

On Site Application of *Pseudomonas Aeruginosa* ETL-1942 and *Bacillus Cereus* ETL-1949 in Decolorization and Degradation of Remazol Black-B

Maulin P Shah*

Industrial Waste Water Research Laboratory Division of Applied & Environmental Microbiology Enviro Technology Limited Plot No: 2413/14 GIDC, Ankleshwar-393002 Gujarat, India

*Corresponding author: shahmp@uniphos.com

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Abstract In the present study an attempt was made to examine the potential of two bacterial strains for decolorization of Remazol Black-B. The strain, isolated from textile effluent treatment plant was characterized on the basis of morphological, biochemical & genotypic characteristics & it was identified as *Pseudomonas aeruginosa* & *Bacillus cereus*. The effect of pH, temperature and initial concentration of dye was studied with an aim to determine the optimal conditions. The bacterial strains used in the study were *Pseudomonas aeruginosa*. ETL-1942 & *Bacillus cereus* ETL-1949. Out of this *Pseudomonas aeruginosa*. ETL-1942 emerged out to be most potent decolorizer, being selected for further studies. The selected bacterium shows higher decolorization in static condition as compared to shaking condition. The optimum pH was 7.0. It shows good decolorization efficiency even in alkaline region. The optimum temperature was 37°C. The strain could decolorize Remazol Black-B (250 mg/l) by 94% within 24 h under static condition, pH 7.0, temperature of 37°C and initial dye concentration of 250 mg/l. Biodegradation and decolorization was confirmed using UV-VIS spectrophotometry, thin layer chromatography (TLC) and fourier transform infrared spectroscopy (FTIR) analysis. The study confirmed the potential of *Pseudomonas aeruginosa* ETL-1942 in the bioremediation of Remazol Black-B.

Keywords: *pseudomonas, bacillus, acid orange, bioremediation, static, shaking*

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1. Introduction

Effluent discharged from the textile industries has variable characteristics in terms of pH, dissolved oxygen, organic, and inorganic chemical content, etc. Together with industrialization, awareness towards the environmental problems arising due to effluent discharge is of critical importance. Pollution caused by dye effluent is mainly due to durability of the dyes in wastewater [16]. Existing effluent treatment procedures utilize pH neutralization, coagulation followed by biological treatment, but they are unable to remove recalcitrant dyes completely from effluents. This is because of the color fastness, stability, and resistance of dyes to degradation [3]. Dyes are difficult to biodegrade because of its synthetic origin and complex aromatic molecular structures which make them stable [15]. Bioremediation is the microbial clean up approach, microbes can acclimatize themselves to toxic wastes and new resistant strains develop naturally, which can transform various toxic chemicals to less harmful forms. Several reports have suggested that the degradation of complex organic substances can be brought about by bacterial enzymes

[5,9,10,23,32,34]. Different dyes used in textile industry usually have a synthetic origin and complex aromatic molecular structures which make them more stable and more difficult to be biodegraded. Due to their ease of manufacturing methodology, azo dye accounts for almost 80% of annual production of commercial dyes all over the world. There are over 10, 000 commercially available dyes with a production of over 7×10^5 tons per year [15]. Azo dyes, containing one or more azo bond (-N=N-), account for 60-70% of all textile dyestuffs used (6). It is estimated that about 10-15% of the total production of colorants is lost during their synthesis and dyeing Processes [14,22]. Whereas, in the case of reactive dyes almost 50% of the initial dye load is found in the dye bath effluents. Colored industrial effluent is the most obvious indicator of water pollution and the discharge of highly colored synthetic dye effluents is aesthetically displeasing and cause considerable damage to the aquatic life. Although several physical-chemical methods have been used to eliminate the colored effluents in wastewater, they are generally expensive, produce large amounts of sludge. More often these conventional modes of treatment lead to the formation of some harmful side products. Interest is therefore now focused on the microbial biodegradation of dyes as a better alternative [2]. Some microorganisms,

including bacteria, fungi and algae, can degrade or absorb a wide range of dyes [25]. The biological mode of treatment of dye bath effluents offers distinct advantages over the conventional modes of treatment. This method is more economical and leads to less accumulation of relatively harmless sludge. Most importantly, biological treatment of dye bath effluents is eco friendly. It causes mineralization of dyes to simpler inorganic compounds which are not lethal to life forms. The basic step in the decolorization and degradation of azo dyes is breakdown of azo bonds, leading to removal of color. Azo dyes are known to undergo reductive cleavage whereas the resultant aromatic amines are metabolized under aerobic conditions [19]. So for complete mineralization of azo dyes the microbial population forming part of treatment system should be able to work efficiently. In view of these problems the most potent bacterial culture was selected in this study for maximum decolorization of Remazol Black-B (azo dye), being selected.

2. Material & Methods

2.1. Sample Collection and Analysis

Ankleshwar Textile Industries, Gujarat is one of the most industrialized cities in India. It is chosen for effluent sample collection. The Effluent sample was collected from the middle point of the area. Standard procedures (Spot and Grab) were followed during sampling. The Temperature and pH were determined at the sampling site. The pH was determined by using pH meter (Hanna digital pH meter) and temperature with laboratory thermometer. The sample was transported to laboratory at 4°C as in accordance with the standard methods [33]. 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-1800). BOD was determined by employing evaporation method by DO meter while COD was measured by COD instrument directly.

2.2. Dyestuff and Chemicals

The textile dye, Remazol Black-B was generous gift from local textile industry, Ankleshwar, Gujarat, India and used for this study without any further purification. All the other chemicals used were of the highest purity available and of analytical grade.

2.3. Experimental Methods

The bacterial cultures were transferred to fresh nutrient medium containing Remazol Black-B (250 mg/l) and were incubated at 37°C, under static condition for 3 days. After 3 day, aliquots (5 ml) of the culture media were withdrawn, centrifuged at 10,000 rpm for 10 minutes in a centrifuge at room temperature to separate the bacterial cell mass. The supernatant was used for analysis of decolorization and all the experiments were repeated in triplicates. Absorbance of the supernatant withdrawn at different time intervals were measured at the Absorbance

maximum wavelength for the dye Acid Orange 10 (λ_{\max} =480 nm) in the visible region on a Shimadzu UV-Visible spectrophotometer (UV 1800). The percentage of decolorization was calculated from the difference between initial and final values using the following formula:

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

The bacterial strain giving maximum decolorization values was selected and used for further decolorization experiments. Changes in Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD) were also studied using Standard Methods for Examination of Water and Wastewater APHA, 1995.

2.4. Conical Flask Assay

Conical flask assay was performed for the detection of decolorizing activity of bacteria. The nutrient broth containing Remazol Black-B was autoclaved at 121°C for 15 minutes. 5% inoculums of the selected culture showing maximum decolorizing activity was added to nutrient broth flasks containing Remazol Black-B (250 mg l⁻¹). The flasks were covered with Aluminum foils and were incubated at 37°C for 3 days. The flasks were observed for decolorization of the azo dye present in the medium.

2.5. Optimization of Parameters

In an attempt to study the effect of static and shaking (130 rpm) condition, the selected, most potent decolorizing bacterial culture, was cultivated for 24 h in nutrient broth and amended separately with 250 mg/l of Remazol Black-B. To determine the effect of pH on decolorization, the fully grown culture was inoculated in conical flasks containing 100 ml nutrient broth of varying pH (5-10) and was amended with 250 mg/l of Remazol Black-B. The pH values were adjusted using 1N NaOH and 1N HCl. In the similar fashion, the optimum temperature of dye decolorization by selected bacterium was determined by evaluating the dye decolorization at 20, 30, 37, 40, 45 and 50°C. After different time intervals, aliquot (5 ml) of the culture media was withdrawn and supernatants obtained after centrifugation was used for analysis of decolorization by Shimadzu UV-Visible spectrophotometer (UV 1800), according to the methods explained earlier.

2.6. Concentration Studies

The selected culture was cultivated for 24 h in conical flask containing 100 ml nutrient broth. After 24 h the media was amended with the dye Remazol Black-B at a concentration of 100, 250, 500, 750 and 1000 mg/l separately to study the effect of increasing dye concentration on percentage dye decolorization.

2.7. Biodecolourization and Biodegradation Analysis

The analysis was done using UV-VIS spectrometry, TLC and FTIR. The supernatants obtained after decolorization were extracted with dichloromethane and dried over anhydrous Na₂SO₄ and evaporated to dryness.

The residue obtained was first examined by Thin Layer Chromatography (TLC). It is further subjected to FTIR spectroscopy.

2.8. Analytical Methods

Absorbance of the supernatant withdrawn at different time intervals were measured at the maximum absorption wavelength for the dye Remazol Black-B (λ_{max} -597 nm) in the visible region on a Shimadzu UV-Visible spectrophotometer (UV 1800). The percentage of decolorization was calculated from the difference between initial and final absorbance values.

2.9. Phylogenetic Analysis

Almost the full length of 16S rRNA genes of bacteria was amplified by PCR with following sets of primers 5'-GAGTTTGATCCTGGCTCAG-3' and 5'-AAGGAGGTGATCCA GCC-3' corresponding to the positions 9 to 27 and 1525 to 1541, respectively, in the 16S rRNA gene sequence of *Escherichia coli* [35]. PCR products were sequenced directly using ABI PRISM Big Dye Terminator Cycle Sequencing Kit on an ABI 3100 DNA sequencer following the manufacturer's instruction. Multiple alignments of the sequences were performed, and a neighbor joining phylogenetic tree [36,37] was constructed using the latest version (ver. 1.8) of the CLUSTAL W program [38]. Similarity values of the sequences were calculated by using the GENETYX computer program.

2.10. Extraction of Biotransformed Products

The supernatants after decolorization, which might contain biotransformation products of the dyes, were extracted with dichloromethane and dried over anhydrous sodium sulfate. The solvent was evaporated and the residue was first examined by thin layer chromatography. Fractions were collected and subjected to IR spectroscopy.

3. Results & Discussion

3.1. Physico-Chemical Characterization of Textile Effluent

Industrial effluent is not stable and it varies often in a wide range depending upon the process practiced. South Asian countries are experiencing severe environmental problems due to rapid industrialization. This phenomenon is very common where the polluting industries like textile dyeing, leather tanning, paper and pulp processing, sugar manufacturing, etc. thrive as clusters. Among these the Textile industries are large industrial consumers of waters as well as producers of wastewater. The effluent discharged by this industry leads to serious pollution of groundwater, soils and ultimately affects the livelihood of the poor [17]. The physico-chemical characterization of the collected textile effluent sample from Textile Industries, Ankleshwar showed a high load of pollution indicators. Colour is contributed to a water body by the dissolved compounds (dyes and pigments). The effluent color was black due to mixture of various dyes and sample was slightly alkaline when compared to the acidic pH of

the dyeing effluent in a previous study [30]. The pH of the effluent alters the physico-chemical properties of water which in turn adversely affects aquatic life, plant and humans. The soil permeability gets affected resulting in polluting underground resources of water [31]. The temperature of the effluent was high in comparison with the temperature of another effluent in one study [21]. High temperature decreases the solubility of gases in water which is ultimately expressed as high BOD/COD. The values of BOD and COD were within the permissible limits in the present sample in comparison to the very high values of BOD and COD in one effluent study. TDS and TSS values of effluent sample was high than the permissible limits. Sediments rate is drastically increased because of High value of Total Dissolved Solids which reduces the light penetration into water and ultimately decrease the photosynthesis. The decrease in photosynthetic rate reduces the DO level of wastewater which results in decreased purification of wastewater by microorganisms [13]. The current sample exhibited high values of heavy metals which was of the same order of magnitude reported in another effluent sample [20]. The nutrients of the surrounding soils are depleted as a result of high value of heavy metals thereby affecting soil fertility. High chloride contents are harmful for agricultural crops if such wastes containing high chlorides are used for irrigation purposes. [1]. Majority of the textile effluent samples have permissible limits of sulphate ions. The effluent showed phenolic contents greater than 0.1 ppm which is though permissible limit of the phenolic compounds still these compounds are very toxic to fish even at very low concentrations [12]. The bleaching and dyeing process are the main causes of pollutants which include caustic soda, hypochlorite and peroxides.

3.2. Identification of Strains ETL-1942 and ETL-1949

The results of 16S rDNA sequence alignment and phylogenetic tree analysis revealed that 16S rDNA sequence of strain ETL-1942 was 100% identical to that of Figure 1. The DNA-DNA hybridization between strain ETL-1942 and a reference strain *P. aeruginosa* JCM 5962T was 96%. The taxonomic characteristics of strain ETL-1942 were mostly the same as those of *P. aeruginosa* JCM 5962T, that is, tests for production of catalase and oxidase, reduction of NO₃ to NO₂, and hydrolysis of casein and gelatin are positive, but o-nitrophenyl-b-D-galactopyranoside (ONPG) test and hydrolysis of starch were negative for the both strains. However, strain ETL-1942 was able to hydrolyze neither lipids (supplied as tributyrin), maltose nor D-mannose, all of which were hydrolyzed by *P. aeruginosa* JCM 5962T. To verify identification of ETL-1949 as *B. cereus* or *B. thuringiensis*, this strain was characterized with phenotypic analysis. Strains ETL-1949 was positive for catalase, oxidase, positive for urease and the Voges-Proskauer reaction and did not hydrolyze starch and tween 80. *Bacillus cereus* and *Bacillus spp.* are widely distributed in nature (Drobniewski, 1993). Species of the genus *Bacillus* are rods, which sporulate in aerobic conditions. The endospores are resistant to heat, dehydration, or other physical and chemical stresses. According to The Bergey's manual of systematic

bacteriology and considering the physiological and biochemical tests performed, the strain was tentatively named as *Bacillus* sp. strain ETL-1949. To confirm the identity of the isolate, PCR amplification and sequencing of the 16S rRNA gene were done. Dendrogram (Figure 2) showing phylogenetic relationships derived from 16S rRNA gene sequence analysis of strain ETL-1949 with respect to *Bacillus* species with validly published names.

The tree was constructed using the neighbour-joining (Felsenstein, 1993). Among the described sub species, the closest relative of isolate ETL-1949 was *Bacillus cereus*. The strain ETL-1949 was a spore-forming Gram-positive rod shaped, facultative anaerobic bacterium, which was motile by means of one or two subpolar flagella. This strain grew well at various concentrations of NaCl ranging from 0 up to 9% (w/v).

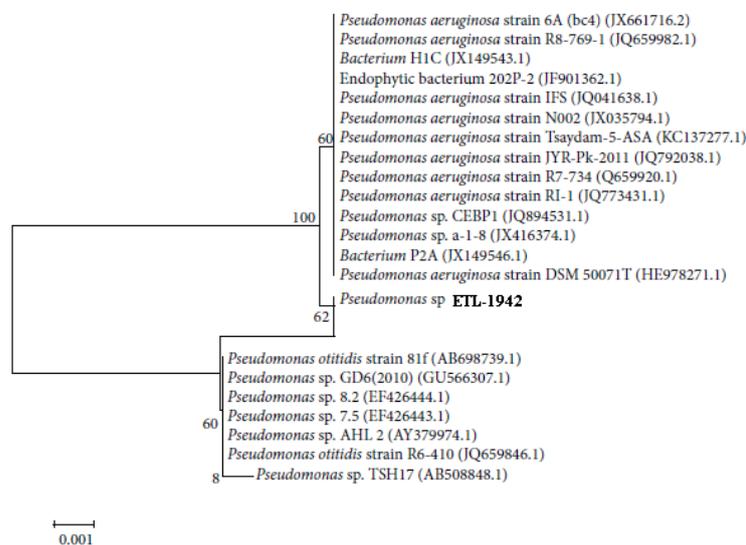


Figure 1. Phylogram (neighbor-joining method) showing genetic relationship between strain ETL-1942 and other related reference microorganisms based on the 16S rRNA gene sequence analysis

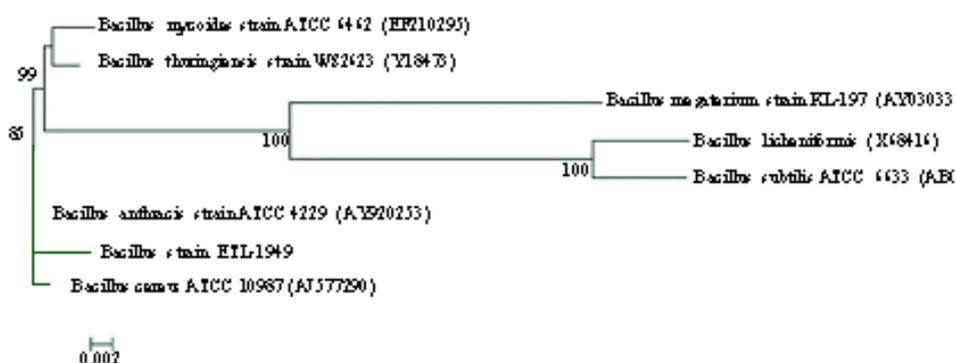


Figure 2. Phylogram (neighbor-joining method) showing genetic relationship between strain ETL-1949 and other related reference microorganisms based on the 16S rRNA gene sequence analysis

3.3. Evaluation of Optimum Conditions

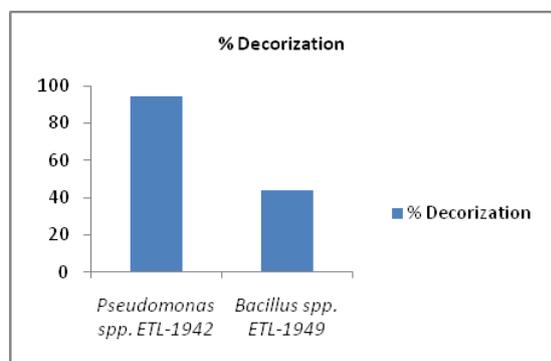


Figure 3. Percentage decolorization of Remazol Black-B (250 mg/l) by *Pseudomonas* spp. ETL-1942 & *Bacillus* spp. ETL-1949 at 370C

The dye decolorization of azo dye Remazol Black-B was studied under static condition with an initial dye

concentration of 250 mg/l using isolated bacterial culture of *Pseudomonas aeruginosa*. ETL-1942 & *Bacillus cereus*. ETL-1949. *Bacillus cereus*. ETL-1949 shows a percentage decolorization value of 48%. and *Pseudomonas aeruginosa*. ETL-1942 shows 94% decolorization (Figure 3). Thus, *Pseudomonas aeruginosa*. ETL-1942 was selected for further decolorization experiments, with getting aware of its decolorizing potential. It was observed that under static anoxic conditions, the dye decolorization of Remazol Black-B was 94% within 24 h as compared to 44% under agitation, respectively (Figure 4). Hence, Static conditions were preferred to investigate bacterial dye decolorization in further experiments. The result bears similarity with those of studies on *Pseudomonas desmolyticum* and *Pseudomonas luteola*. It was found out that under agitation conditions, presence of oxygen deprives the azoreductase from obtaining electrons needed for cleavage of azo dyes. Whereas under static conditions, these electrons are available to azoreductase from NADH

to decolorize azo dyes [7,28]. The optimum pH for *Pseudomonas spp.* ETL-1942 was pH 7.0. However, *Pseudomonas spp.* ETL-1942 was also capable of decolorizing the dye over a pH range of 7-9 with a good efficiency (Figure 5). Majority of the azo dye reducing bacterial species reported [8,18] and [29] so far were able to reduce the dye at pH near 7. The decolorization of the dye, Remazol Black-B was studied with temperature range of 20 to 50°C. The optimum temperature for *Pseudomonas aeruginosa*. ETL-1942 was 37°C (Figure 6). Considerable decrease in Chemical Oxidation Demand (COD) and Biochemical Oxygen Demand (BOD) was also observed. The values for Reduction in COD were 82% and BOD was 70% respectively (Figure 7).

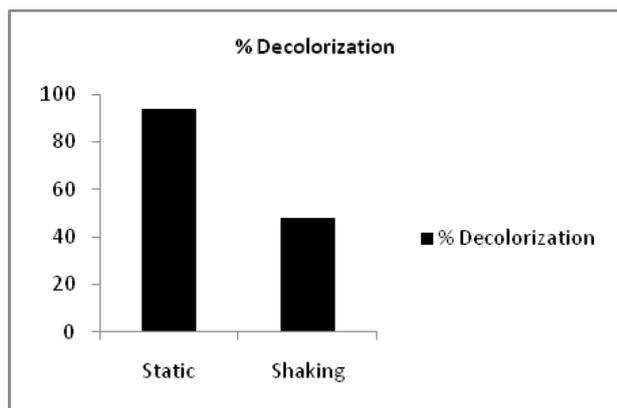


Figure 4. Effect of Static & Shaking condition on% decolorization of Remazol Black-B by *Pseudomonas aeruginosa*. ETL-1942

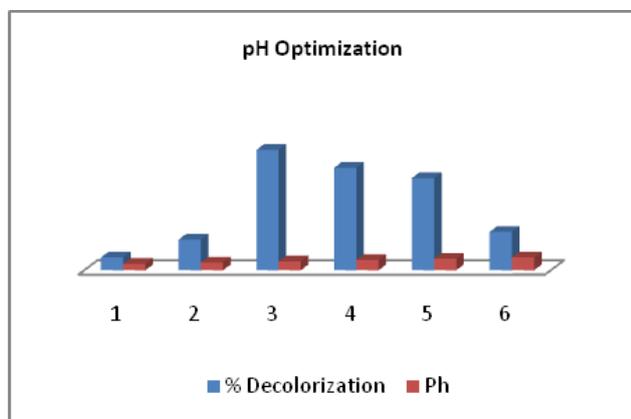


Figure 5. Effect of pH on Remazol Black-B decolorization by *Pseudomonas aeruginosa*. ETL-1942

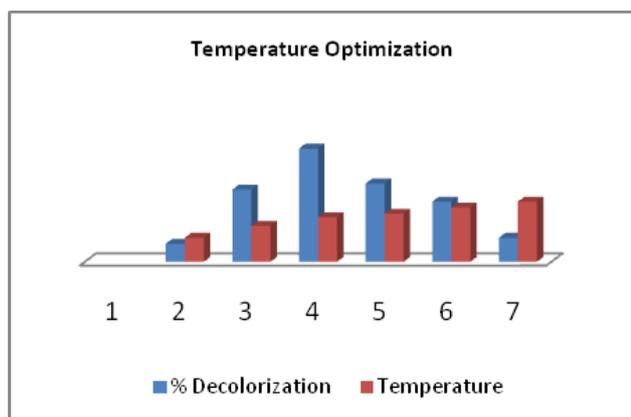


Figure 6. Effect of Temperature on Remazol Black-B decolorization by *Pseudomonas aeruginosa*. ETL-1942

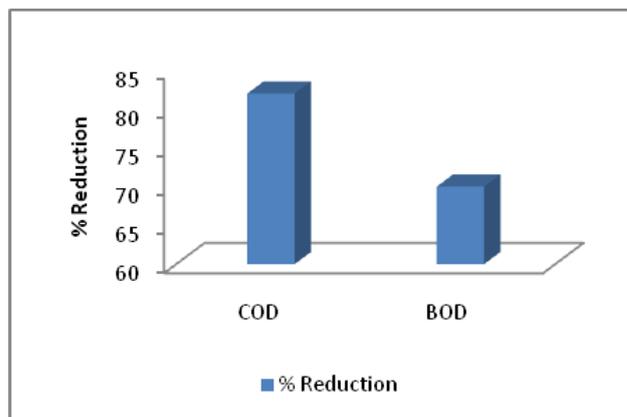


Figure 7. Percentage Reduction in COD & BOD values of dye caused by *Pseudomonas aeruginosa*. ETL-1942

3.4. Effect of Different Concentration of Dye on Decolorization

Percentage decolorization of Remazol Black-B by *Pseudomonas aeruginosa*. ETL-1942 was found to vary with initial concentrations (100-1000 mg/l) when studied up to 48 h. The 94% decolorization of Remazol Black-B was observed within 12, 16 and 24 h for the dye concentration of 100, 250 and 500 mg/l, respectively at optimum conditions of pH 7.0, temperature 37°C and under static batch study. However, for the dye Remazol Black-B concentration of 750 mg/l, maximum decolorization of 62% and for dye concentration of 1000 mg/l, only 50% of decolorization was achieved (Figure 8). This is because of the toxic nature of azo dyes. The Percentage decolorization is found to be decreasing with increase in dye concentration as evident from Figure 6. The dye decolorizing potential of *Pseudomonas aeruginosa*. ETL-1942 was quite high and it decolorizes the dye with better efficiency even at high concentrations of Remazol Black-B. *Pseudomonas aeruginosa*. ETL-1942 was able to decolorize the dye at a concentration much higher than other bacterial strains [4,11,24]. The *Pseudomonas aeruginosa*. ETL-1942 could tolerate Remazol Black-B up to 1 g/l which is in contrast to the toxic effect reported for the azo dye Remazol Black-B in concentrations within 0.037-0.051 mM [27]. This observation is of significance for bioremediation since it indicates potential of *Pseudomonas aeruginosa*. ETL-1942 to withstand high concentration of azo dye. Due to its high degrading potential, *Pseudomonas aeruginosa*. ETL-1942 could be used successfully in treatment of textile waste waters as they contain high concentration of azo dyes.

3.5. Identification of Metabolic Intermediates

Inoculation of *Pseudomonas aeruginosa*. ETL-1942 to media containing azo dyes resulted in the decolorization of the dye, Remazol Black-B. The Biodecolourization was confirmed by UV-VIS spectrum. The absorbance analyzed from 300 nm to 800 nm. The initial dye solution showed high peak at the wavelength of 597 nm. The decolorized sample showed lowering of peak to a minimal absorbance value for dye concentration 250 mg/l, which indicates that the decolorization is due to dye degradation (Figure 7). The dye degradation by *Pseudomonas aeruginosa*. ETL-

1942 was further supported by TLC analysis. The spots observed in the initial dye solution was different from the spot observed in the supernatant obtained after decolorization (Figure 9). The supernatant obtained after dye decolorization was different from the original dye which was suggested by different Rf values. This clearly indicates that decolorization was due to degradation of dyes into intermediate products.

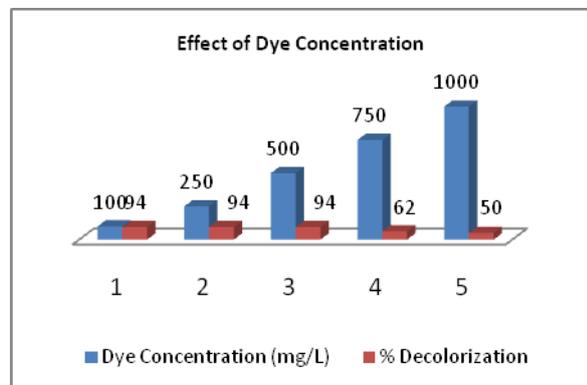


Figure 8. Effect of increasing dye concentration (Static condition) on the Percentage decolorization of Remazol Black-B by *Pseudomonas aeruginosa*. ETL-1942



Figure 9. TLC experiments shows different Rf values of control dye solution and decolorized samples.

3.6. Spectroscopic Analysis

Incubation of the azo dyes, with *Pseudomonas aeruginosa*. ETL-1942 resulted in the decolorization of the dye Remazol Black-B and the biotransformed metabolites were characterized by FT-IR. The results of FT-IR analysis of the dye Remazol Black-B and sample obtained after decolorization showed various peaks (Figure 10). The results of FT-IR analysis of Acid orange dye and sample obtained after decolorization showed various peaks. The FT-IR spectra of Remazol Black-B dye (Figure 10) displays peaks at 3483, 2929, 1660 and 1440 cm^{-1} , for $-\text{OH}$ stretching vibration, aromatic $-\text{CH}$ stretching vibration, $-\text{C}=\text{C}-$ stretching and $-\text{N}=\text{N}-$ stretching vibration, respectively. While peak near 1065 cm^{-1} is for $-\text{S}=\text{O}$, indicates sulfoxide nature of the dye. The IR spectra of degradation product displays peak at 3263 cm^{-1} for $-\text{OH}$ stretching. During the degradation of aromatic amines of Remazol Black-B there is formation of aromatic aldehyde as an intermediate which was confirmed by the spot test using 2, 4-dinitrophenyl hydrazine reagent which indicated color test due to presence of aldehyde. Besides the signal in IR at 1660 and

2929 cm^{-1} , which corresponds to aldehyde and a signal at 2869 cm^{-1} for $-\text{CH}$ stretching is similar to that of vanillin. Thus aldehyde, one of the intermediate, formed during degradation of Remazol Black-B is confirmed. The naphthalene part of the dye was further biodegraded with opening of one ring, the formation of aldehyde as one of the intermediate is confirmed from the IR data.

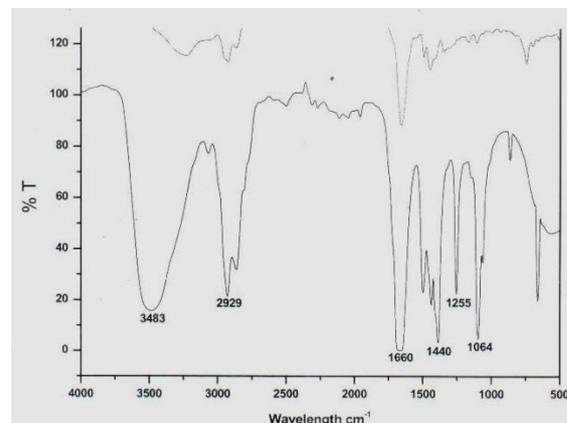


Figure 10. FTIR Spectrum of Acid orange 10 and its degradation product

4. Conclusion

The present study confirms the ability of newly isolated bacterial culture *Pseudomonas aeruginosa*. ETL-1942 to decolorize the textile dye Remazol Black-B with decolorization efficiency of 94%, thus suggesting its application for decolorization of dye bearing industrial wastewaters. Although decolorization is a challenging process to both the textile industry and the waste water treatment, the result of this findings and literature suggest a great potential for bacteria to be used to remove color from dye wastewaters. Interestingly, the bacterial species used in carrying out the decolorization of Remazol Black-B in this study was isolated from the textile dye industry waste effluent. The ability of the strain to tolerate, decolorize azo dyes at high concentration gives it an advantage for treatment of textile industry waste waters. However, potential of the strain needs to be demonstrated for its application in treatment of real dye bearing waste waters using appropriate bioreactors.

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