Microbial Degradation of Reactive Orange M2R Dye by an Application of Bacterial Consortium ETL-A: Bioremedial Approach towards Sustainable Environment

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Abstract: Textile and Textile dyestuff industries discharge effluent having high Chemical Oxygen Demand (COD) and colour, making it difficult to treat the effluent completely. Several Physical, Chemical and Biological treatment methods are available to treat such an effluent. However, in recent years biological treatment with focus on bacteria have been drawing a tremendous attention due to their ability to degrade complex structured dyes and hence treat waste water. In the present study, Soil and Water samples collected from textile dye effluent contaminated sites of Ankleshwar, Gujarat was studied for screening and isolation of bacteria capable of decolorizing and degrading textile dyes. A bacterial consortium ETL-A was selected on the basis of rapid dye decolorization. Bacterial consortium exhibited 93% decolorization ability within 30 hours under static conditions at 35°C in presence of Glucose and Yeast Extract as cosubstrates. 16S rRNA gene amplification was carried out for the sequencing and identification of these strains. The degradation of dye was confirmed by HPTLC and FTIR analysis. Considerable decrease in COD of the dye (above 85%) was indicative of conversion of complex dye into simple oxidizable products.

Keywords: Reactive orange M2R, Biodegradation, HPTLC, FTIR, COD, ETL-A.

1. INTRODUCTION

Worldwide, dye wastewater has become one of the main sources of severe pollution problems due to the greater demand for textile products and the proportional increase in production and applications of synthetic dyes [Santos et al., 2007]. It is estimated that over 10,000 different dyes and pigments are used industrially and over 0.7 million tons of synthetic dyes are produced annually worldwide [Robinson et al., 2001]. In the textile industry, up to 200,000 tons of these dyes are lost to effluents every year during dyeing and finishing operations as a result of inefficiency in the dyeing process [Zollinger et al., 1987, Ji et al., 2008]. Unfortunately, most of these dyes escape conventional wastewater treatment processes and persist in the environment as a result of their high stability against light, temperature, water, detergents, chemicals, and microbial attack [Couto., 2009]. Notwithstanding, industries are required to eliminate color from their effluents containing dyes, before disposal into water bodies, due to environmental legislation [Neil et al., 1999]. Several reports have also shown that textile dyes and effluents have toxic effects on plants which perform important ecological functions such as providing a habitat for wildlife, protecting soil from erosion, and providing the organic matter that is so significant to soil fertility [Ghodake et al., 2009]. Consequently, it is pertinent to develop efficient treatment strategies for removal of color from dye wastewater. Various physicochemical methods, such as adsorption on activated carbon, electrocoagulation, flocculation, froth flotation, ion exchange, membrane filtration, ozonation, and reverse osmosis have been used for decolorization of dyes in wastewater [Robinson et al., 2001, Gupta et al., 2004]. However, these methods are less efficient, costly, of limited applicability, and produce wastes, which are difficult to dispose of [Daneshwar et al., 2007]. On the contrary, biological processes provide a low-cost, environmentally benign, and efficient alternative for the treatment of dye wastewater [Ali et al., 2009]. Decolorization by biological means may take place in two ways: either by adsorption (or biosorption) on the microbial biomass or biodegradation by the cells [Zhou et al., 1993]. Biosorption involves the entrapment of dyes in the matrix of the adsorbent (microbial biomass) without destruction of the pollutant, whereas in biodegradation, the original dye structure is
fragmented into smaller compounds resulting in the decolorization of synthetic dyes. Several researchers have described the use of microorganisms as biosorption agents in the removal of pollutants from wastewater [Gupta & Rastogi., 2008, 2009, Won et al., 2005]. However, due to operational ease and facile adaptability of microorganisms to a given set of conditions, the biodegradation mechanism is considered efficacious in comparison to biosorption for treatment of dye wastewater [Singh & Arora., 2011]. Over the past few decades, numerous microorganisms have been isolated and characterized for degradation of various synthetic dyes, but most of the reports have dealt mainly with decolorization of azo dyes [Tony et al., 2009, Gou et al., 2009, Telke et al., 2008]. In the present study, a consortium was selected from four consortiums isolated from soil and water samples collected from areas of textile dyeing and printing in Ankleshwar, Gujarat, India and its ability to degrade Reactive Orange M2R (ROM2R) a monoazo dye was analyzed. The consortium’s ability to remove Chemical oxygen demand was measured to ensure the ability of the consortium to convert complex dye to simple non toxic compound. The degraded product after the treatment with consortium ETL-A was analyzed by HPTLC and FTIR.

2. MATERIALS AND METHODS

Chemicals & Dyes
The textile dyes that are manufactured and used mostly for dyeing and printing were selected for the study. The common name of all dyes has been used for convenience; the dyes were procured from local textile industry, Ankleshwar, Gujarat, India. The dyes were Remazol Brilliant Red 5, Reactive Orange M2R, Reactive Red 195, Red HE8B, Reactive Blue 59, Remazol Brilliant Blue R, Remazol Black B, Reactive Black 8. A monoazo dye Reactive Orange M2R (ROM2R) was selected as model dye for all experiments.

Growth Medium
Bushnell and Haas medium (BHM) (HiMedia) containing the following in gl-1: MgSO4, 0.2; K2HP04, 1.0; CaCl2, 0.02; FeCl3, 0.05; NH4NO3, 1.0 supplemented with Glucose (0.1% w/v), Yeast extract (0.3% w/v) and 100ppm (mgl-1) ROM2R was used for enrichment of organisms in soil and water sample.

Sample Collection
Soil and water samples were collected from areas of textile dyeing and printing in Ankleshwar, Gujarat, India was used for screening of dye decolorizing bacteria.

Enrichment of Dye Degrading Organisms
BHM media containing dye (ROM2R 100 mgl-1 ) as sole source of carbon and energy (without cosubstrate) (100 ml) was taken in 250 ml Erlenmeyer flasks and was inoculated with 10 ml of soil suspension (10% w/v) and incubated under static condition at 37 °C. Repeated transfers were carried out in fresh dye containing media till stable dye decolorizing cultures were obtained showing consistent growth and decolorization during successive transfer. Same was repeated for water samples. Co-substrates such as glucose and Yeast Extract were also used along with dye in BHM media.

Identification by Gram Staining and 16SrRNA
The cultures that showed consistent decolorization were subjected to separation on dye containing agar plates and were submitted to Bangalore Genei, Bangalore, India for identification by 16S rRNA sequencing method.

COD Reduction during Decolorization
The decolorization medium was observed for the change in Chemical Oxygen Demand at different time intervals. For determining change in COD, titrimetric procedure was followed in which supernatant refluxed with potassium dichromate, in presence of silver sulfate, mercury sulfate and concentrated H2SO4 was titrated with Ferrous Ammonium Sulfate (FAS) using ferroin indicator (APHA, 2005).

Dye Decolorization under Static and Shaking Conditions
Bushnell and Haas Broth (BHB) 100ml in 250ml Erlenmeyer flasks along with glucose (0.1%w/v) and Yeast Extract (0.3%w/v) amended with 100 ppm of ROM2R was inoculated with bacterial culture (5% w/v) and incubated at 37°C under static or shaking conditions. All the decolorization experiments were performed in triplicates.
Analysis of Growth and Decolorization of ROM2R

Culture broth of 5 ml was collected from each flask at different time intervals of incubation. These samples were centrifuged at 8000 rpm for 10 minutes and supernatant was analysed by UV-Vis spectrophotometer (Shimadzu UV-1800, Japan) at λmax of respective dye (λmax of ROM2R=488nm). The cell pellet obtained upon centrifugation of 5 ml culture was resuspended in 5 ml distilled water and its absorbance was studied at 660 nm. The uninoculated flask containing the dye (ROM2R) was used as reference to correct the abiotic colour disappearance and the uninoculated medium without dye was used as blank. The efficiency of dye decolorization was determined based on the reduction of the absorbance in relation to the zero time absorbance expressed as percentage. The decolorization efficiency was expressed as follows:

\[
\text{% decolorization} = \left(\frac{\text{Ai} - \text{Af}}{\text{Ai}}\right) \times 100
\]

Where \(\text{Ai}\) = Initial Absorbance,
\(\text{Af}\) = Final Absorbance

HPTLC Analysis of the Degraded Product

Degradation of dyes was monitored on precoated TLC silica gel plates. The culture supernatants at different periods during decolorization were used for analysis. A 10μl of the sample was spotted on TLC plates using a micro syringe. The solvent system used was n-propanol, methyl ethyl ketone, Ammonium hydroxide (4:3:3 v/v). The dye chromatogram was observed in daylight and in UV light (254nm).

Fourier Transform Infra Red Spectroscopy Analysis of Degraded Product

Metabolites produced by biodegradation of the ROM2R were extracted with equal volumes of ethyl acetate. The extracts were dried over anhydrous Na2SO4 evaporated to dryness in rotary evaporator and analysed. FTIR analysis was carried out on Thermo Scientific 6700 set up, frequency range 4000cm\(^{-1}\) to 400cm\(^{-1}\).

Spectrum of Dyes Decolorized by the Culture

Mixture of various dyes is present in industrial effluents therefore ability of the consortium VSS to decolorize different dyes was studied.

3. Results and Discussions

Screening pattern of Dye Decolorizing Bacterial Cultures

Isolation of bacteria was carried out from textile dye contaminated soil and water samples from various sites of Ankleshwar GIDC, Gujarat, India for decolorization and degradation of textile dyes. Four different bacterial consortium namely ETL-A, ETL-B, ETL-C, ETL-4 were isolated by enrichment culture technique using dye ROM2R (100 ppm) and also by adding glucose and Yeast Extract as co-substrates. It was evident from the results that addition of co-substrate is essential for decolorization. The same was reported by (Nigam et al., 1996). Of all the bacterial consortia obtained, bacterial consortium ETL-A was found to be the most efficient with maximum decolorization under static conditions at 37 °C. Four different bacterial species were obtained upon isolation on dye agar plates from the mixed consortium ETL-A. When each pure culture was tested individually for its decolorizing ability in liquid medium and in combination with other pure cultures, none of the cultures showed decolorization even on extended incubation. But, when all the four bacterial cultures were mixed and inoculated together in a liquid medium, complete decolorization of ROM2R was observed, thus suggesting role of all four bacterial species in decolorization. Chen et al., 2006, Bafana et al., 2007, Yang et al., 2009, Patil et al., 2010, Phugare et al., 2011 reported synchronized action of microorganisms for the biodegradation of textile dye.

Change in COD during Decolorization

All four bacterial consortia isolated by enrichment culture technique were studied for change in COD. Mixed results were obtained with different consortia. Consortium ETL-D showed minimum COD reduction of 38% and 60% of ROM2R dye in 24 hours and 48 hours of inoculation respectively. Consortium ETL-A showed maximum COD reduction of 67% and 83 % of ROM2R dye in 24 hours and 48 hours of inoculation respectively. All the other consortia showed COD reductions in between the COD reduction by ETL-D and ETL-A (Figure 1). High rate in COD reduction is considered as an indicator of mineralization. (Hassan et al., 2002).
Identification of Cultures Present in Consortium VSS by Gram Staining and 16S rRNA Technique

ETL-A consortium obtained after enrichment culture technique was subjected to gram staining. Organism present in consortium ETL-A was identified by 16SrRNA technique. The consortium contains Bacillus subtilis, Stenotrophomonas sp., Pseudomonas stutzeri and Pseudomonas aeruginosa.

Decolorization Ability and Growth Pattern of Consortium ETL-A under Static/Shaking Conditions

Decolorization ability and growth of the bacterial cultures under static/shaking conditions was obtained by spectrophotometric method and the results are represented in Figure 2 and 3. The consortium ETL-A exhibited decolorization up to 93% within 30 hours under static condition, whereas under shaking condition, the culture showed 20% decolorization of ROM2R dye in 30 hours. Under shaking condition, the culture though showed faster growth, it showed lesser decolorization. The results clearly indicated that decolorization was not depended on biomass concentration but was significantly correlated with dissolved oxygen. The azo reductase driven bacterial decolorization of azo dyes is normally inhibited by the presence of oxygen primarily due to competition in oxidation of reduced electron carriers (e.g. NADH) with either Oxygen or Azo group as the electron receptor (Chang et al, 2000).

Fig 1. Reduction in COD

Fig 2. Decolorization of Reactive Orange M2R by Mixed Bacterial Consortium
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**Fig 3.** Decolourization of M2R by Mixed Bacterial Consortium ETL-A under Static Condition.

**HPTLC Analysis**

Spectrophotometric analysis at 488 nm of uninoculated medium (Figure 4) containing ROM2R showed a simultaneous decrease in peak of samples withdrawn at various time intervals. Evidence of the removal of dye can be observed with absorbance at λmax being virtually zero after 48 hours of inoculation with ETL-A consortium (b) and shift of peak towards the U.V. region as shown in Figure 4. HPTLC results showed the formation of new peaks as observed and compared to control dye (Figure 5, 6, 7). The results obtained justify that the chromophoric groups were totally removed from the intact dye structure. The multiple bands obtained in the metabolite lane confirmed the biodegradation of the azo dye as compared to single band of control dye (Figure 7). The difference in Rf values (data not shown) of control dye and metabolites supports the FTIR data which suggests biodegradation of ROM2R. The similar results were reported by Chaube et al, 2010.

**Fig 4.** Spectrophotometric Analysis (a) Uninoculated media containing ROM2R (b) Media Containing ROM2R inoculated with ETL-A Consortium
Fig 5. HPTLC Profile of ROM2R

Fig 6. Metabolites of ROM2R Dye after Degradation

Fig 7. HPTLC Plate Exposed under UV Light, Control Dye Track(II) and Metabolites Track (I)
FTIR Analysis

Results of FTIR analysis of control and sample obtained after decolorization showed various peaks (Figure 8 and 9). The FTIR spectrum of control ROM2R displays peak at 3440.1 cm\(^{-1}\) for the intramolecular hydrogen bonding aromatic \(-\mathrm{OH}\) and \(-\mathrm{O-H}\) stretching, peak at 1622.7 cm\(^{-1}\) and 1555.4 cm\(^{-1}\) for \(-\mathrm{C=C}\) stretching of aromatic rings, 1466.5 cm\(^{-1}\) and 1394.6 cm\(^{-1}\)–\(\mathrm{C-H}\) stretching of alkyl acetics, peak at 1329.8 cm\(^{-1}\) and 1187.5 cm\(^{-1}\) for \(-\mathrm{C-N}\) stretching due to amines, peak at 845.2 cm\(^{-1}\), 799.9 cm\(^{-1}\), 763.3 cm\(^{-1}\) for \(-\mathrm{C-H}\) stretching, 617.8 cm\(^{-1}\)–\(\mathrm{C-Cl}\) stretching. The degradation metabolites of ROM2R using consortium ETL-A showed peak at 3442.1 cm\(^{-1}\) for \(-\mathrm{O-H}\) stretching, peak at 1644.8 cm\(^{-1}\)–\(\mathrm{C=C}\) stretching of alkenes, 1400.1 cm\(^{-1}\) for \(-\mathrm{C-H}\) stretching due to \(-\mathrm{CH}_3\), peak at 1110.4 cm\(^{-1}\), 619.3 cm\(^{-1}\), 476.9 cm\(^{-1}\) for \(-\mathrm{C-Cl}\) stretching indicating presence of alkyl chloride. Here peak at 1392.8 cm\(^{-1}\) is missing which confirms absence of amines (Figure 7) and formation of new peaks suggests the biotransformation of Reactive Orange M2R dye. The similar results were reported by Srivastava et al., 2012.

![Fig 8. FTIR Spectra of Reactive Orange M2R](image1)

![Fig 9. FTIR Spectra of Degradation Metabolites of Reactive Orange M2R](image2)

Spectrum of Dyes Decolorized by the Bacterial Consortium

Industrial effluents consist of a mixture of various dyes. Ability of consortium ETL-A to decolorize different dyes was studied (Figure 10). There was rapid degradation observed for all dyes (100 mg/l\(^{-1}\)) used in the study within 24 hours. Reactive Orange M2R, Remazol Brilliant Red 5, Reactive Red 195,
Red HE8B, Reactive Blue 59 showed decolorization in the range of 90-97 %. Remazol Brilliant Blue R and Remazol Black B showed decolorization in the range of 50-86%. Reactive Black 8 showed decolorization in the range of 22-40%. The percent decolorization varied which could be due to the structural differences of all dyes or due to the toxic effect of dyes (Khehra et al, 2005).

![Spectrum of Dyes Decolorized by Consortium ETL-A](image)

### 4. CONCLUSION

An extensive research study demonstrates efficient decolorization of seven out of eight dyes tested at pH 7 and 37 °C temperature by consortium ETL-A. The Consortium isolated grows best in static conditions, where in oxygen can be easily depleted, thus creating conditions favorable for decolorization of azo dyes. Reduction in COD of dye after treatment with ETL-A indicates degradation of the dye. HPTLC and FTIR results also confirm the dye degradation. High decolorization extent and complete mineralization of azo dye show the potential of this bacterial consortium to be used in the treatment of industrial effluents.

### REFERENCES


