Exploitation of Two Consortiums in Microbial Degradation and Decolorization of Remazol Black and Acid Orange

Shah M*

Industrial Waste Water Research Laboratory, Division of Applied & Environmental Microbiology, Environmental Technology Limited, Gujarat, India

Abstract

Different soil samples were collected from effluent treatment plants were used as a source for the isolation of 16 morphologically distinct bacteria capable of decolourising textile dye, Acid orange. Decolourisation assay was performed using two textile dyes - Acid oranje and emazol black to screen for high dye decolourising bacterial isolates. 5 Bacterial isolates showed high decolourisation of Acid orange and a consortium of these 5 colonies called A was developed. 3 Bacterial isolates showed high decolourisation of Remazol black and a consortium of these 3 isolates, B, was developed. The screened bacterial isolates were identified as Pseudomonas sp., Alcaligenes sp., Rhodococcus sp., Stenotrophomonas sp., Proteobacterium sp., and Bacillus sp. by 16S rDNA analysis. A exhibited high decolourisation percentage of 200 mg/l of Acid orange in the presence of anhydrous sodium acetate under static condition at pH 7 and temperature of 40°C within 48 hours. B showed high decolourisation of 200 mg/l of Remazol black in the presence of galactose and a combination of straw and beef extract under static condition at pH 7 and temperature of 30°C within 48 hours. LC-MS analysis of the degraded product of Acid orange by A showed the presence of 1-{(3-amino-5-[(aminoxy) sulphonyl] phenyl} ethanol and 7, 8-amino-3-[(aminoxy) sulphonyl] naphthalene-1-ol. LC-MS analysis of the degraded product of Remazol black by B showed the presence of 1.1’-diazene-1,2-diyldinapthelen-2-ol and sodium-4-amino-naphthalene-1-sulphonate.

Keywords: Pseudomonas; Alcaligenes; Stenotrophomonas; Rhodococcus; Remazol black; Acid orange

Introduction

The growth of the world population, the development of various industries, and the use of fertilizers and pesticides in modern agriculture has overloaded not only the water resources but also the atmosphere and the soil with pollutants [1-4]. In the last few decades the handling of wastewater appeared to be one of the most important. Textile industry which is one of the largest water consumers in the world produces the wastewater comprising of various recalcitrant agents such as dye, sizing agents and dyeing aid. Therefore it has to be really concerned in releasing these types of wastewater to the environment. In the disposal of textile wastewater, color is of very important due to the aesthetic deterioration as well as the obstruction of penetration of dissolved oxygen and sun light into natural water bodies [5-9]. The degradation of the environment due to the discharge of polluting wastewater from industrial sources is a real problem in several countries. This situation is even worse in developing countries like India where little or no treatment is carried out before the discharge [10]. In spite of the many steps taken to maintain and improve the quality of surface and groundwater, the quantities of wastewater generated by these industries continue to increase and municipalities and industries are confronted with an urgent need to develop safe and feasible alternative practices for wastewater management. Bioremediation is a pollution-control technology that uses natural biological species to catalyze the degradation or transformation of various toxic chemicals to less harmful forms. Xenobiotic compounds are not naturally available and hence the locally occurring microorganisms cannot readily degrade them. Hazardous materials may render harm to humans, livestock, wildlife, crops or native plants through handling, ingestion, application to land or other distributions of the contaminated materials into the environment. The textile industry leaves about 50% of the textile azo dyes in free state to be discharged in the factory effluent and eventually to the surrounding environment. Azo compounds constitute the largest and the most diverse group of synthetic dyes and are widely used in a number of industries such as textile, food, cosmetics and paper printing [11]. The reactive azo dyes-containing effluents cause serious environmental pollution. Therefore, industrial effluents containing azo dyes must be treated before discharging into the environment to remove the dye toxicity from textile effluents [12]. Our study deals with the isolation of bacteria, development of mixed bacterial consortium for static degradation of textile dyes, Acid orange and Remazol black. 16S rDNA analysis has been carried out to determine the bacterial isolates. A degradation pathway has been elucidated for these sulphonated azo dyes using bacterial consortium.

Materials and Methods

Collection and laboratory analysis

The Effluent sample was collected from the middle point of the area. The Temperature and pH were determined at the sampling site. The pH was determined by using pH meter (Cyber scan pH meter) and temperature with laboratory thermometer. The sample was transported to laboratory at 4°C as in accordance with the standard methods [13]. The physicochemical parameters such as (Colour, Biological Oxidation Demand (BOD) Chemical Oxygen Demand (COD), Total Suspended Solids (TSS), and Total Dissolved Solids (TDS) were determined as soon as the sample was brought to the laboratory. Sample colour was analyzed by spectrophotometer (Shimadzu UV-1700). BOD was

*Corresponding author: Shah M, Industrial Waste Water Research Laboratory, Division of Applied & Environmental Microbiology, Environmental Technology Limited, Gujarat, India, Tel: 91-9099965504; E-mail: shahmp@uniphos.com

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Dyes and chemicals

The textile dye, Remazol Black and Acid orange was obtained from local textile industry. The stock solution of dyes was filter sterilized and added to the growth medium in the required concentration. All the chemicals used were of analytical grade and procured from Himedia Pvt Limited. The Chemical Structure of Remazol Black and Acid orange is shown in Figure 1.

Isolation and screening

The Textile Effluent was collected in sterile collection tubes from the sludge and wastewater of the ditches at industrial site. The sample collected from the textile mill was screened for Acid orange decolorizing bacterial strains by inoculating 10 ml of sludge solution into 250 ml Erlenmeyer flask containing 100 ml nutrient broth (gL–1 Peptone-5, Meat extract-1, Yeast extract-2, NaCl-5, pH-7). The flasks were incubated at 35°C under shaking conditions (140 rpm). After 48 h of incubation, 1.0 ml of the culture broth was appropriately diluted and plated on Nutrient Agar containing 20 mg L⁻¹ Acid orange. The Morphologically distinct bacterial isolates showing clear zones around their colonies due to decolorization of dye were selected for further studies. The pure culture stocks of these isolates were stored at 4°C on Nutrient Agar slopes containing 1000 mg L⁻¹ of Acid orange. These test tubes were incubated with different dye concentrations (50 mg/l, 100 mg/l, 200 mg/l and 400 mg/l), under different oxygen conditions (aerobic, anaerobic and static), at different temperatures (10°C, 20°C, 30°C, 40°C, 50°C and 60°C) and at different pH (2 to 9). At various time intervals (24 and 48 h), the percentage of decolourisation was calculated. Each experiment was repeated for three times and mean value was taken for result analysis

Decolorization study

To study the decolorization, used medium was minimal medium containing following ingredients per liter: KNO₃: 5 g, (NH₄)₂SO₄: 1g, K₂HPO₄·3H₂O: 0.87 g, KH₂PO₄: 0.54 g, MgSO₄·7H₂O: 0.2 g, CaCl₂·2H₂O: 0.02 g, FeSO₄·7H₂O: 0.01 g, MnSO₄·H₂O:0.005 g supplemented with 2.5%-10% NaCl and dye as sole carbon source.

Effect of different physico-chemical parameters on decolourisation

Aliquot of 0.1 ml of each consortium (A and B) and each of the bacterial isolates was inoculated into test tubes containing LB Media amended with dyes (Acid red and Mordant black). These test tubes were incubated with different dye concentrations (50 mg/l, 100 mg/l, 200 mg/l and 400 mg/l), under different oxygen conditions (aerobic, anaerobic and static), at different temperatures (10°C, 20°C, 30°C, 40°C, 50°C and 60°C) and at different pH (2 to 9). At various time intervals (24 and 48 h), the percentage of decolourisation was calculated. Each experiment was repeated for three times and mean value was taken for result analysis

Decolorization measurement

For decolorization measurement, sample (1.5 ml) of the culture media was taken at different time intervals, well agitated prior to being subjected for BLAST analysis to identify the bacterial isolate (http://blast.ncbi.nlm.nih.gov/Blast.cgi).
sampling (due to the insolubility of dye) and centrifuged at 7500 rpm for 5 min with a micro refrigerated centrifuge at 20°C to separate bacterial cell mass. Decolorization rate was calculated from the difference between initial and final absorbance values at the absorbance maxima of the dye (490 nm) on a UV-vis spectrophotometer (Shimadzu, Japan). Uninoculated culture media with and without added dyes were used as controls.

**Metabolites extraction**

About 100 ml of Acid orange (200 mg/ml) containing LB Media with 0.1 ml of 24 hour grown culture of A was incubated for 24 hours and 48 hours. Using a separating funnel, the organic compounds were extracted using equal amount of ethyl acetate from the incubated dye culture (24 and 48 hour cultures). The ethyl acetate phase was then subjected to evaporation at 78°C to obtain a powdery residue of the organic compound (69).

**LC MS analysis**

The powdery residue was then dissolved in acetonitrile (HPLC Grade). LC-MS analysis was performed using a Finnigan model Mass Spectrometer (Thermo Electron Corporation, USA) using C-18 column from Waters. The cartridges were conditioned with pure acetonitrile, washed with deionized water (0.1% Formic Acid) and the elution took place with 70% acetonitrile, containing 0.1% formic acid. The flow rate was 0.8 ml/min. The ion trap detector with atmospheric pressure electro-spray ionization (API-ESI) source used for quantification in negative ionization mode. Operating conditions were dry with temperature of 325°C, Capillary voltage 3500V, Nebulizer 14 psi, dry gas Helium 5.0 l/min. Ion trap full scan analyses were conducted from m/z 200-1400 with an upper full time of 300 minutes. The nebulizer gas flow and the curtain gas flow (Nitrogen gas) were set at 4800, 40, +70V respectively. Instrumentation control and data acquisitions were performed with data analysis for Mass Spectroscopy (X caliber, USA).

**Results**

**Characterization of textile effluent**

The effluent sample collected from a small scale Textile Industries, Ankleshwar, Gujarat, India was dark red in color, with pungent smell and pH of slightly above neutral level and was within the permissible limits (Table 1). The temperature of the effluent was high. Total Suspended Solids (TSS) and Total Dissolved Solids (TDS) in the textile effluent were very high. The solids present in ground water, besides effecting the growth of the plants directly, also affect the soil structure, permeability and aeration, indirectly effecting the plant growth. The Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD) values were within the permissible limits in the effluent sample. Different bacterial strains isolated from the textile effluent were screened for their ability to decolorize the textile Azo dye (Toludine Red) and the potential strains were characterized morphologically and biochemically.

**Screening for high dye decolourising bacterial isolates and development of bacterial consortium**

Upon serial dilution, 16 morphologically distinct colonies were isolated and named as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16. Each of these isolates was subjected for dye decolourisation assay. At the end of 48 hours, in the case of Acid orange, isolates 6, 13, 7, 15 and 16 showed more than 70% decolourisation. Isolates 1, 4, 2, 10, 12, 11 and 14 showed a moderate decolourisation. No decolourisation was observed for 8 and 9. Thus, consortium, A, of 6, 7, 13, 15 and 16 was developed (Table 2).

In the case of Remazol black (Table 2), the isolates 6, 14 and 16 showed a high decolourisation of more than 65%. 10 and 12 showed moderate decolourisation percentage. 4, 3 and 8 showed negligible decolourisation percentage. 1, 2, 7, 9, 11, 13 and 15 showed no decolourisation. Thus, consortium, B, consisting of 6, 14 and 16 was developed.

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Table 1: Physico-chemical characterization of the textile effluent collected from Textile Industries, Ankleshwar.

| S.No | Parameter | Unit  | Effluent  |
|------|-----------|-------|-----------|
| 1    | Color     | -     | Dark red  |
| 2    | Smell     | -     | pungent   |
| 3    | Temperature | °C | 38        |
| 4    | PH        | -     | 8.0       |
| 5    | TDS       | mg/l  | 7500      |
| 6    | TSS       | mg/l  | 2800      |
| 7    | COD       | mg/l  | 700       |
| 8    | BOD       | mg/l  | 220       |

Table 2: Decolorization Assay using 500 mg/L Dye.

| Sample | % Decolorization with Acid Orange | % Decolorization with Remazol Black |
|--------|----------------------------------|-------------------------------------|
| 1      | 38.24                            | -                                   |
| 2      | 57.28                            | -                                   |
| 3      | 46.56                            | 17.36                               |
| 4      | 23.68                            | 9.6                                 |
| 5      | 51.04                            | 4.18                                |
| 6      | 59.52                            | 71.17                               |
| 7      | 74.88                            | -                                   |
| 8      | -                                | 17.04                               |
| 9      | -                                | -                                   |
| 10     | 23.36                            | 36.6                                |
| 11     | 40.64                            | -                                   |
| 12     | 45.12                            | 34.4                                |
| 13     | 74.72                            | -                                   |
| 14     | 43.04                            | 65.27                               |
| 15     | 74.72                            | -                                   |
| 16     | 87.2                             | 69.13                               |
Characterization and identification of screened bacterial isolates

DNA was isolated from each of the screened bacterial isolates - 6, 7, 13, 15, 16 and 14. The presence of DNA was analysed in 0.8% agarose by gel electrophoresis. The gel in Figure 2 shows the DNA isolated from the screened bacterial isolates. 16S rDNA sequence of each of the samples was amplified using universal primers. The image of the gel in Figure 3 shows the amplified 16S rDNA bands of the screened bacterial isolates. In comparison with 1 kb molecular marker, the size of amplified product was identified as 1.5 kb. Each of the amplified products was further purified by DNA Elution kit (Bioserve Biotechnologies India Private Limited) and was subjected to cycle sequencing. The sequence obtained by cycle sequencing was subjected to BLAST analysis.

Decolorization mechanism

Decolorization of Rhodococcus was qualitatively monitored by using a UV-vis spectrophotometer. The UV- vis spectra (200-800 nm) of the supernatant at 4th day of decolorization are shown in Figure 4. According to Hui Wang et al. [14] decolorization of dyes may be due to adsorption or biodegradation mechanisms. In case of adsorption the UV-vis absorption peaks decrease approximately in proportion to each other whereas if dye removal is due to biodegradation, either the major visible light absorbance peak disappears or a new peak appears. Comparing the control and sample spectrum it was understood that as Acid orange was decolorized, its absorbance peak at 490 nm in the UV range remained indicating that decolorization of Acid orange was occurred under degradation mechanism rather than surface adsorption.

Effect of different dye concentration on decolourisation

As shown in Table 3, it was found that as the concentration of dye increases, the decolourisation percentage of the dye decreases. Taking this observation into account and also the concentration of dye discharged by the textile and dye industries, 200 mg/l of dye was selected as the optimum concentration for further studies.

Effect of oxygen on the decolourisation

As shown in Table 4, under aerobic conditions, A showed a decolourisation percentage of 85.6 after 48 hours in LB Media. Individual bacterial isolates 13 and 16 could decolourise at a faster rate compared to the rest of the isolates and the consortium. 13 and 16 also showed maximum decolourisation percentage of about 90 after 48 hours. As shown in Table 5, under aerobic conditions, in the case of Remazol black, the individual bacterial isolate 16 could decolourise at a faster rate. However, the consortium as well as each of the individual isolates showed a decolourisation percentage of more than 75 after 48 hours. Under anaerobic condition, the consortium, A, could decolourise at a faster rate than the individual bacterial isolates. In the case of Remazol Black, the consortium, B, as well as the individual bacterial isolates showed a decolourisation percentage of more than 75 after 48 hours. The individual isolate 14 could decolourise at a faster rate. Under static condition, the consortium, A, as well as the individual bacterial isolates showed a decolourisation of more than 70%. Maximum decolourisation was shown by 7 after 48 hours, followed by 13 and 15. Consortium A could decolourise up to 92.2%. However, 13 could decolourise at a faster rate. In the case of Remazol black, each of the bacterial isolates as well as the consortium, B, showed a decolourisation of more than 75%. 14 showed maximum decolourisation efficiency in 48 hours. However, 16 could decolourise at a faster rate (Figures 5 and 6).

Effect of carbon source

As shown in Table 5, in the case of A, which was used against the dye, Acid orange, sodium acetate (anhydrous) showed maximum decolourisation of about 99.9%. Casein, maltose and galactose showed a decolourisation percent of more than 80. In the case of B Table 6 which was used against Remazol black, decolourisation in the presence of galactose was high with a decolourisation percentage of 91. The presence of dextrose and fructose also showed high decolourisation efficiency. Paddy straw along with beef extract acted as a nitrogen source which could bring about high decolourisation of 89.4% (Figure 7).

Effect of temperature on the decolourisation

As shown in Table 7, A showed a significant percentage of decolourisation of Acid orange between 20°C and 40°C. However, maximum decolourisation of about 98% was observed at 40°C. B showed high percentage decolourisation of Remazol black between 20°C and 40°C. However, the highest percentage of decolourisation of about 97 was observed at 30°C. Hence, the optimum temperature for the decolourisation of Mordant black by Con B was determined to be at 30°C (Figures 8 and 9).

pH effect

As depicted in the Table 8, A showed a maximum decolourisation of 91.1% at pH 7. Hence, the optimum pH for the decolourisation
Acid orange by the consortium, A, was 7. Significant percentage of decolourisation of Remazol black, greater than 60% was observed in the case of B between pH ranges 6 to 7. However, the highest decolourisation percentage of 95% was observed at pH 7 and hence, it was taken as the optimum pH for the decolourisation of Remazol black by B (Figure 10).

LC-MS analysis

As shown in Figure 11 the LC-MS analysis of Acid orange degradation taken at 0th hour and 24th hour. The chromatogram of Acid orange (0th hour) showed a peak at retention time of 7.9 with an m/z (mass/charge) ratio of 513.01, which corresponds to the molecular weight of Acid orange. In the chromatogram taken at 24 hour incubation, the original peak disappeared and new peaks with varied retention time appeared. Mass spectra showed two distinct peaks with m/z value of 261 and 279, which corresponds 1-[3-amino-5-[(aminoxy) sulphonyl] phenyl] ethanol of molecular mass of 259 and 7,8-amino-3-[(aminoxy) sulphonyl] naphthalene-1-ol of molecular mass of 281. As shown in Figure 12 the LC-MS analysis of Remazol black degradation taken at 0th hour and 24th hour. The chromatogram of Remazol Black (0th hour) showed a peak at retention time of 8.18 with a m/z (mass/charge) ratio of 413.16, which corresponds to the molecular weight of Remazol Black. In the chromatogram taken at 24 hour incubation, the original peak disappeared and new peaks with varied retention time appeared. Mass spectrum showed distinct peaks with m/z value of 381.16, 245.16 and 317.22, which correspond to 1',1-diazene-1,2-diylidinaphthalen-2-ol-sulphide ion of molecular mass 381, 1',1'-diazene-1,2-diyldinaphthalen-2-ol of molecular mass 245 and sodium-4-amino-naphthalene-1-sulphonate of molecular mass 317.

Discussion

The decolourisation reaction of the azo dyes by the bacteria is the structure of azo dye. Colour removal is related to the number of azo bonds in the dye molecule. Dyes with simple structure and low molecular weights exhibit greater decolourisation in the presence of bacteria, whereas colour removal is more difficult with highly substituted high molecular weight dyes [15]. In this study, total 6 bacterial colonies were screened from the 16 bacterial isolates obtained during serial dilution. Each of the bacterial colonies exhibited different decolourisation efficiency. Hence, it was inferred that the colour removal process is also dependent on the potential of the bacteria to attack the dye molecule leading to its decolourisation. In 2002, Navitan Blue S5R was tested for decolourisation with 5 different bacterial species - Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas putida, Flavobacterium multivorum and Ochrobacterium anthropi. Pseudomonas aeruginosa was the best degrader, able to degrade up to 1200 mg/l and was used for further studies [16]. Another report stated the isolation of four bacterial species that were capable of growing...
on Methyl red, of which one of them *Vibrio logei* showed the highest Methyl red degrading activity [17].

From the decolourisation assay results obtained, two consortia were developed and were used for further studies. Mixed culture or consortium has the capacity to decolourise at a higher rate in comparison to the individual bacterial colonies. This is due to the synergistic effect of the bacterial isolates in dye decolourisation. It has been stated that the individual strains may attack the dye molecule at different positions or may use the decomposition products produced by the other strain, thereby, bringing about synchrony in decolourisation of the azo dye [18]. A consortium GB containing *Galactomyces* and *Bacillus* sp. could decolourise Brilliant Blue G to a large extent [19]. The species identified in our experiment by this analysis are *Pseudomonas* sp., *Alcaligenes* sp., *Proteobacterium*, *Rhodococcus* sp, *Bacillus* sp and *Stenotrophomonas*. It has been reported earlier that *Pseudomonas aeruginosa* has the capacity to degrade Navitan Fast Blue [19]. *Acinetobacter calcoaceticum* has been reported to decolourise diazo dye, Direct Brown MR [20]. *Proteobacterium* has been reported to decolourise Indigo carmine [21]. In both the cases, the percentage decolourisation of the dye decreases with increase in dye concentration. The optimum concentration of the dye for both the consortia was zeroed in to 200 mg/l and was maintained for the rest of the decolourisation experiments. The decrease in percent decolourisation with increase in dye concentration can be attributed to reasons such as the toxic nature of the dye on the microbial cells.

### Table 7: Effect of carbon/nitrogen source on decolourisation of Remazol Black by Consortium B.

| Carbon/Nitrogen sources | % Decolourisation |
|-------------------------|-------------------|
| Maltose                 | 91.5 ± 0.55       |
| Dextrose                | 81.2 ± 1.20       |
| Galactose               | 91.7 ± 0.45       |
| Yeast extract           | 60.83 ± 0.56      |
| Lactose                 | 26.9 ± 2.93       |
| Casein                  | 74.05 ± 0.79      |
| Fructose                | 89.37 ± 1.35      |
| Starch                  | 15.05 ± 4.51      |
| Ammonium sulphate       | 4.2 ± 0.77        |
| Paddy straw             | 70.6 ± 3.29       |
| Sodium nitrate          | 42.6 ± 0.76       |
| Beef extract            | 71.6 ± 1.73       |
| Sodium acetate          | 0 ± 0.22          |
| Straw+Beef extract      | 84.8 ± 2.88       |

### Table 8: Effect of Temperature on decolorization of two dye by two consortium.

| Temperature (°C) | % Decolorization of Acid Orange by A | % Decolorization of Remazol Black by B |
|------------------|------------------------------------|---------------------------------------|
| 10               | 0 ± 1.18                           | 0 ± 0.09                              |
| 20               | 49 ± 0.728                         | 73.12 ± 2.9                           |
| 30               | 65.8 ± 1.52                        | 96.82 ± 1.18                          |
| 40               | 96.7 ± 2.12                        | 88.2 ± 1.108                          |
| 50               | 8.20 ± 1.18                        | 2.10 ± 1.42                           |
| 60               | 2.21 ± 1.86                        | 0 ± 0.08                              |

### Figure 7: Effect of Carbon and Nitrogen Source.

### Figure 8: Effect of carbon/nitrogen source on decolourisation of Remazol black by consortium B.

### Figure 9: (a) Effect of Temperature of decolorization of Acid Orange by A (b) Effect of Temperature of decolorization of Remazol Black by B.

### Figure 10: (a) Effect of pH on decolorization of Acid orange by A (b) Effect of pH on decolorization of Remazol Black by B.
of the dye [19], depletion of carbon source \textit{in vitro}, thereby pressurizing the bacteria to survive with the dye as its sole source of carbon.

Dyes are deficient in carbon source [22] and hence, it is difficult for the bacteria to utilize it as its sole source of carbon for its survival. Higher the dye concentration, the longer the time required to remove the colour. In this part of decolourisation experiment, it was inferred that static condition was the best condition for both the consortia. Though anaerobic condition showed better results, static condition was chosen for further studies owing to difficulties in maintaining the culture. During the dye reduction stage, if the extra cellular environment is aerobic, the high redox potential electron acceptor, oxygen, may inhibit the dye reduction mechanism. This is because the electron liberated from the oxidation of electron donors by the cells are preferentially used.

![Figure 11:](image)

(a) Chromatogram of Acid Orange at 0 Hour
(b) Mass Spectrum of Acid Orange at 0 Hour
(c) Chromatograph of Acid Orange after 24 Hour decolorized culture
(d) Mass Spectrum of Acid Orange after 24 Hour decolorized culture
to reduce oxygen rather than the azo dye, and the reduction product, water, is not a reductant [23]. Also, the postulated intermediates of the dye reduction reaction, which include the hydrazine form of the dye and the azo anion free radical form of the dye, tend to be reoxidised by molecular oxygen [24].

It has been stated that however, the inhibition of azo dye reduction under aerobic conditions tends only to be a temporary effect rather than an irreversible effect. If the air is replaced with oxygen free nitrogen, the reducing activity is restored and occurs at a similar rate to that which was observed under continuous anaerobic conditions. It has been suggested

Figure 11: (a) Chromatogram of Remazol black at 0th hour
(b) Mass spectrum of Remazol black at 0th hour
(c) Chromatogram of 24 hour decolourised culture of Remazol black
(d) Mass spectrum of 24 hour decolourised culture of Remazol Black e) Pathway of degradation
that if a sequential anaerobic-aerobic system is employed for wastewater treatment, the amines can be mineralized under aerobic conditions by a hydroxylation pathway involving a ring opening mechanism [25]. In both the decolourisation studies, lack of carbon supplements in minimal media did not show significant decolourisation. This can be attributed to the fact that the bacteria utilize the carbon sources to release NADH, which are the co-factors for the enzyme, azoreductase. Azoreductase brings about reductive cleavage of the azo bond in dyes, thereby causing decolourisation. Minimal media, devoid of any carbon source, hence, does not act as an effective medium for the bacteria to bring about the decolourisation of dyes. It was concluded that neither of the individual bacterial isolates nor the consortia could decolourise utilizing the dye as its sole source of carbon for its growth.

As stated earlier, dyes are deficient sources of carbon. It has been stated that the oxidation of organic electron donors and/or hydrogen is coupled to the colour removal process. The addition of electron donors such as glucose or acetate ions apparently stimulates the reductive cleavage of azo bonds [26]. The thermodynamics of the different electron donors are different and therefore, the reaction rate is likely to get influenced by the type of electron donor. It was reported that the organic nitrogen sources can regenerate NADH, which acts as an electron donor for the reduction of azo dyes by microorganisms. However, in some cases, the addition of carbon sources seemed to be less effective to promote the decolourisation, probably due to the preference of the cells in assimilating the added carbon sources over using the dye compound as the carbon source [27]. The addition of nitrogen also has another advantage in tannery industries. Ammonium salts are used in the delimiting stage, (one of the unit operations in leather processing) and ammonium salts are one of the constituent of tannery effluents. Since the tannery effluent also contains excessive amount of organic nitrogen, mainly from the hides and skins during leather manufacture, the combined effect of the effluent treatment can have considerable effect on the decolourisation by the biomass [19].

The temperature required to produce the maximum rate of colour removal tends to correspond with the optimum cell culture growth temperature as well as the optimum activity of enzyme within 35°C to 45°C. The dye decolourisation efficiency is negligible at low temperature (10°C and 20°C) due to inactive state of the enzyme. There is negligible decolourisation at high temperature (greater than 45°C). The decline in dye decolourisation efficiency is due to the presence of galactose and a combination of straw and beef extract under static condition at pH 7 and temperature of 30°C within 48 hours. The study of degradation of Acid orange shows the action of azoreductase leading to the generation of 1-3-amino 5-[(aminoxy) sulphonyl] phenyl ethanol and 7, 8-amino-3-[(aminoxy) sulphonyl] naphthalen-1-ol. LC-MS analysis on the degradation of Remazol black shows the presence of 1,1'-diazene-1,2-diyl)dinaphthlen-2-ol, formed as a result of desulphonation and sodium-4-amino-naphthalene-1-sulphonate, formed as a result of azoreductase activity. In both the studies, azoreductase was the key enzyme causing dye degradation.

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