

Microbial Degradation of Reactive Orange M₂R Dye by Bacterial Consortium ETL-A

Shah M*

Industrial Waste Water Research Laboratory, Division of Applied and Environmental Microbiology Lab, India

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 year's 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 h 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

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 [1]. 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 [2]. 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 [3,4]. 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 [5]. Notwithstanding, industries are required to eliminate color from their effluents containing dyes, before disposal into water bodies, due to environmental legislation [6]. 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 [7]. 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, electro coagulation, flocculation, froth flotation, ion exchange, membrane filtration, ozonation and reverse osmosis have been used for decolorization of dyes in wastewater [8,9]. However, these methods are less efficient, costly, of limited applicability, and produce wastes, which are difficult to dispose [10]. On the contrary, biological processes provide a low-cost, environmentally benign, and efficient alternative for the treatment of dye wastewater [11]. Decolorization by biological means may take place in two ways: either by adsorption (or biosorption) on the microbial biomass or biodegradation by the cells [12]. 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 [13-15]. 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 [16]. 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 [17-19]. 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 nontoxic compound. The degraded product after the treatment with consortium ETL-A was analyzed by HPTLC and FTIR.

Materials and Methods

Chemicals and 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.

*Corresponding author: Shah M, Industrial Waste Water Research Laboratory, Division of Applied and Environmental Microbiology Lab, India, Tel: +91-9099965504; E-mail: shahmp@beil.co.in

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Growth medium

Bushnell and Haas medium (BHM) (HiMedia) containing the following in g^l⁻¹: MgSO₄: 0.2; K₂HPO₄: 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 100 ppm (mg^l⁻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 mg^l⁻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 16 SrRNA

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) 100 ml in 250 ml 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 λ_{max} of respective dye (λ_{max} of ROM₂R=488 nm). 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:

$$\% \text{ decolorization} = [(A_i - A_f) / A_i] \times 100$$

Where A_i=Initial Absorbance,

A_f=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 10 μl 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 day light and in UV light (254 nm).

Fourier transform infrared 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 4000 cm⁻¹ to 400 cm⁻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.

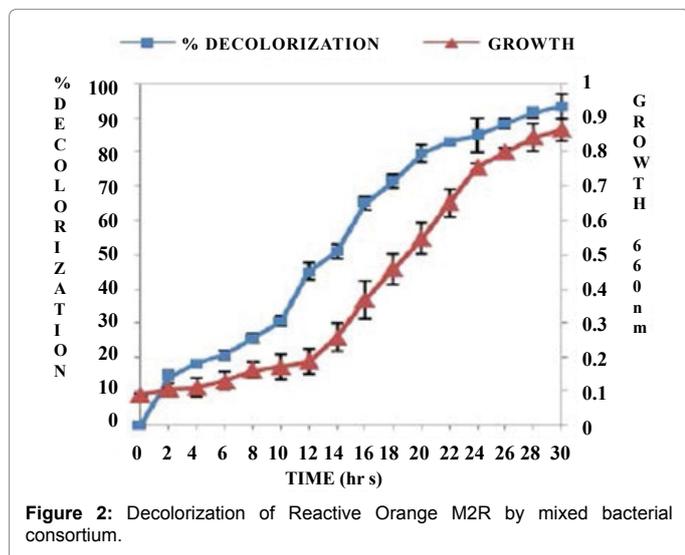
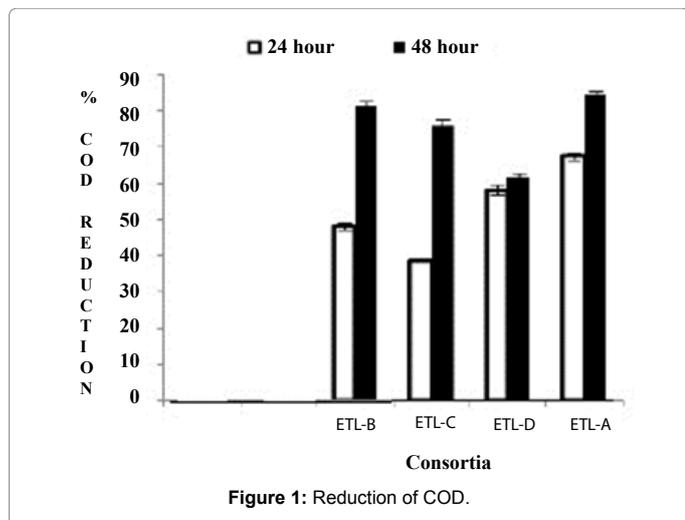
Results and Discussion

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. [16]. Of all the bacterial consortia obtained, bacterial consortium ETL-A was found to be the most efficient with maximum decolorization efficiency 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. Researchers reported synchronized action of microorganisms for the biodegradation of textile dye [19-23].

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 h and 48 h of inoculation respectively. Consortium ETL-A showed maximum COD reduction of 67% and 83% of ROM₂R dye in 24 h and 48 h 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 [24].



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 (Figures 2 and 3). The consortium ETL-A exhibited decolorization up to 93% within 30 h under static condition, whereas under shaking condition, the culture showed 20% decolorization of ROM₂R dye in 30 h. 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

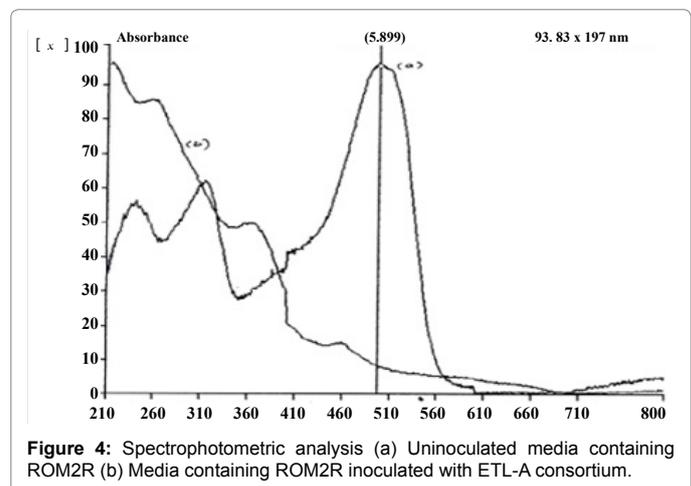
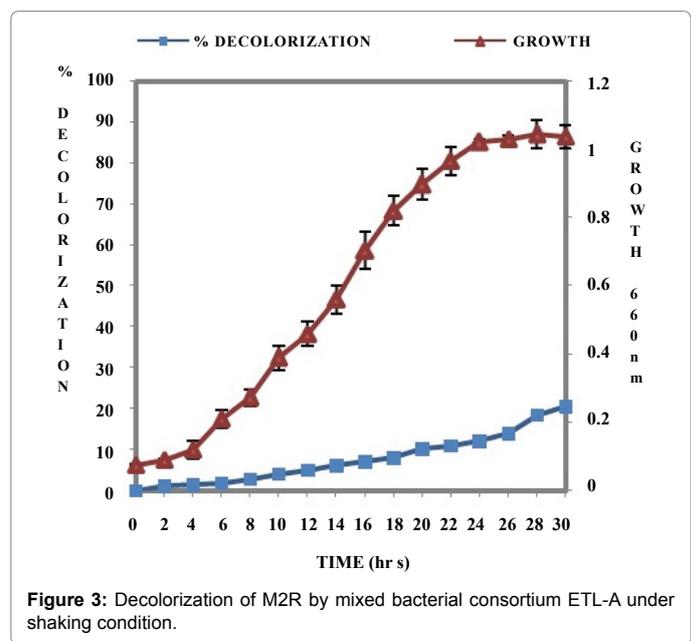
competition in oxidation of reduced electron carriers (e.g. NADH) with either Oxygen or Azo group as the electron receptor [25].

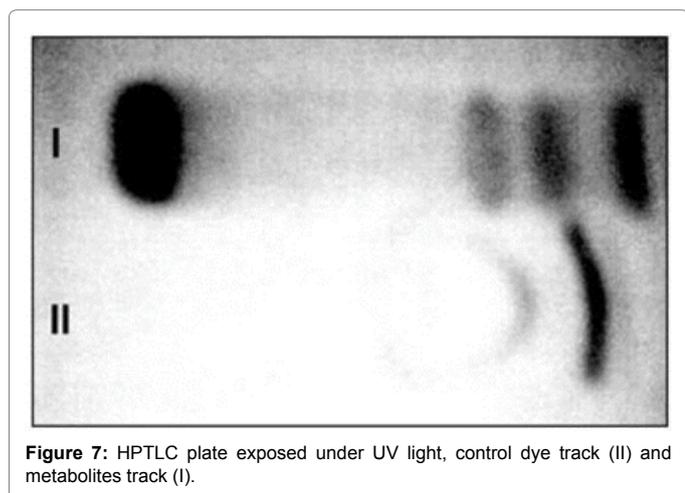
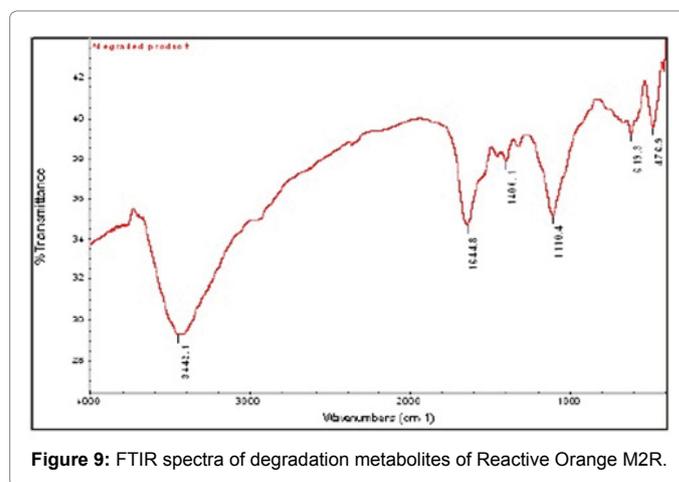
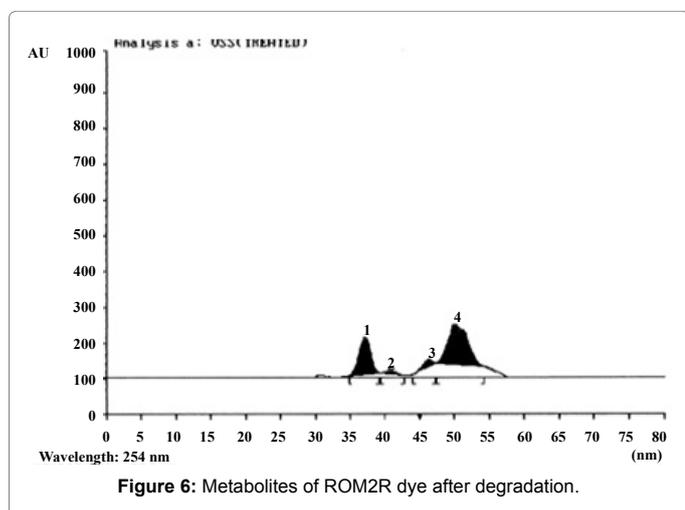
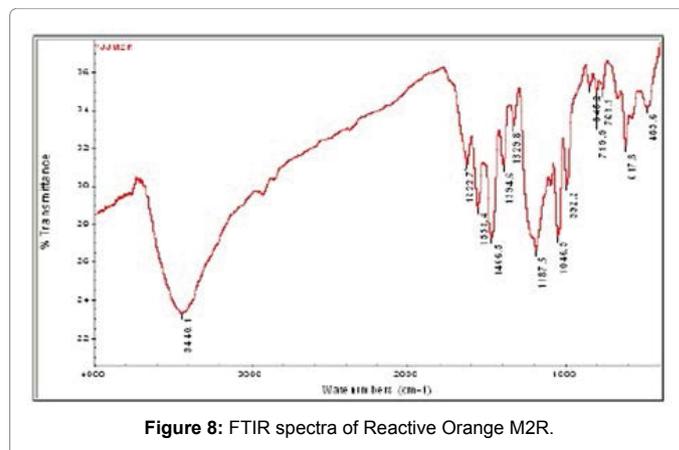
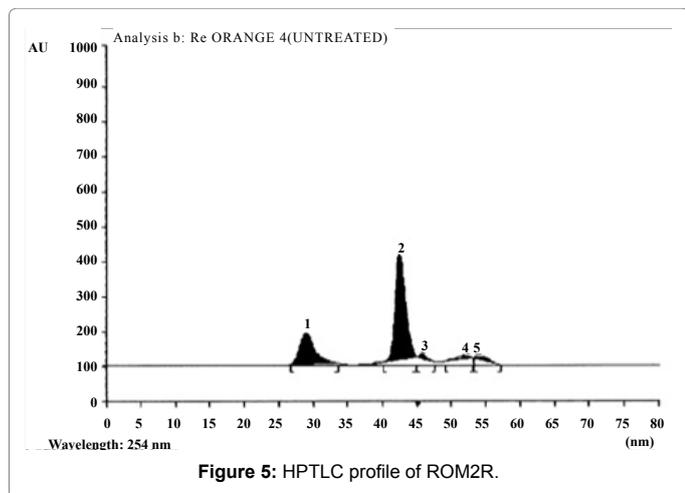
HPTLC analysis

Spectrophotometric analysis at 488 nm of uninoculated medium containing ROM₂R showed a simultaneous decrease in peak of samples withdrawn at various time intervals (Figure 4). Evidence of the removal of dye can be observed with absorbance at λ_{max} being virtually zero after 48 h 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 (Figures 5-7). 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 R_f values (data not shown) of control dye and metabolites supports the FTIR data which suggests biodegradation of ROM₂R.

FTIR analysis

Results of FTIR analysis of control and sample obtained after





decolorization showed various peaks (Figures 8 and 9). The FTIR spectrum of control ROM₂R displays peak at 3440.1 cm⁻¹ for the intramolecular hydrogen bonding aromatic -OH and -O-H stretching, peak at 1622.7 cm⁻¹ and 1555.4 cm⁻¹ for -C=C stretching of aromatic rings, 1466.5 cm⁻¹ and 1394.6 cm⁻¹ -C-H stretching of alkyl acetals, peak at 1329.8 cm⁻¹ and 1187.5 cm⁻¹ for -C-N stretching due to amines,

peak at 845.2 cm⁻¹, 799.9 cm⁻¹, 763.3 cm⁻¹ for -C-H stretching, 617.8 cm⁻¹ -C-Cl stretching. The degradation metabolites of ROM₂R using consortium ETL-A showed peak at 3442.1 cm⁻¹ for -O-H- stretching, peak at 1644.8 cm⁻¹ -C=C stretching of alkenes, 1400.1 cm⁻¹ for -C-H stretching due to -CH₃, peak at 1110.4 cm⁻¹, 619.3 cm⁻¹, 476.9 cm⁻¹ for -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 Zhou et al. [26].

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 h. 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 [27,28].

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

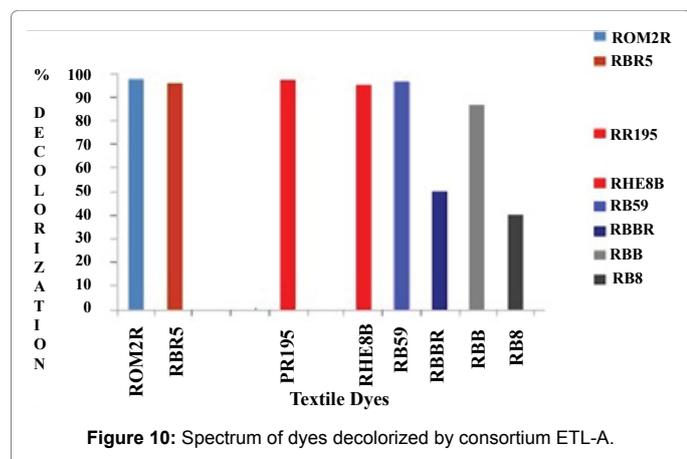


Figure 10: Spectrum of dyes decolorized by consortium ETL-A.

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.

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