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Efficacy of *Rhodococcus rhodochrous* in Microbial Degradation of Toludine Dye

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Abstract

An efficacy of *Rhodococcus rhodochrous* for the treatment of toludine red was investigated by using dye as sole carbon source. Optimization of various parameters such as pH, temperature, NaCl percentage (w/v) and dye concentration was studied by using one factor at a time method. *Rhodococcus rhodochrous* was capable of decolorizing Toluidine Red at a pH and temperature range of 6.5-9.5 and 25°C-40°C respectively and the best decolourization was achieved at 25ppm dye concentration, pH 6.5, temperature 35°C and 5% (w/v) NaCl. Optimized UV-Vis and HPLC analysis proposed that decolourization was occurred under biodegradation mechanism rather than bio sorption. GC/MS analysis was carried out to identify degradation products. Final compounds formed from dye degradation appeared at m/z 193 and m/z 77 which were identified as 2-(2-carboxy vinyl) benzoic acid and Benzene respectively. Related pathway for biodegradation mechanism was proposed. Findings revealed that salt tolerant bacteria, *Rhodococcus rhodochrous* was potential for removal of Toluidine Red at salty condition.

Keywords: *Rhodococcus*; Toludine Red; Benzene; HPCL

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 dying 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 azodyes-containing effluents cause serious environmental pollution. Therefore, industrial effluents containing azodyes must be treated before discharging into the environment to remove the dye toxicity from textile effluents [12]. This study aims to investigate the potential of bacterial cultures isolated from industrial dye effluent for decolorization of a textile dye, Crystal violet. Dye decolourization by bacterial cultures was optimized with respect to various nutritional sources (carbon and nitrogen), environmental parameters (temperature, pH).

Materials and Methods

Sampling and analysis of effluent

Ankleshwar is one of the most industrialized cities in India. It is known as the chemical hub and was 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 (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 determined by employing evaporation method by DO meter while COD was measured by COD instrument directly.

Dyes and chemicals

The textile dye, Toludine Red (λ max 507 nm) was obtained from

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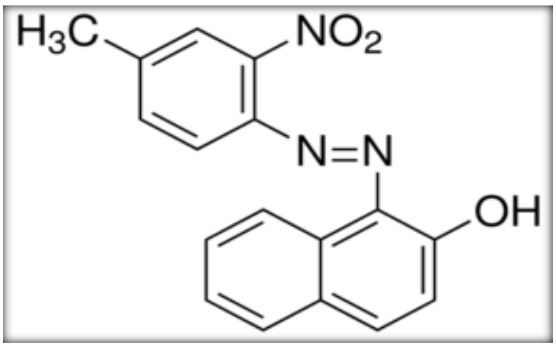

Figure 1: Chemical Structure of Toludine Red Chemical Structure of Toludine Red.

Merck. Acetonitril used in high performance liquid chromatography (HPLC) analysis was of HPLC grade. Stock solution of Toluidine Red was prepared by dissolving dye powder in dimethyl formamyde (DMF) as solvent (1mg/ml), using an ultra sonic bath. 0.25% tween 80 was added to dye stock solution due to the insolubility of the dye. The Chemical Structure of Toludine Red is shown in Figure 1.

Isolation, screening and identification

The Textile Effluent was collected in sterile collection tubes from the sludge and wastewater of the ditches at industrial site located in Ankleshwar Textile Industries, Ankleshwar. The sample collected from the textile mill was screened for Toludine Red decolorizing bacterial strains by inoculating 10ml of sludge solution into 250ml. Erlenmeyer flask containing 100ml 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 (140rpm). After 48h of incubation, 1.0 ml. Of the culture broth was appropriately diluted and plated on Nutrient Agar containing 20 mg L^{-1} Toludine Red. 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 Toludine Red. These isolates were screened for their ability to decolorize Toludine Red in liquid culture. The Screening process in liquid media was carried out by inoculating a loop full of cultures exhibiting clear zones into Nutrient broth containing Toludine Red under static conditions. After 24h of incubation, 1ml. of cell suspension was transferred to fresh nutrient broth containing Toludine Red to screen the strains with color removing ability. The Screening procedure in liquid medium was continued until complete decolourization of broth. A small amount of decolorized broth was transferred to nutrient agar plates containing Toludine Red (50 mg L^{-1}). The bacterial isolate which tolerated higher concentration of the Azo dye was isolated by streaking plate method. The Azo dye decolorizing bacteria was identified from several aspects including Molecular identification, morphology characters, biochemical tests as described in Bergey's manual of determinative bacteriology (Indole, Methyl Red, Voges-Proskauer test, Citrate, Catalase, Oxidase, Nitrate Reduction test, Hydrolysis of Casein, Starch, Urea and Gelatine). Assimilation of various sugars such as D-glucose, D-fructose, galactose, mannitol and D-maltose as sole carbon source were determined by inoculating the isolate into carbohydrate broth supplemented with respective carbon source. After inoculation the tubes were incubated at 37°C for 24-48h.

Phylogenetic analysis

The genomic DNA was extracted by using Genomic DNA isolation kit (HiPurATM Bacterial and Yeast Genomic DNA Miniprep Purification spin kit, HIMEDIA) as per manufacturer's instructions. Amplification of 16S rRNA gene and its purification was performed according to Saha and Chakrabarti [15]. For sequencing, three separate primers were used namely 27f (5´AGAGTTTGATC CTGGCTCAG 3´), 530f (5´ GTGCCAGCM GCCGCGGTAA 3´), and 1492r (5´ TACGGY TACCTTGTTACGACTT 3´). The GenBank Accession number for 16S rRNA gene of the strain BUPNP1 is KF652059. Phylogenetic analysis and tree was constructed according to Saha et al. [16]

Decolorization study

To study the decolorization, used medium was minimal medium containing following ingredients per liter: KNO_3: 5g, $(NH4)2SO_4$: 1g, $K_2HPO_4.3H_2O$: 0.87g, KH_2PO_4: 0.54g, $MgSO_4.7H_2O$: 0.2g, $CaCl_2.2H_2O$: 0.02g, $FeSO_4.7H_2O$: 0.01g, $MnSO_4.H_2O$:0.005g supplemented with 2.5%-10% NaCl and dye as sole carbon source.

Experimental process

In 35 ml tubes, 15 ml of the sterilized decolorizing medium was inoculated with 1% of 1.5×10^8 cfu ml 1 of the bacterial suspension in distilled water plus 3% (w/v) NaCl, according to the method of Asad et al. [14]. Optimization under different culture conditions was done using one factor at a time (OFAT) method with the basic condition of 25 ppm Toluidine Red, pH 8.5, 5% (w/v) NaCl and temperature 35°C. This method is based on changing one parameter at a time and maintaining the pre optimized at constant level. Different decolorization efficiencies were obtained at different dye concentrations (10ppm to 25ppm with 5ppm interval), initial pHs (4.5,6.5,7.5,8.5,9.5,10.5), NaCl percentages in decolorization media (2.5%(w/v) to 10%(w/v) with 2.5%(w/v) interval) and incubation temperatures (25°C to 40°C with 5°C interval). Adjusting the amount of pH was done with NaOH 1N and HCl 1N. Dye stock solution was sterilized separately before adding to decolorization culture and incubation was carried out at static condition. All experiments were performed in triplicate with un inoculated culture as control.

Decolorization measurement

For decolorization measurement , sample (1.5ml) of the culture media was taken at different time intervals, well agitated prior to 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 (490nm) on a UV-vis spectrophotometer (Shimdzu, Japan). Un inoculated culture media with and without added dyes were used as controls.

Metabolites extraction and analysis

UV Visible spectrophotometer was applied to monitor the decolorization process whereas biodegradation was analyzed by HPLC. Identification of metabolites was carried out by GC/MS analysis. After 10 days of incubation, 5 ml sample collected from the culture was extracted with two equal volume of ethyl acetate. The extract was dried in a rotary evaporator and the remaining solvent was evaporated at room temperature overnight. The residue was dissolved in 0.5 ml HPLC grade acetonitrile and used for HPLC and GC/MS analysis. High performance liquid chromatography (HPLC) analysis was carried out on C18 column (symmetry, 406 mm × 250 mm, Knauer, K2500,

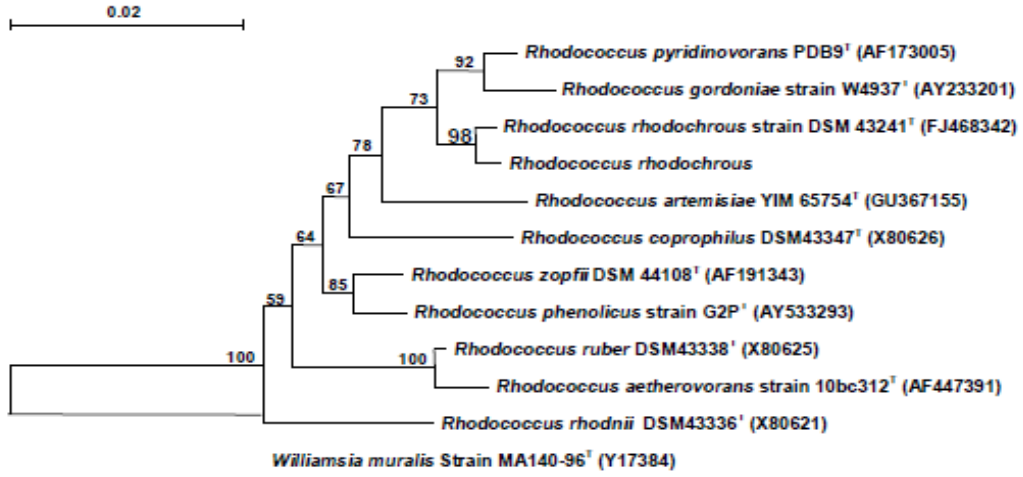

Figure 2: Neighbour-joining phylogenetic tree based on 16S rRNA gene sequence analysis.

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

Germany) using acetonitrile: water containing 0.1% formic acid (50:50) as mobile phase with flow rate of 1 ml/min for 10 min and UV detector at 254 nm at room temperature. GC/MS analysis of metabolites was carried out using a HP6890 GC coupled with a HP-5973 MS detector (Hewllet Packard, USA). Gas chromatography was done with a HP-5MS column (30m long×0.25mm i.d.) in a temperature programming mode. The injector temperature was maintained at 250°C. The initial temperature of column was 60°C kept constant for 3 min then increased up to 250°C with 10 °C /min rate and held at 250°C for 3min. Helium was used as carrier gas at a flow rate of 1 ml/min. The ionization voltage was 70 eV. Degradation products were identified on the basis of mass spectra in the wiley 275 spectral library stored in the computer software (ChemStation) of the GC/MS.

Results and Discussion

Physicochemical 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.

Phylogenetic analysis

Based on 16S rDNA gene sequence analysis, carried out using various online tools available at website of Ribosomal Data project (RDP), Release-10 *(www.rdp.cme.msu),* the strain was classified as a member of the genus *Rhodococcus* (Figure 2). The strain showed closest sequence similarity with *Rhodococcus rhodochrous*^{T} (99.5%), followed by *Rhodococcus pyridinovorans*^{T} (99.1%) and *Rhodococcus gordoniae*^{T} (98.3%). The phylogenetic analysis shows that the strain is a member of *Rhodococcus sp.* However in absence of overall genome relatedness data (+) the species status remains undetermined. Figure 2 is showing the relative position of isolates among various representative members of the genus *Rhodococcus* (having greater than 96% sequence identity). Bootstrap values (as percentages of 500 replications) are shown at nodes. Bar, 0.02 substitutions per site. The sequence of *Williamsia muralis* strain MA140-96T was used as an out group. The tree was generated using TREECON software using NJ method (Kimura model). The phenotypic properties of the strain are listed in Table 2.

Initial dye concentration effect

The utmost decolorization skill of isolated organism was investigated through testing against different initial dye concentrations (10-25 ppm, pH 8.5, temperature 35°C, 5% (w/v) NaCl) when studied up to 210 hr of incubation (Figure 3). At 25, 20 ppm dye concentrations, the highest dye removal was 75%, 70% respectively. The required time to reach maximum decolorization extent for 25 ppm dye was 144 hr whereas it was 96hr for 20 ppm dye concentration. At 15, 10 ppm of preliminary dye concentrations, the maximum decolorization was 60%, 42% respectively. Required time to reach a maximum color removal at 15 ppm dye concentration was 96 hr whereas it was 48hr for 10 ppm dye. No decolorization was observed in 5ppm of Toluidine Red due to the derisory carbon source essential for bacterial commencement and insolubility of the dye. Obtained results showed that decolorization increased with increasing initial dye concentration and increased dye concentration required more time to reach maximum decolorization extent. Similar results were also obtained earlier in case of other dyes [17,18]. Direct relationship between dye concentration and color

Morphology and biochemical tests		Sugar utilization profile			
Gram's character	+	Lactose	-	Sorbitol	+
Motility	-	Xylose	-	Mannitol	-
Endospore formation	-	Maltose	+	Adonitol	-
Catalase	+	Fructose	+	Arabitol	-
Oxidase	-	Dextrose	+	Erythriol	-
Methyl red test	+	Galactose	+	α-Methyl-D-glucoside	-
Voges-Proskaur test	-	Raffinose	+	Rhamnose	-
Nitrate reduction	+	Trehalose	-	Cellobiose	-
Indole production	+	Melibiose	-	Melezitose	+
Phosphate solubilization	-	Sucrose	+	α-Methyl-D-mannoside	-
Caesin hydrolysis	-	L-Arabinose	+	Xylitol	-
Starch hydrolysis	-	Mannose	-	o-Nitrophenyl β-galactoside	-
		Inulin	-	Esculin hydrolysis	-
		Sodium gluconate	-	D-Arabinose	+
		Glycerol	+	Citrate	-
		Salicin	-	Malonate	-
		Dulcitol	-	Sorbose	-
		Inositol	+		

Table 2: Morphology, Biochemical Test for the identification of isolate.

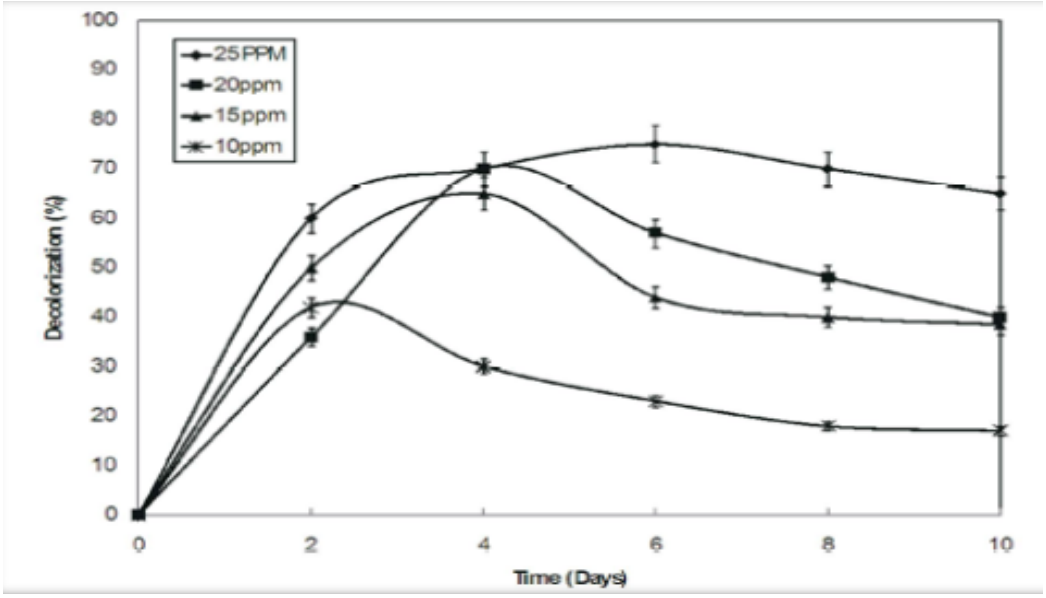

Figure 3: Dye concentration effect.

removal could be attributed to the fact that bacterial strain used dye as sole carbon source for its growth and activity. Chang and Lin showed that decolorization rate increased when initial dye concentration rose up to a certain extent and then levelled off [19]. Results clearly indicated that the best decolorization was obtained at 25ppm dye concentration therefore; 25ppm dye was used for further bacterial dye removal experiments.

pH effect

Effect of pH (4.5,6.5,7.5,8.5,9.5,10.5,35°C, 5% (w/v) NaCl) on decolorization of Toluidine Red at 25 ppm dye concentration was studied up to 240 hr of incubation (Figure 4). NaOH and HCl 1N were used for pH adjustment. The best decolorization was achieved at pH 6.5 with 80% decolorization after 96 hr of incubation. This could be due to the fact that bacterial cultures generally exhibit maximum dye removal at pH values near neutrality or slightly acidic [20-21]. *Rhdodococcus* was competent of decolorizing Toluidine Red within a wide range of pH (6.5-9.5), in contrast with common decolorizing bacteria that have a narrow pH range [14]. According to obtained results, the rate of color removal was negligible at strongly acidic (pH 4.5) or strongly alkaline (pH 10.5) conditions (30% at pH 4.5 and 33% at pH 10.5). Same result was obtained earlier with *Jadhav and Kalme* who found the best pH for decolorization of direct red 5B with *Comamonas sp*. UVS was 6.5 [22]. Guo et al. reported that the maximum ability of salt tolerant bacteria, CAS, to decolorize K-2BP was in the pH range of 6-9 and temperature range of 30-37°C [23]. Further decolorization experiments by Rhodococcus were investigated under pre optimized pH (6.5).

Temperature effect

Effect of temperature on dye removal was studied within a 25°C-

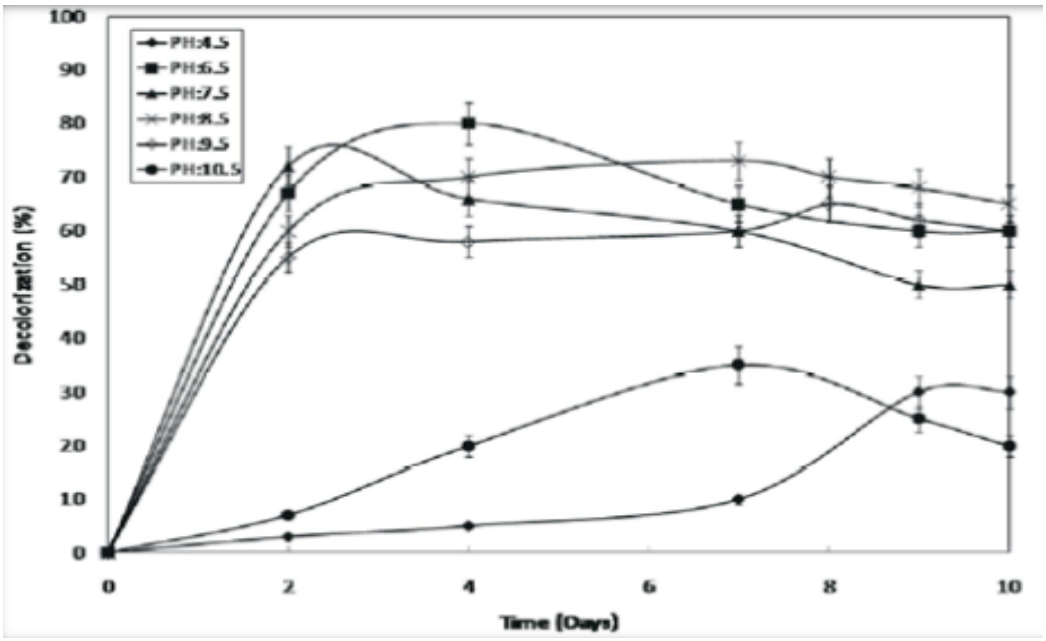

Figure 4: Effect of pH.

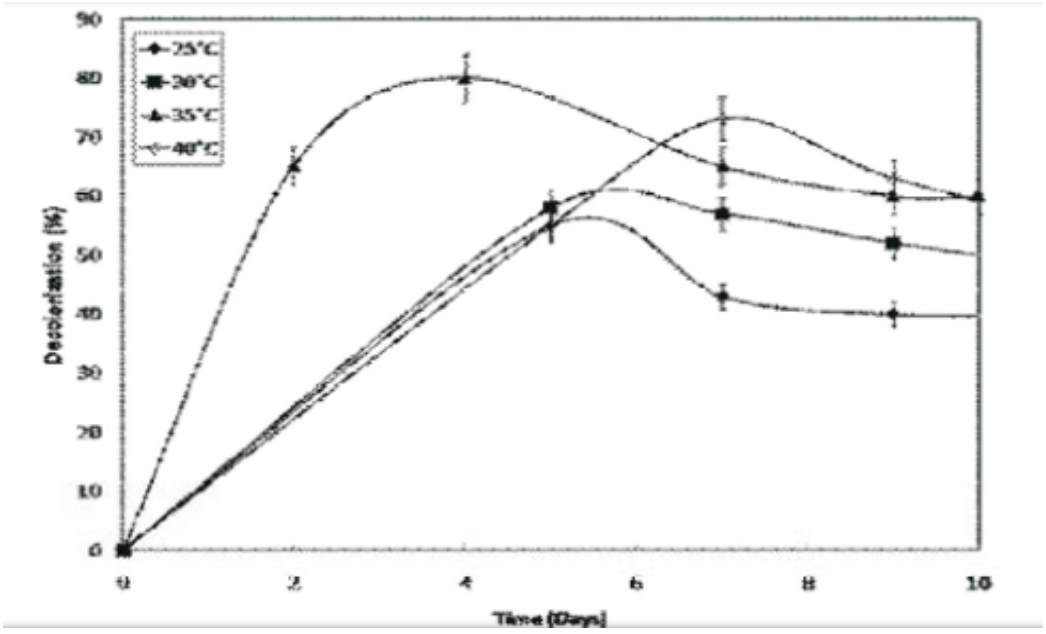

Figure 5: Temperature Effect.

40°C temperature range at obtained optimum pH and 25 ppm dye concentration with 5%(w/v) NaCl (Figure 5). Results denoted that the maximum decolorization extent was obtained at 35°C (80%) but the difference of decolorization percentage was not significant between 40°C and 35°C. Interpretation showed that when temperature increases from 25°C to 35°C decolorization of Toluidine Red increased and then levelled off slowly. Reason behind this could be due to the increase of bacterial growth and enzyme activity with temperature. Same results was obtain with Kolekar in bio treatment of disperse blue 79 and acid orange 10 by Bacillus fusiform is KMK5 [24].

NaCl effect

Performance decolorization of isolate in the presence of NaCl could be due to the shortfall of cell activity or their different salt concentrations (2.5 %(w/v)-10 %(w/v) with 2.5 %(w/v) Interval, pH 6.5, temperature 35°C, 25 ppm Toluidine Red) was tested. Results

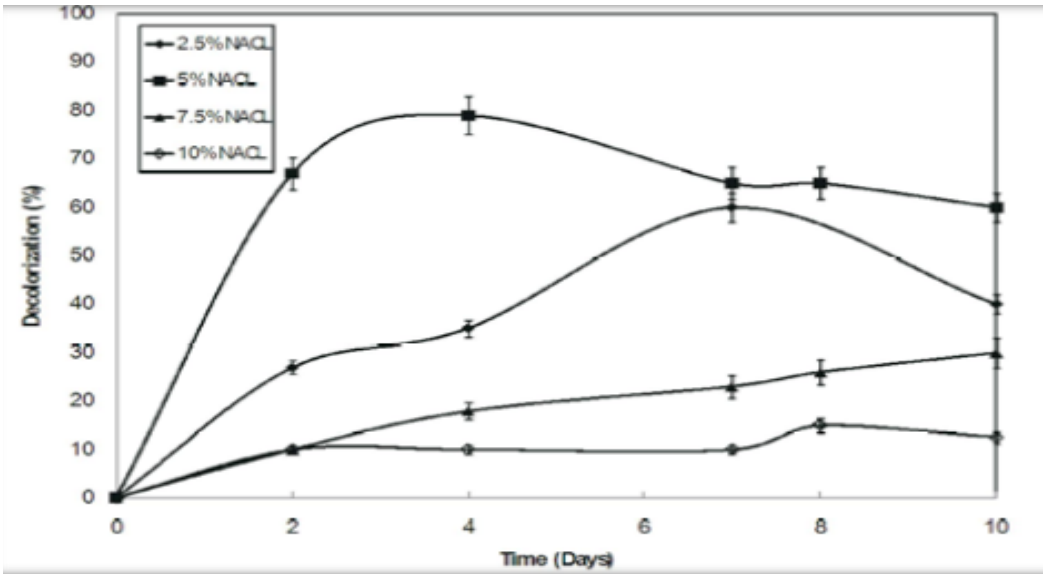

Figure 6: Salinity Effect.

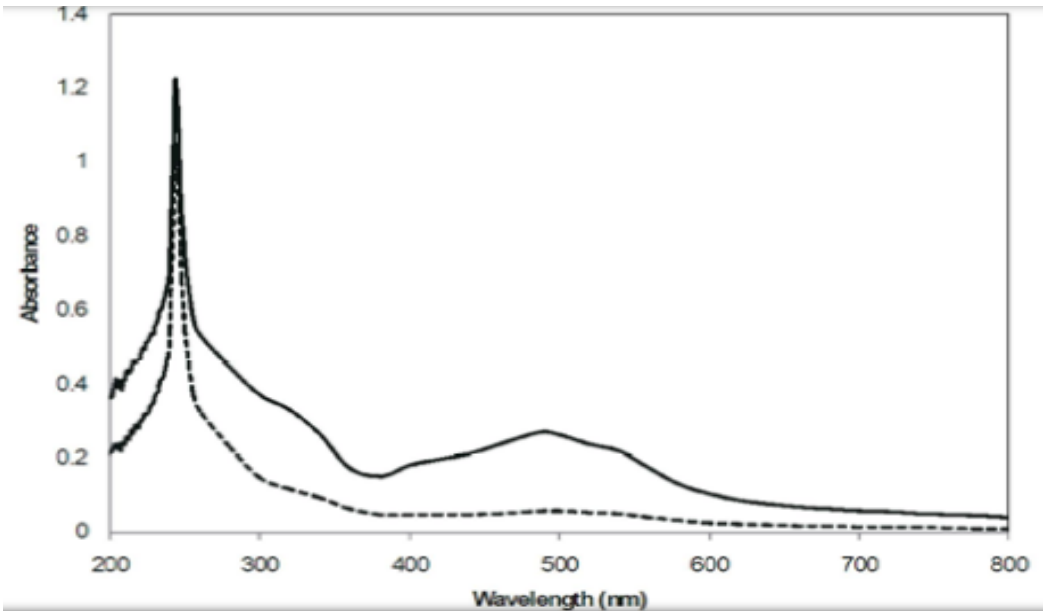

Figure 7: Changes in UV-visible spectra of Toluidine Red (490nm) before and after de-colorization at optimum condition: 0h (—) and 96h (-----).

depicted that decolorization of this strain increased due to the raise in salinity of the culture from 2.5% (w/v)-5 %(w/v) and then levelled off (Figure 6). Similar observation was reported earlier by Guo et al. in bio treatment of Reactive Brilliant Red K-2BP with salt tolerant bacteria, CAS [23]. As shown in Figure. 6 decolorization at 2.5 % (w/v) NaCl reached to its maximum percent (60%) After 7 days of incubation and the final decolorization at 7.5 %(w/v) NaCl was only 30%. Decreasing in decolorization rate at 7.5% (w/v)-10 %(w/v) NaCl could be due to the loss of cell activity or their plasmolysis. Bacterial dye reduction was optimum at 5 % (w/v) salinity in which 80% decolonization was observed after 96hr of incubation. The capability of strain D in dye removal even at high salinity (up to 5 %(w/v) suggested that D2 strain could be used for bio treatment of the effluents with salty condition.

Decolorization mechanism

Decolourization mechanism 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 7. According to Hui Wang et al. [21] 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 Toluidine Red was decolorized, its absorbance peak at 490 nm in the visible region completely disappeared and only 203 nm peak in

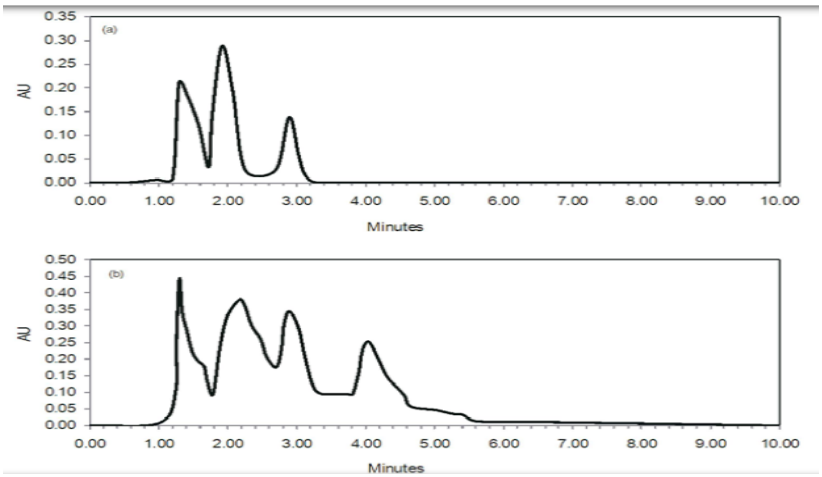

Figure 8: HPLC analysis of (a) Toluidibe Red (b) and products formed after degradation at optimum condition.

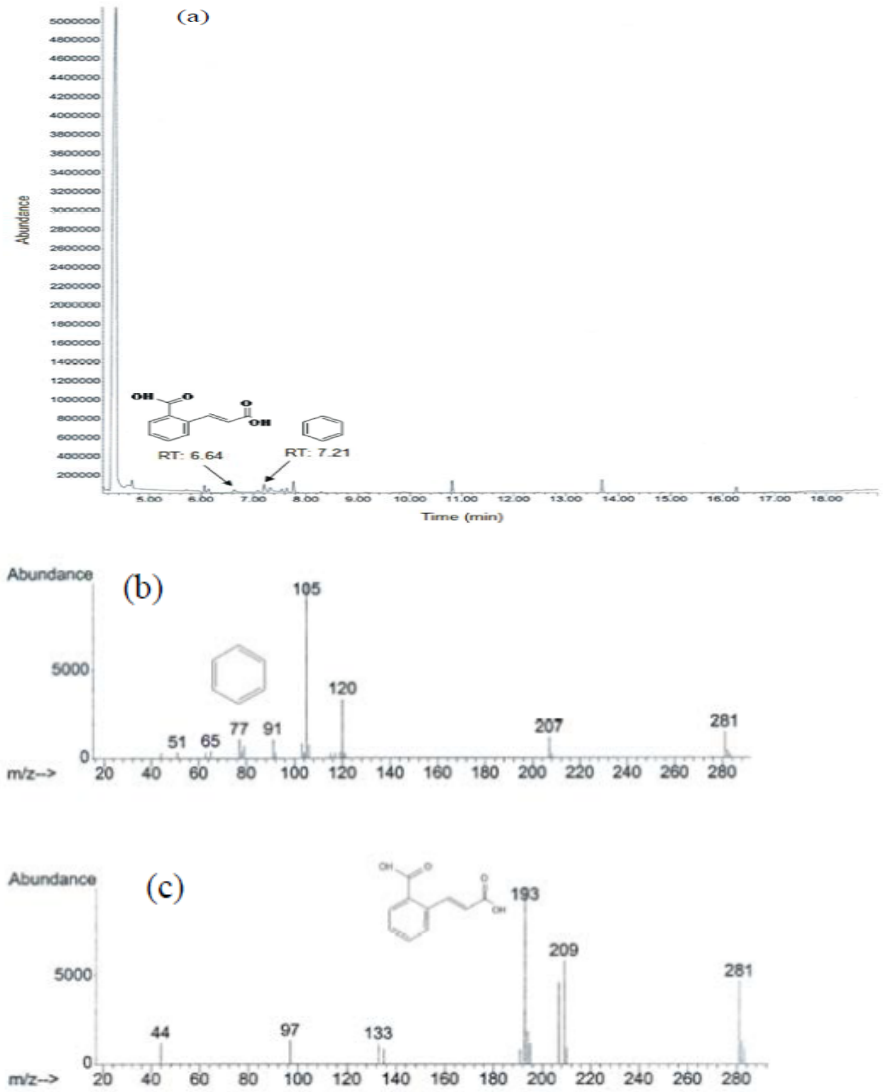

Figure 9: Decolorization products of Toluidine Red (a) Total ion chromatogram of GC/MS analysis (b) Benzene (c) 2-(2-Carboxy-vinyl)-benzoic acid.

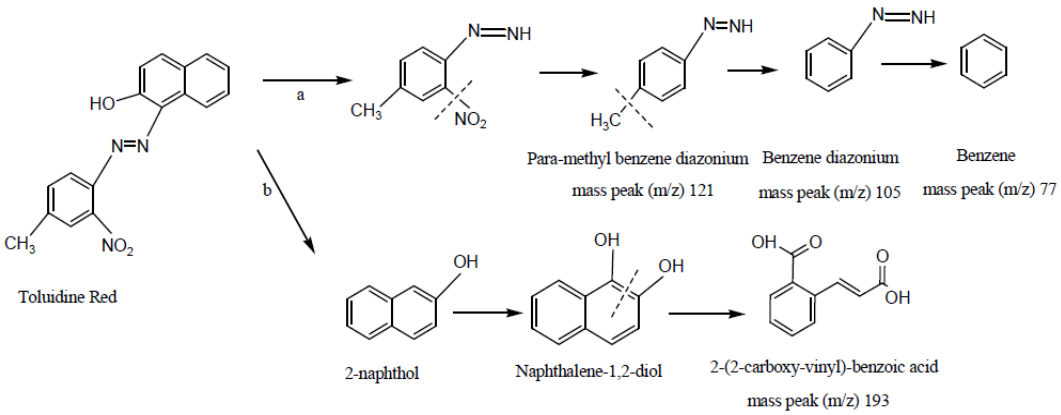

Figure 10: Suggested pathway for biodegradation of Toluidine Red.

the uv range remained indicating that decolorization of Toluidine Red was occurred under degradation mechanism rather than surface adsorption.

Biodegradation analysis

In order to keep an eye on biodegradation product of Toluidine Red, HPLC analysis was done. The HPLC analysis of control sample showed two major peaks at 1.911 min and 1.2 min retention time and a minor peak at 2.887 min retention time. As decolorization completed, the emergence of some new peaks were observed due to biodegradation of parent dye with three major peaks at 1.295 min, 2.172 min and 2.888 min and one minor peak at 4.046 min retention times (Figure 8). Forming of new peaks in comparison to control sample revealed that chemical structure of the azo dye was altered due to the bacterial enzymatic activity and new metabolites were produced in culture supernatant after decolorization process. GC/MS analysis was carried out to identify biodegradation metabolites. Extracted sample with ethyl acetate was injected in to a GC/MS. Total ion chromatogram of Toluidine Red and proposed pathway for biodegradation is shown in Figures 9 and 10 respectively. According to the figures the peak having a retention time of 7.21 min corresponds to para methyl benzene diazonium (m/z 121) after the main degradation of Toluidine Red and then giving up NO_2 group. Cleavage of azo dyes can take place symmetrically or asymmetrically. In this study the main degradation of Toluidine Red was occurred asymmetrically by cleavage between the nitrogen of azo group and the carbon of 2-naphthol ring. The resulted fragment (m/z 121) was subjected to degradation by breaking methyl bond to give rise of m/z peak of 105. Finally benzene fragment at m/z 77 have been obtained from benzene diazonium (m/z 105) by cleavage of azo group (Figures 9b and 10a). The peak having a retention time of 6.64 min was identified as 2-(2-carboxy vinyl) benzoic acid with m/z 193 which was another degradation product of Toluidine Red (Figure 9c and Figure 10b). Results clearly showed that Rhodococcus was able to degrade Toluidine Red due to its enzymatic activity. There was no mass peak in mass spectra attributed to degradation or consumption of tween 80 asco-substrate by microorganism so increasing dye solubility in decolorization media was the main function and role of tween 80 in this study.

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