyl Orange was shown in Fig.1.

Toxicity Analysis of Methyl Orange in *Channa channastriata*

Results of histopathological studies showed marked changes in the sections of gills and skin of Murrel fishes (control and test). The fish exposed to dye showed the clubbing of lamellae, vacuolar degradation, lifting of primary lamella and high level proliferation of epithelial cell. The blood vessels were not properly circulated in the fish. Tubular damage characterized by vacuolated, degenerated, hypertrophied tubular epithelial cells and occlusion of tubular lumen was recorded at all alterations (Fig.2).

However, no notable changes were observed in the histological sections of skin samples (Fig.3). Similarly, exposure of fish, *Oreochromis mossambicus* to sub-lethal concentration of effluent strongly affected the rates of feeding, absorption and conversion. Protein contents of muscle, liver, gill and intestine decreases with increasing concentrations of dye effluent (Amutha *et al*., 2002).

Isolation and Screening of Bacterial Strains Decolorizing Methyl Orange

The results shown in Table.2 revealed that five isolates designated as TVU-M1 to TVU-M5 were found to be effective in decolorizing Methyl Orange. Out of which, TVU-M4 exhibited the highest decolorization efficiency of about 88.24 %.

Based on the Morphological, cultural, biochemical characteristics and 16s r DNA analysis, TVU-M4 isolate was identified as a moderate halotolerant bacteria - *Bacillus* sp TVU-M4. Similarly *Bacillus gordonae* and *B.thuringiensis* exhibited excellent decolorization of Tectilon Blue 4R-01 and Acid Red-119 respectively (Walker and Weatherley, 2000; Dave and Dave, 2009).

HPLC Analysis of Decolorized Sample

To elucidate the exact the exact phenomenon of dye decolorization, the HPLC analysis of dye sample was carried out at 0 h incubation that showed the presence of 1 major peak with retention time of 8.808 min (data not shown). As the decolorization progressed, the biodegradation of parental dye compound was observed with 25 detectable peaks at 24 h extracted metabolites, however major peak was not observed at 8.808 min, clearly indicating the biodegradation of Methyl Orange by *Bacillus* sp. strain TVU-M4. This result was in complete accordance with the findings of Kalyani *et al.* (2009).

FTIR Analysis of Decolorized Sample

Comparison of FTIR spectrum of the control dye with extracted metabolites after complete decolorization clearly indicated the biodegradation of Methyl Orange by *Bacillus* sp. strain TVU-M4 (Data not shown). The results of FT-IR analysis of Methyl Orange parent dye and sample obtained after decolorization showed various peaks. The FT-IR spectra of Methyl Orange parent dye displayed peaks at 3474, 2912, 1567 and 1424 cm-1, for –OH stretching vibration, aromatic -CH stretching vibration, -C=C- stretching and -N=N- stretching vibration respectively. However the FT-IR spectra of degradation product displayed peaks at different positions indicating the complete breakdown of Methyl Orange.

Phytotoxicity Assay

Phytotoxicity tests were performed in order to assess the toxicity of the untreated and treated Acid Orange-10 dye samples (Fig.4). *S. vulgare* seeds treated with tap water showed 100% germination, the mean plumule length of 27.23±1.05 cm and the mean radical length of 6.17±0.95 cm.

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Fig.1 Chemical Structure of Methyl Orange

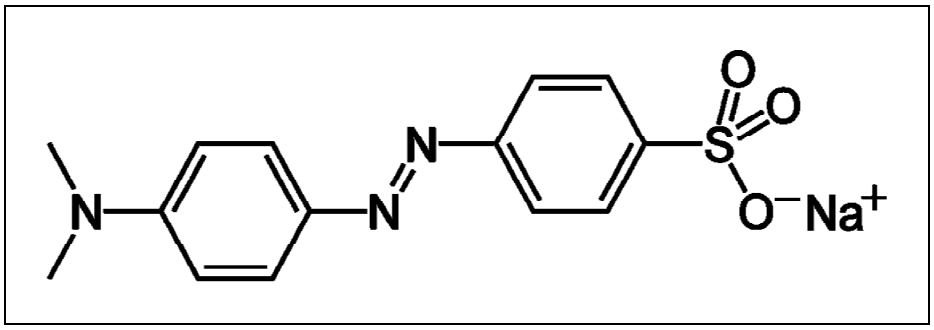


Table.1 Physico-chemical characteristics of Tannery effluent

Parameter	S1	S2	S3	Permissible limit
pH	8.8	8.2	8.0	6.0-8.0
Color	Dark Blue	Blackish Blue	Dark Brown	850
TS (mg/ L)	2,400	2,600	2,720	2,200
TDS (mg/ L)	2,300	2,840	2,240	2,100
TSS (mg/ L)	281	298	372	100
DO (mg/ L)	2.8	3.2	3.0	4.0-6.0
BOD (mg/ L)	240	140	172	30
COD (mg/ L)	360	444	326	250
Sulfate (mg/ L)	2,290	2,212	2,261	1,000
Magnesium (mg/ L)	250	220	310	200
Phosphate (mg/ L)	5.5	6.2	4.8	5.0
Nitrate (mg/ L)	11.60	12.2	10.80	10
Fluoride (mg/ L)	4.0	3.8	2.8	2.0
Phenol (mg/ L)	6.0	5.6	4.9	1.0
Oil and grease	15.9	14.3	16.2	10

Table.2 Bacterial Strains Decolorizing Methyl Orange (TVU-M1 to M5)

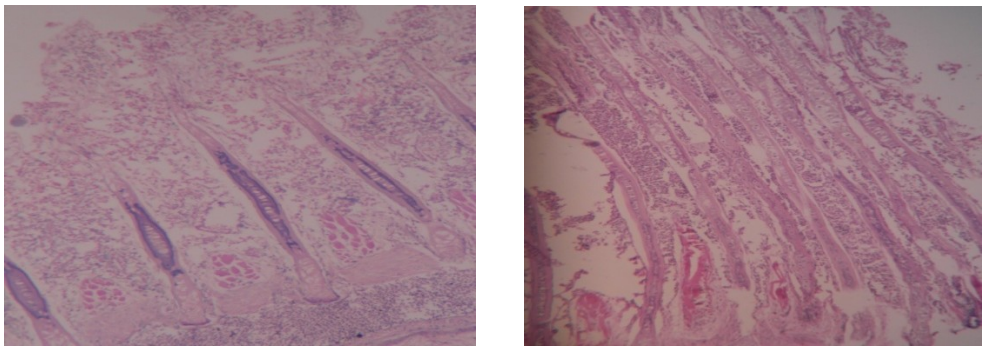
Sl. No	Isolates	Sample Collection Site	% of Decolorization
1	TVU-M1	S2	71.65 %
2	TVU-M2	S3	64.76 %
3	TVU-M3	S2	62.76 %
4	TVU-M4	S1	88.24 %
5	TVU-M5	S1	61.08 %

Note: The isolates are considered for the table only with 50% decolorization ability.

Toxicity analysis of Methyl Orange in *Channa channastriata*

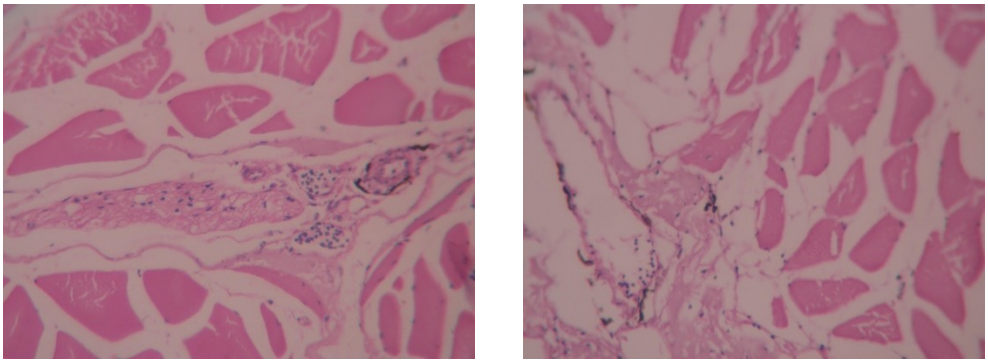
(a) (b)

Fig.2 *Channa channastriata* (Murrel Fish) (a. Control: b. Test)



(a) (b)

Fig.3 Histological section of *Channa channastriata* gills (a. Control: b. Test sample)



(a) (b)

Fig.4 Histological section of *Channa channastriata* skin (a.Control: b.Test sample)

Water (control) Dye sample Treated Sample

Fig.5 Phytotoxicity study in Monocot plant (*Sorghum vulgare*)

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