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Isolation and Development of a Bacterial Consortium for Biodegradation of Textile Dyes Reactive Red 31 and Reactive Black 5

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Abstract: *Azo dyes represents largest group and most versatile among synthetic dyes. Its Extensiveuse in industries of paper, photography, dying, printing, cosmetics and their subsequent release in industrial effluents by traditional methods of treatment is causing serious environmental problems worlwide. In the present studies a mixed bacterial consortium (azo 8) was isolated from dye polluted soil from valsad and G.I.D.C., Vapi, Gujarat, India, by enrichment technique using Nutrient Broth/Agar and Bushnell and Haas Medium (BHM) incorporated with azo dyes Reactive Red 31 and Reactive Black 5.The consortium shows complete decolorization within 24 – 36 hours in static condition. The presence of laccase suggests its role in the degrdation of dyes. The biodegradation was monitored by TLC, HPLC and LC-MS. The results suggest that locally developed bacterial consortium azo 8 can be useful tool and must be exploited as bioremediation agents to reduce dye pollutants.*

Keywords: *Bacterial consortium, Reactive Azo Dyes, Biodegradation, Laccase, LC-MS.*

I. Introduction

Textile dye wastewater has become one of the main reasons behind severe pollution problems due to the greater demand for textile products and increase in production and application of synthetic dyes [1]. Azo dyes account for the majority of all textile dyestuffs produced and are the most commonly used synthetic dyes in the textile, food, paper making, color paper printing, leather and cosmetic industries [2]. Dyes are broadly classified into several types. Based on the chemical structure of chromophoric group synthetic dyes are classified as azo dyes, anthraquinone dyes, etc. These dyes have an adverse effect on the environment. The dyes are toxic and carcinogenic due to their high COD (Chemical Oxygen Demand) values. Azo dyes are considered as electron deficient xenobiotic components because they possess azo (N=N) and sulphonic (-SO3) electron withdrawing groups, generating electron deficiency and making the component less susceptible to oxidative catabolism by bacteria.

Azo dyes are the largest group of dyes used in the textile industry [3]. The discharge of these sulfonated azo dyes has not only a negative aesthetic effect but also some azo dyes and their degradation products, sulfonated and unsulfonated aromatic amines are toxic or even carcinogenic [4]. Much of these studies have emphasized only the decolorization/degradation of dye wastewater, not discussing much about the metabolites released by the cleavage of azo group. There is a need of novel mechanism for the removal of dyes from textile wastewater. Compared with chemical/physical methods, biological processes have received much more attentions due to cost effectiveness, lower sludge production and environmental friendliness [5], [6].

Nowadays trends of using mixed microbial cultures known as consortium system as compared to individual strains as a treatment of dyes and dyestuffs. This consortium system for treatment possess higher degree of biodegradation and ionization due to synergistic metabolic activities of microbial population and provide noticeable advantages over the individual pure culture in the degradation of textile dye stuffs[6], [7], [8]. The present study deals with a defined three bacterial strains, designated as consortium azo 8, was capable of degrading azo Reactive Red 31 and Reactive Black 5 under static condition. The dye degradation was studied by laccase activity and by TLC, HPLC and LC-MS analysis.

II. Materials and Methods

2.1Samples

Dye contaminated Soil samples were collected from Valsad and G.I.D.C., Vapi, Gujarat, (India) and kept in cold condition and used without any pretreatment.

2.2 Dyes

The textile azo dyes Reactive Red 31 and Reactive Black 5 dyes were procured from Atul Industries, Atul, Gujarat.

2.3 Screening method of dye degrading bacteria

Isolation of the microbial strains was carried out from the soil samples by enrichment culture technique using Nutrient broth/ Nutrient agar and Bushnell and Haas Medium (BHM) containing 0.05 % Starch, 0.05 % Yeast extract and 100 ppm Reactive Red 31 and Reactive Black 5. Morphologically distinct bacterial strains showing decolorization on every successive transfer were selected for development of efficient bacterial consortium for azo dye decolorization.

2.4 Decolorization studies

All the decolorization experiments were performed in triplicates. Decolorization study of Rective Red 31 and Reactive Black 5 were carried out by addition of 100 ppm of dye in 250 ml Erlenmeyer flask containing 100 ml of BHM medium containing 24 hr. old culture of all three bacterial isolates (mix consortium). All the studies were carried at Room Temperature and at pH 7. Samples (2 ml) were withdrawn after various time intervals to monitor decolorization rate. Aliquots withdrawn were directly measured at 544 nm for Reactive Red 31 and 597 nm for Reactive Black 5. The above mentioned protocol was followed for different studies viz. effect of shaking and static conditions, effect of increasing dye concentrations on decolorization. The percentage decolorization was calculated [6] as follows:

$$\% \text{ Decolorization} = \frac{\text{Initial absorbance} - \text{Observed absorbance}}{\text{Initial absorbance}} \times 100$$

2.5 Identification of the Bacterial consortium

A 16S rRNA analysis method was used to identify the selected bacterial strains. The nearly full-length 16S RNA gene was amplified by PCR with forward primer 8F and reversed primer Universal 1492R. Purified PCR products were sequenced using the ABI PRISM 3730XL Sequencer and the ABI PRISM BigDye Terminator Cycle Sequencing ready-reaction kit.

2.6 Effect of Dye Concentration

Various dye concentrations ranging from 100 to 1000 mg /lit were added to BHM in order to examine the effect of varying initial dye concentrations on decolorization.

2.7 Effect of Shaking and Static condition

Decolorization of dyes was studied using different culture conditions like agitation and stationary conditions to find out the pattern of decolorization / degradation. Influence of provided shaking condition was studied on orbital environmental shaker agitating at 150 rpm at room temperature. For stationary condition experiment was conducted without agitation at RT.

2.8. Determination of factors responsible for dye degradation (Enzyme analysis)

Decolorization is mainly a biological process, either a direct enzymatically catalyzed reaction involving non-specific enzymes or a reaction with enzymatically reduced electron carriers. To study the possible enzymes involved in decolorization ***Plate screening method*** was used. Spot inoculation of culture in sterilized Nutrient agar plates having added 0.02% Guaiacol plates is incubated at 30ºC for 4 days.

2.9 Preparation of cell free extract

The consortium azo 8 and individual were grown separately in the BHM with 0.05% YE for 24 hours at RT and then transferred to the same media incorporated with 100 ppm Reactive dyes concentration and incubated and monitored for decolorization. After decolorization, each decolorized culture and azo 8 were centrifuged at 7000 rpm for 20minutes at 4°C (Eppendorf 5430R). The supernatant was filtered through 0.45mm filter (Millipore) and the resulting cell free filtrate culture supernatant was designated as the Crude Culture Fraction (CCF), used for the assay of crude extracellular enzymatic activity. The cell pellets were suspended in potassium phosphate buffer (PPB) (50 mM, pH 7.4) containing 0.1g ml^{-1} of lysozyme then were incubated at RT for 30 min and cells were subjected to sonication (Frontline Sonicator - 250) constant 250 Hz for 6 cycles, each cycle for 60 secondand similar interval was kept between each cycle at 4°C[9]. The cell lysate was mixed with equal amount of PP (Protein Precipitation) buffer. The protein pellet were dissolved in a minimum quantity of 20mM potassium phosphate buffer (pH 7.4) and the protein was loaded on to a Sephadex G-75 column (size 1 cm x 20 cm), purification column and centrifuged at 10,000rpm for 1 minute. The column was washed with washed buffer and protein were eluted using elute buffer and (Protein purification kit) used as the source of intracellular partially purified proteins.

2.10 Laccase enzyme assay

Assay of the enzyme laccase was using reaction mixture contained 1 ml 0.1mM Guaiacol, 3 ml 10 mM Sodium phosphate buffer (SPB) pH 7.0 with 1ml crude filtrate and/or partially purified protein as source of laccase in total volume of 5 mL at Room Temperature. The change in absorbance of the reaction mixture

containing guaiacol was monitored at 470 nm at every 1 min interval for total 5 min. Enzyme activity was calculated using extinction coefficient and µg of Guaiacol oxidized/minute. Specific activity was calculated was expressed in units per milligram protein.

2.11 TLC analysis

Thin Layer Chromatography was used for separation of degraded product of azo dye. The solvent system used was Butanol: Water: Acetic acid, (5:3:2).Resulted TLC plates were resolved with 0.2% Ninhydrin spray.

2.12 HPLC analysis

HPLC analysis was carried out model Agilent 1200 series, on Hypersil BDS C18 column (250 x 4.6 mm, 5 micron particle size, Pheniminex). The mobile phase was HPLC grade Acetonitrile and phosphate buffer (90:10 v/v) with flow rate was 1 ml/min. The decolorized culture medium was centrifuged at 10,000 rpm for 15 min and supernatant was collected and filter through 0.45 PVDF filter and then analyzed. The purity was checked by PDA detector, isocratic system and 45 min run time [10].

2.13 LC-MS analysis

LC-MS of biodegraded reactive dyes were carried out using Model AB Sciex API 2000 by comparing with control dyes. The LC-MS analysis was done with auto (2mm/sec) speed. The decolorized culture medium was centrifuged at 10,000 rpm for 15 min and supernatant was collected and filter through 0.45 PVDF filter and then used with methanol for LC-MS analysis. 20 µl of prepared sample and dyes of 100 ppm concentration were injected. Operating conditions were dry with temperature of 325ºC, capillary voltage 3500V. The ion trap detector with atmospheric pressure electro – spray ionization (API – ESI) source was used for in negative ionization mode. Ion trap full scan analyses were conducted from m/z 120-1000 with an upper full time of cps.

III. Results and Discussion

3.1 Screening of dye degrading organisms

The main objective of this study was to screen out bacterial strains capable of decolorization and biodegradation of Reactive Red 31 and Reactive Black 5 azo dyes. Present study resulted in isolation of three strains all capable of degrading azo dye. All strains isolated from dye polluted soil indicates the natural adaptation of these bacteria to survive in the presence of toxic dye. All three bacterial isolates were developed in consortium able to degrade dye at wide range of temperature RT to 40°C and decolorization at optimum at neutral to acidic pH.

3.2 Identification of bacterial consortium

The three bacteria in the consortium belongs to Escherichia coli (Azo 6), Bacillus cereus (Azo 9) and Bacillus infantis (Azo 12). The similar study was conducted by [11] for identification of bacterial consortium PMB11, a combination of three bacteria.

3.3Effect of different dye concentration

The mix consortia have shown decolorization activity at concentration between 100-1000 ppm. The decolorization activity of the cultures was strongly inhibited at higher concentration of dye, (Fig 3.2). It has been proposed that dye concentration can influence the efficiency of microbial decolorization through combination of factor including the toxicity imposed by dye at higher concentration [12]. Thus consortium azo 8, could decolorize dye much above the reported dye concentration in wastewaters, and can be successfully employed for treatment of textile effluents.

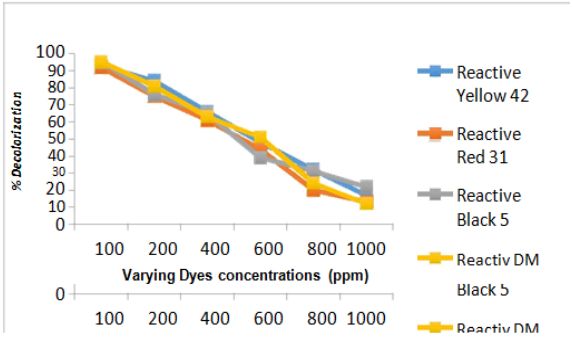


Figure: 3.3 Effect of different dyes concentration on percent decolorization

3.4 Effect of static and shaking condition

Under static condition consortium culture exhibited remarkable decolorization / degradation, 84% of Reactive Red 31 (Figure 3.4) within 36 hours while 86% (maximum) of Reactive Black 5 (Figure 3.4) in 30hrs of incubation. While comparable reduced decolorization was recorded under the shaking condition even after the 48 hours of incubation. Further incubation did show very little changes in decolorization activity under shaking condition.

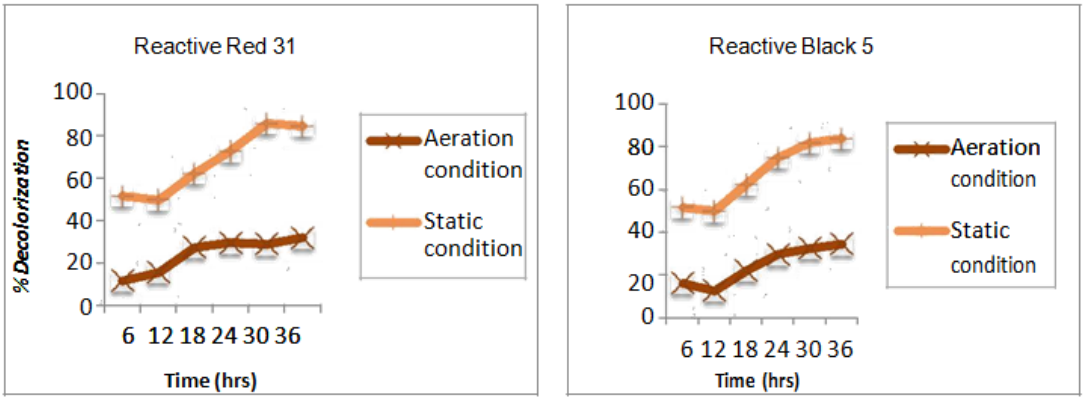


Figure 3.4: Effect of shaking and static condition on percent decolorization

3.5 Determination of factors responsible for dye degradation (Enzyme analysis)

Plate screening method show dark brown zone was observed around colonies producing laccase enzyme responsible for dye degradation (photo plate 3.5). Extracellular oxidoreductases, including lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase produced by wood-decomposing microorganism, are directly involved in the degradation of lignin in their natural lignocellulosic substrates and various xenobiotic compounds including dyes [13]. The production of lignolytic enzymes is observed as a colorless halo around microbial growth [14]. So, the Azo dyes degradation capability of consortium azo 8 is due to laccase initially and primarily confirmed by plate screening method.

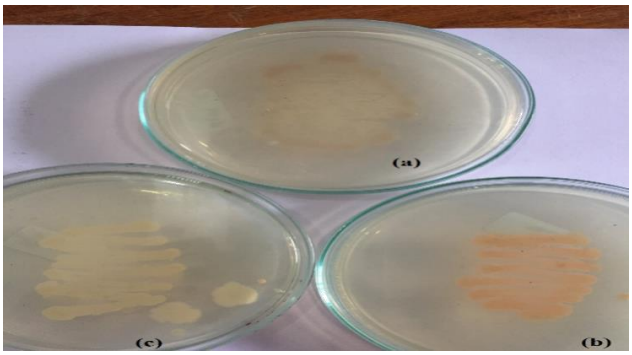


Photo plate 4: Screening of Laccase producing bacteria using Guaiacol

3.6 Laccase enzyme assay

The protein concentration after cell lysis was estimated as 900g/ml by Folin-Lowry protein estimation method. The enzymatic analysis of consortium azo 8 (after decolorization) for Reactive Red 31 was calculated (Table: 3.6.1) 2.04 and 4.43 units/mg of protein from crude filtrate and partially purified enzymes respectively, and for Reactive Black 5 was calculated (Table: 3.6.2) 3.03 from crude filtrate and 4.10 units/mg of protein from partially purified enzymes. Similar studies were reported by [15] and [16].

Table: 3.6.1 Enzymatic activity with Reactive Red 31 dye of Induced state (36 hours decolorization)

Enzymatic activity of	**Laccase**	
	CCF	**PP**
Azo6	0.02	0.80
Azo12	1.67	2.20
Azo9	1.25	2.35
azo 8(BC)	2.04	4.43

Specific Enzyme activity- Units/mg of protein. Azo6: *Escherichia coli* Azo12: *Bacillus infantis* Azo9: *Bacillus cereus* azo 8: Bacterial consortium (BC)

Table: 3.6.2Enzymatic activity with Reactive Black 5 dye of Induced state (36 hours decolorization)

Enzymatic activity of	**Laccase**	
	CCF	**PP**
Azo6	0.18	0.79
Azo12	0.98	1.98
Azo9	1.12	2.46
azo 8(BC)	3.03	4.10

Specific Enzyme activity- Units/mg of protein. Azo6: *Escherichia coli* Azo12: *Bacillus infantis* Azo9: *Bacillus cereus* azo 8: Bacterial consortium (BC).

3.7 TLC analysis

The degraded metabolites of Reactive Red 31 and Reactive Black 5 were analyzed by TLC. TLC patterns of control dye sample and treated sample of both dyes having different spot. R*f* value of control Reactive Red 31 – 0.68 was not present in treated sample pattern with R*f* value 0.42 (Plate 3.7(a)). While, R*f* value of control Reactive Black 5 – 0.26 was not present in treated sample pattern with R*f* value 0.43 (Plate 3.7(b)).

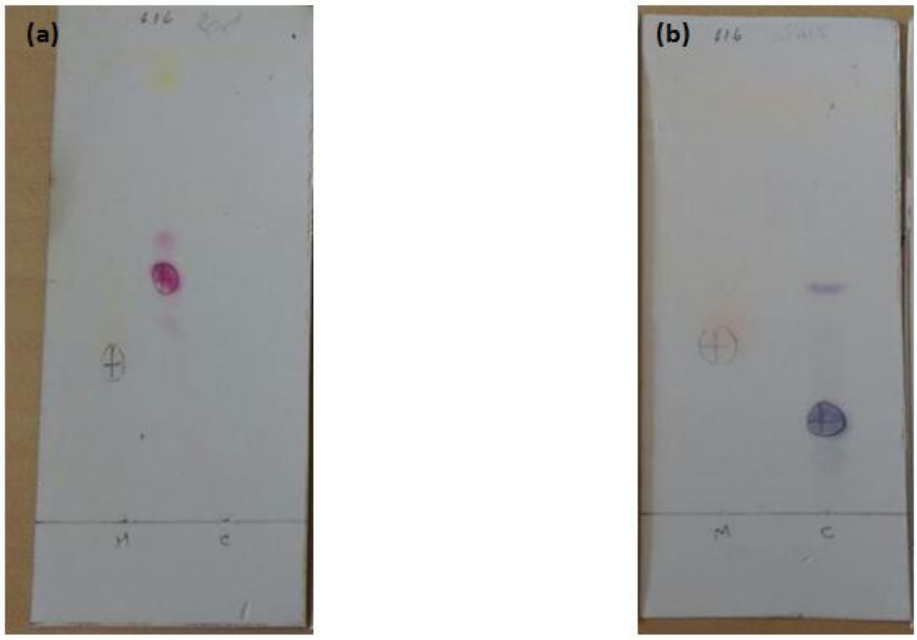
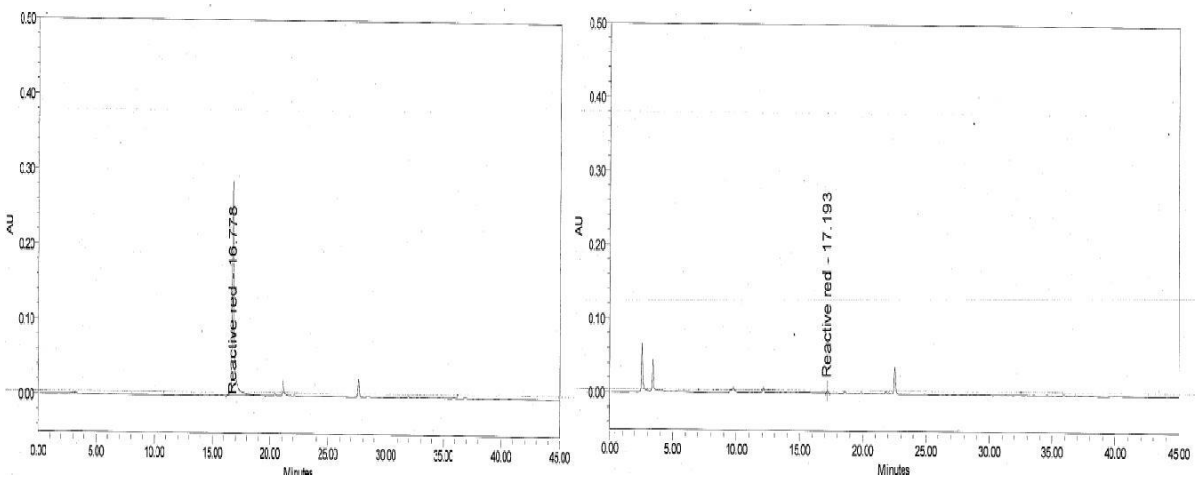


Plate 3.7: (a) TLC analysis of the treated Reactive Red 31 (b) TLC analysis of the treated Reactive Black 5

3.8 HPLC analysis

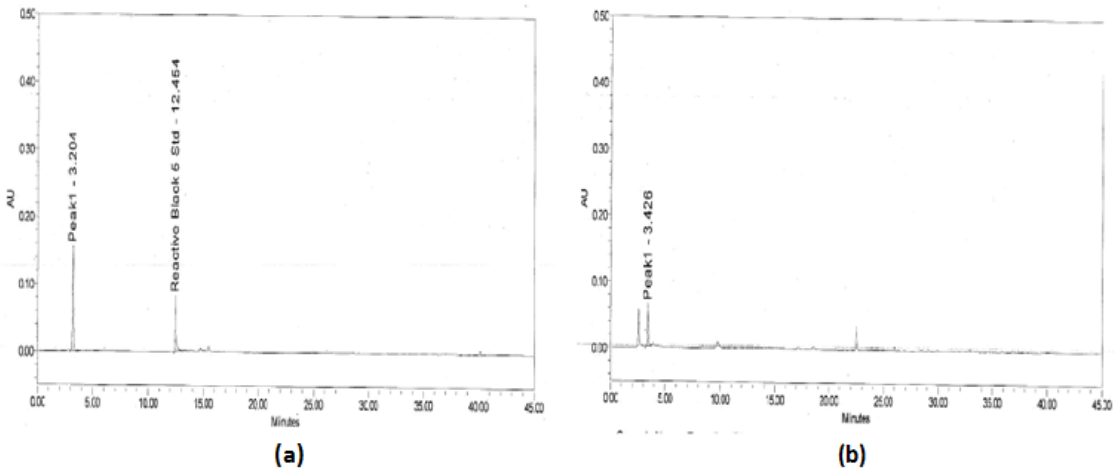
The metabolites obtained after complete decolorization of Reactive Red 31 and Reactive Black 5 were centrifuged and filter through 0.45 PVDF, dissolved in HPLC grade methanol and used for analysis. HPLC chromatogram of control Reactive Red 31 showed major peak at retention time 16.778 min (Figure 3.8.1 (a)). Decolorized of Reactive Red 31 shows two peaks at retention time 2.550 min and 3.410 min (Figure 3.8.1 (b)) suggest the degradation of Reactive Red 31



(a) **(b)**

Plate 3.8.1: HPLC elution profiles of, Control Reactive Red 31 (a), treated sample of Reactive Red 31 (b)

HPLC chromatogram of control Reactive Black 5 showed major peak at retention time 12.454 min and 3.204 (Figure 3.8.2 (a)). Decolorized treated sample of Reactive Black 5 shows two peaks at retention time 2.450 min and 3.426 min (Figure 3.8.2 (b)) suggest the degradation of Reactive Black 5. Similar studies carried out by [17] HPLC analysis of a dye Remazol Black B and its degraded products. Reported peaks of metabolites shifted towards lower retention time compared to the control peak at quite higher retention time which suggested the formation of less aromatic amines and more polar compounds by biodegradation process of bacteria. Identification of metabolites should be the objective of further detail investigations.

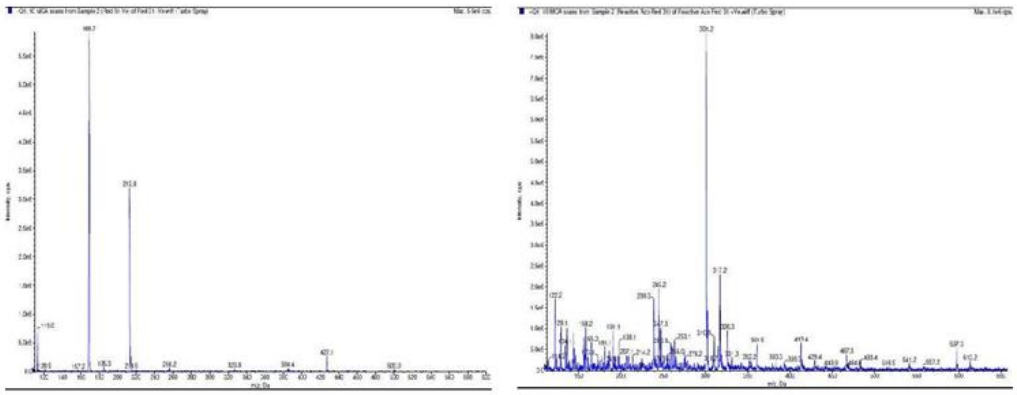


(a) **(b)**

Plate 3.8.2: HPLC elution profiles of, Control Reactive Red 31 (a), treated sample of Reactive Red 31 (b)

3.9 LC-MS analysis

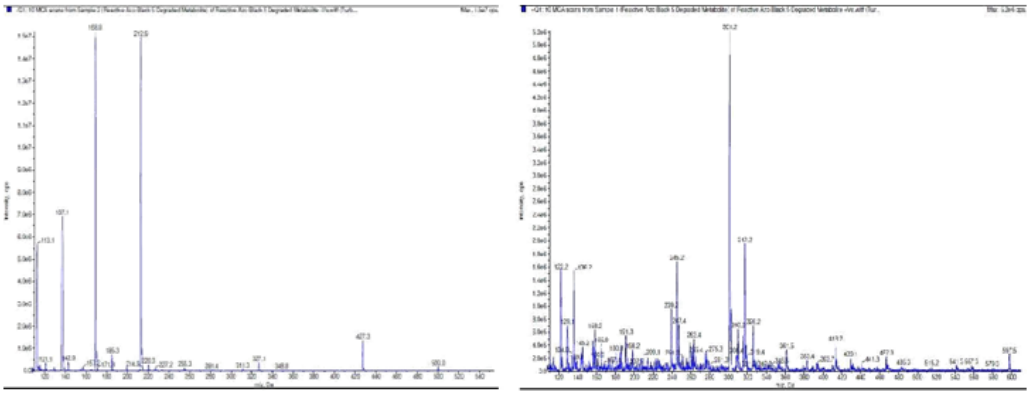
The products of degradation were analyzed and the spectrum of LC-MS of the Control Reactive Red 31 and after complete decolorization Red 31 treated sample shown in the (Plate 3.9.1 (a) and (b)) respectively. The chromatogram of control Reactive Red 31 showed base peak of 168.7 m/z (Plate 3.9.1 (a)), while in the case of decolorized culture of Reactive Red 31, the mass spectrometric showed significant peak of 301.2 m/z. The other all peak presents in the spectrum of control Red 31 dyes were absents in the treated sample, indicating the entire dye molecule has been decomposed to colorless low molecular weight fragments by consortium azo 8.



(a) (b)

Plate 3.9.1**:** (a) LC-MS spectra of Reactive Red 31 dye control, (b) treated sample of Reactive Red 31

The products of degradation were analyzed and the spectrum of LC-MS of the Control Reactive Black 5 and after complete decolorization Reactive Black 5 treated sample shown in the (Plate 3.9.2 (a) and (b)) respectively. The chromatogram of control Reactive Black 5 showed base peak of 168.8 m/z, and 212.9 m/z (Plate 3.9.2 (a)), while in the case of decolorized culture of Reactive Red 31, the mass spectrometric showed similar significant peak of 301.2 m/z. The other all peak presents in the spectrum of control Reactive Black 5 dyes were absents in the treated sample, indicating the entire dye molecule has been decomposed to colorless low molecular weight unidentified fragments by consortium azo 8. [18] reported similar studies LC-MS analysis of the dyes and their degraded products confirmed degradation of Reactive Black 5 by *Aeromonas punctate* and Reactive Red 120 dye by *Pseudomonas aeruginosa*.



(a) (b)

Plate 3.9.2**:** (a) LC-MS spectra of Reactive Black 5 dye control, (b) treated sample of Reactive Black 5

IV. Conclusion

In the present study, a potential azo dye degrading consortium was developed and identified by 16S rRNA sequencing. The identified culture belonged to Bacillus species and Escherichia species. The study of laccase enzyme in bacterial consortium azo 8 resulted in increased enzymatic activities compared to individual confirmed its involvement in dye decolonization. TLC analysis confirmed the dyes decolonization was due to its degradation. Further, HPLC and LC-MS analysis confirmed the biodegradation of reactive Red 31 and Reactive Black 5, and indicates the degraded metabolites were of simple and non-toxic.

As a conclusion this study reports that the potential developed consortium azo 8 could be successfully applied in contaminated industrial effluents and in the safe bioremediation of textile effluents.

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