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Biodegradation of Azo Dye Reactive Red BL by Alcaligenes Sp. AA09

[1]Ajay Kumar Pandey, [2]Vinay Dubey
[1, 2,] *(Department of Biotechnology, School Of Bioengineering, SRM University, Kattankulatur, Chennai, Tamilnadu 603203, India)*

Abstract- *A dye-decolorizing bacterium strain was isolated from activated sludge of a textile printing wastewater treatment plant of Perundurai. The strain was identified as Alcaligenes sp. AA09 based on Gram staining and sequence analysis of 16S rDNA gene. Alcaligenes sp. AA09 decolorized more than 90% of the azo dye reactive red BL within 24 h under aerobic condition at pH 7.0 and temperature 25^{0}C with 50-200mg/l dye concentration. A 60Kd NADH/NADPH-dependent, oxygen sensitive, soluble cytosolic enzyme, azoreductase was responsible for degradation of reactive red BL azo dye. The bacterial dye degradation was confirmed by analysis of the degraded products using TLC and GC-MS analysis.*

Key Words- *Biodegrdation, Reactive Red BL, Aerobic, Decolorization, Azo dyes, Azoreductase, phytotoxicity*

I. Introduction

Global industrialization has resulted in the release of large amounts of potentially toxic, mutagenic and xenobiotic compounds into the biosphere [1-2]. Cleaning up of the environment by the removal of hazardous contaminants is a crucial and challenging problem needing numerous approaches to reach long-lasting suitable solutions [3]. The textile industries are using synthetic dyes with ease of production, fastness and variety in colour compared to natural dyes and daily discharging millions of liters of untreated effluent containing harmful dye wash into receiving water bodies posing serious health problems [4-6]. An average textile mill produces 60 x 10^4 m of fabric and discharges approximately 1.5 million liters of effluent per day in India [3-9]. Azo dyes, with a total annual production 7 x10^5 metric tons worldwide production are commercially available and are typically 5-10% of this amount is discharged into environment which is usually recalcitrant to conventional wastewater treatment methods [10]. Presence of dyes in aqueous ecosystem diminishes the photosynthesis by impeding the light penetration into deeper layer thereby deteriorating the water quality and lowering the gas solubility [11-13]. To avoid these problems, the effluent from textile industries must be treated before their discharge. During the past three decades, several physical, chemical and biological decolorization methods have been accepted by the paper and textile industries [12-15]. Wide range o microorganisms including bacteria [9], fungi, yeasts, actinomycetes and algae capable of degrading azo dyes have been reported of them bacterial and fungal cultures have found to cause decolourization of various azo dyes to a great extent [15-16]. Azoreductase enzymes responsible for decolourisation of azo dyes are purified from several bacterial strains [16-18].

The present investigation was aimed to exploit the biodegradation abilities of indigenous microbial flora for remediative purposes. In the present study we screened and isolated bacterial strains organisms for the ability to decolorize azo dyes present in the textile effluents as well as the commercially available azo dye reactive red BL. The dye degraded products after the microbial treatment would be analyzed by TLC and GC-MS. Further the study of enzyme involved in dye degradation and partial purification of the same was carried out.

II. Materials And Methods

2.1. Dyes and Media

Azo dye Reactive Red BL was provided by Department of textile industry, Anna University, Chennai, India. All media components and chemicals used in the studies were of analytical grade.

2.2. Sample Collection

The textile dye effluent samples were collected from Common effluent treatment plant, Perundurai in sterile plastic containers. The samples were brought to the laboratory and processed within 48 h.

2.3. Isolation of bacteria from Textile effluent

Serial dilution of textile dye effluent sample has been done from 10^{-1} to 10^{-6} by using distilled water. One hundred µl of effluent sample was inoculated on LB medium by using sterilized glass rod spreader and incubated at 25^{o}C for 48 h. After 48 h of incubation morphologically distinct colonies were picked and transferred in 1 ml LB broth and incubated at 25^{0}C for 48h.The pure culture was prepared and stored at 4^{0}C for further studies.

2.4. Decolorization of textile dye effluent by isolated bacterial strains

For checking the dye effluent decolorization by bacterial strains, decolorization assay was performed, in which the bacterial colonies from pure culture were transferred to 25 ml LB broth and allowed to grow for 48h at 37^0C. The effluent was mixed with the bacterial culture at 1:1(v/v) and 3:1(v/v) ratio and incubated at 30°C under shaking conditions for 48 hrs. Uninoculated effluent with broth served as control.Decolorization was determined in 5 ml aliquots from each flask after 48 hrs of incubation. The culture was centrifuged at 10,000 rpm for 15 min to remove the cells. The absorbance of the supernatants was measured at 533 nm using spectrophotometer at regular intervals of 1h during the decolorization process.

2.5. Enrichment of dye decolorizing bacterial strains

The bacterial strains capable of decolorizing the textile effluent efficiently were enriched by growing into conical flasks containing 100mL (pH 7.0) of MSM medium (gL^{-1}): (Na_2HPO_4 , 1.264; KH_2PO_4, 0.326; NH_4Cl, 1.0; $MgSO_4$, 0.098; $CaCl_2$. $2H_2O$, 0.044; $FeSO_4.7\ H_2O$, 0.01 and NaCl, 0.1) amended with 50mg/L dye as the sole source of C and N. The MSM medium containing dye were inoculated with the strains that showed efficient decolorization and incubated at 25^0C for 24 hrs under static condition, cell suspension from each flask were plated onto MSM agar medium and incubated at 30^0C for 48 hrs. Morphologically distinct colonies were selected and were purified by streaking twice on LB agar medium. Single colony were transferred on to agar slant and stored at 4^0C for further studies [8].

2.6. Dye decolorization assay

The decolorization assay of azo dye, Reactive Red BL was carried out in 100ml MSM medium at 200 mg/L dye concentration with inoculum concentration 10% v/v at 25°C and 150 rpm. Two types of control were used, uninoculated sterile medium supplemented with dye (abiotic control) while in other inoculated medium in which dye has been omitted (biotic control). After incubation at shaking condition to a sampling time 3h aliquot (2ml), culture media was withdrawn, centrifuged at 10,000 rpm for 10 min to separate the bacterial cell mass and clear supernatant.Decolorization of dyes was determined by monitoring the decrease in absorbance at the maximum absorption wavelength ($\lambda_{max.}$)at 518 nm for Reactive Red BL in an UV-Visible scanning spectrophotometer. Decolorization activity was calculated by applying the following formula [2]:

$$\text{Decolorization (\%)} = \frac{A_0 - A_t}{A_0} \times 100$$

A_0- Initial Absorbance

A_t – Observed Absorbance

2.7. Effect of different concentrations of dye on decolorization

To check the efficiency of dye decolorization by the bacterial isolate in question decolorization assay was carried out at different concentration of dye Reactive Red BL, 50 mg/L, 100 mg/L, 200 mg/L, 300 mg/L, 400mg/L, 500 mg/L. Samples were withdrawn at the interval of 3h and subjected to centrifugation at 10,000 rpm and supernatant was used to determine the decolorization of dye by UV-Vis spectrophotometer at 518 nm and the % decolourization was calculated [2].

2.8. Effect of incubation conditions on dye decolorization

In a conical flask 100ml LB broth, 200mg/L dye and inoculum (10% v/v) were added. One set of flasks were incubated at 25^OC under static conditions and the other at same temperature on an orbital shaker at 150rpm. Appropriate controls were used as mentioned above. Samples were withdrawn at regular interval of 3h, centrifuged at 10,000 rpm for 10 min and the absorbance of supernatant was read at 518 nm in a spectrophotometer and the % decolorization was calculated.

2.9. Effect of yeast extract as a co substrate

In this experiment dye concentration was gradually increased from 100 mg/L to 500 mg/L while yeast extract concentration decreased from 400 mg/L. To the flask containing 500 mg/L dye, no yeast extract was added. Two controls have been used, one is abiotic without inoculation while other with 500mg/lit yeast with inoculation in which dye has been omitted. The inoculated flasks were incubated at 25^OC in stationary condition. Concentration of dye has been determined by taking absorbance at 518 nm in regular intervals in a spectrophometer and decolorization calculated [19-20].

2.10. Effect of pH on decolorization

For determination of pH dependence of bacterial bio-decolorization, medium was supplemented with Reactive Red BL dye at low concentration of dye 200 mg/lit. The medium pH was adjusted between 5.0 to 9.0 with hydrochloric acid and sodium hydroxide and inoculated with 10% (v/v) bacterial culture and incubated at 25^{O}c temperature. Samples have been collected from regular time intervals up to 24 hrs. Decolorization % was calculated based on the OD values at 518 nm [21].

2.11. Identification and characterizationof Dye decolorizing bacterial strain

The dye decolorizing bacterium was found to be a Gram-negative, rod-shaped and motile. For identification of bacterial strain the genomic DNA was extracted from culture [17-18]. The universal primers were used and PCR amplification was performed in total reaction volume 20 µL. The PCR programming used were 94 °C for 5 min, 30 cycles at 94 °C for 1 min, 54 °C for 1 min and 72 °C for 3 min and final extension was at 72 °C for 10 min. The amplified PCR product was detected on 0.8% agarose gel in TBE buffer. Nucleotide sequences were determined by DNA sequencing method. The sequences of 16S r DNA gene of the bacterial strain were subjected to BLAST search tool in gene database of NCBI.

2.12. Azoreductase activity in crude protein extract

The culture was grown in minimal medium containing 25mg l^{-1} of Reactive Red BL until the dye was completely decolorized. Cells were harvested by centrifugation, washed with 50 mM phosphate buffer (pH 7) and resuspended in the same buffer. Cells were then disrupted by sonication (four cycles of 15 s, 75% amplitude;), and cell debris was removed by centrifugation at 4°C. Supernatant was used as the crude protein extract. The enzyme assay mixture was assayed as consisted of 50 mM phosphate buffer (pH 7), 2 mM NADH, 20 µM Reactive Red BL, and 0.1 ml of crude protein extract in a total volume of 1 ml. Dye decolorization was monitored spectrophotometrically at 518 nm. Azoreductase activity in the presence of NADPH was measured in a similar manner by the addition of 2 and 0.2 mM of NADPH, to the assay mixture. Enzyme activity was expressed in units of micromoles of dye reduced per minute. Protein content of the crude protein extract was determined by Lowry's method using bovine serum albumin as standard for determination of specific activity, i.e., enzyme units per milligram protein.

2.12. Partial purification of Azoreductase

The enzyme responsible for dye decolorization has been partially purified by a combination of different purification processes. Crude extract was subjected to ammonium sulphate ($(NH_4)_2SO_4$) precipitation at 40% saturation to remove impurities, followed by 60% saturation in a second step to precipitate the azoreductase. The precipitated proteins from the second precipitation step have been collected by centrifugation, and the pellet has been dissolved in 30 mL of phosphate buffer (50 mM, pH7.0).The precipitated proteins were desalted by dialysis against phosphate buffer (50 mM, pH7.0) overnight. The desalted solution after dialysis has been filtered through a 0.45 µm filter and was subjected to anion exchange chromatography using DEAE cellulose as column bed in 1.5 cm diameter with bed length 10 cm and applied 1.5 mL aliquots of the resulting solution to the column. Protein was eluted at 5.4 cm/h. with the sample buffer and with step wise increasing concentration of buffer containing NaCl with range 0-200 mM and highest concentration up to 1 M. The 1.5 mL fractions were collected for each concentration of NaCl. The protein concentration and enzyme activity has been determined at every steps of purification.

2.12. SDS-PAGE

In order to ascertain the presence of the enzyme, SDS PAGE was carried out using the sample. Resolving gel and Stacking were prepared and poured into the gel apparatus and allowed to polymerize. Sample buffer (β-merceptoethenol and bromophenol blue) was added into sample with volume 1:5 and the mixture was heated at 100 °C for 2 min and then spun down for a few seconds and then loaded in the wells of acrylamide polymerized gel in gel apparatus. The marker was also loaded in separate well to determine the molecular weight. The electrodes were attached to the gel apparatus and power supply was turned on at 150 V. Electrophoresis was carried out at a constant voltage until the dye reaches about 1 cm from the end of the gel. Power was turned off and the gel was taken off with the help of spacer. The gel was submerged in staining solution and allowed the gel to stain overnight. Gel was then placed in destaining solution to remove background stain. Protein bands were clearly visible after destaining.

2.13. Extraction and analysis of degraded product

The bacterial strain was inoculated in minimal medium containing 100 mg/l of Reactive Red BL and incubated under ambient conditions. After complete decolorization, 40 ml of dye degraded samples was taken and centrifuged at 10000 rpm for 10 min. Then the supernatant was filtered through Whatman #1 filter paper. Filtrate was then extracted three times with diethyl ether pooled and evaporated the extracted product to dryness [21].The degraded products were analyzed by TLC and GC MS. For TLC the solvent system butenol: water: acetic acid = 5:3:2 was poured it into the TLC tank. The extracted sample was dissolved in 0.5 ml of methanol and was loaded on TLC plate of 3.5 × 5.0 cm and allowed to run in a TLC tank. After the run was complete the TLC plate was taken out and air dried. The plate was observed under UV light and the R_f values of the bands were recorded. The extracted metabolites were also analyzed by the Gas chromatography-mass spectrometry carried out with JEOL GC Mate-II fitted with HP5 column.

III. Results and Discussion

3.1. Screening and Identification

The textile dye effluent sample of common textile effluent treatment plant and textile industries from Perundurai and Erode area have given morphologically distinct colonies were picked up, showing decolorization zone on LB agar plates containing Reactive Red BL dye. The isolates were used for textile effluent decolorization assay and the maximum decolorization showing isolate was used for the decolorization in conical flasks. The efficiency of decolorization was calculated. The selected bacterial strain 006/a/PP/I* has shown maximum efficiency in decolorization of the mixture of textile effluent. The flask which contained effluent and broth in 1:1(v/v) ratio showed more decolorization in comparison to 3:1 (v/v) ratio which was 81.1 % and 46 % respectively after 24 h of incubation (Fig.1).

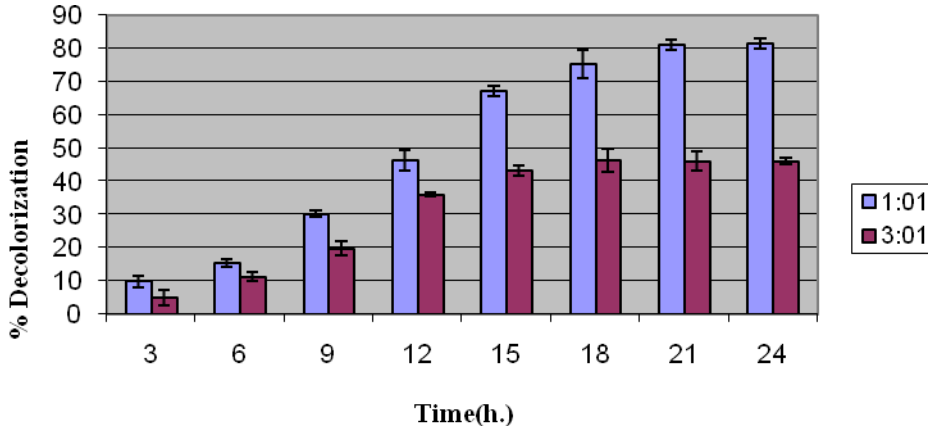

Fig.1. Textile Effluent Decolorization assay

The effect of incubation conditions namely shaking and stationary condition on decolorization of Reactive Red BL by 006/a/PP/I*, revealed that static condition was more suitable for decolorization, where the activity was found to be 95% and at shaking condition it was 84.83%. . The data suggest that in static condition is more appropriate for the decolorization of the dye by the bacterium (fig.2).

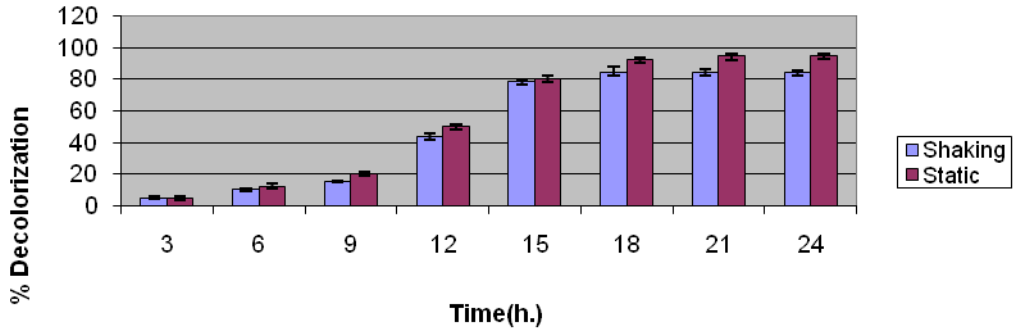

Figure.2. Effect of Shaking and static condition on dye decolorization

Different concentrations of azo dye Reactive Red BL, ranges from 50,100,200,300,400,500mg/lit has been taken for the determination of the effect of dye concentration on the decolorization. In 48 hrs it has been found that with increase in dye concentration the dye decolourizing efficiency of the bacterial strain decreases. The maximum decolorization was found on 50mg/lit concentration and minimum decolorization was found on 500mg/lit concentration (fig.3).

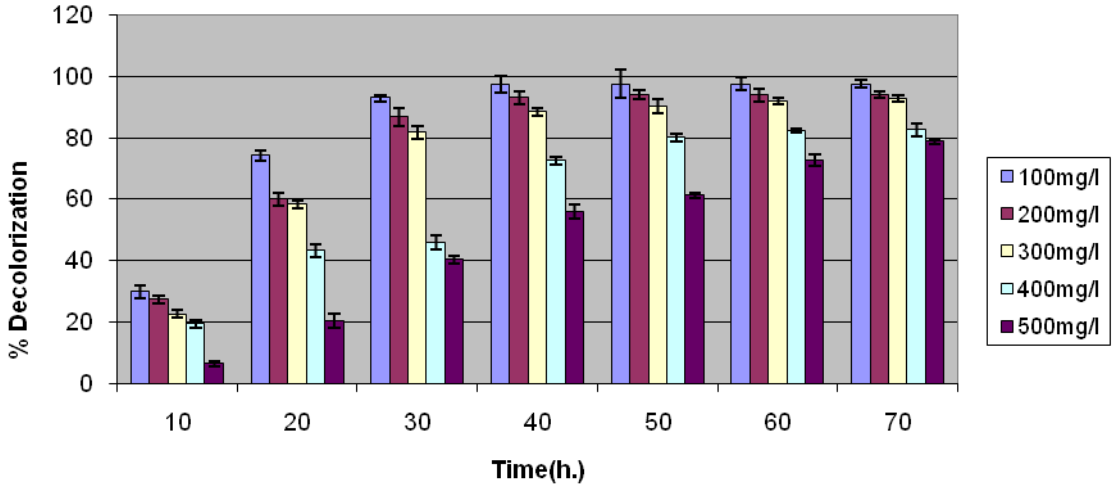

Figure.3. Effect of dye concentration on decolorization efficiency of Alcaligenes sp. AA09

Different concentrations of yeast extract (100–500 mg/l) have been utilized the determination of effect of yeast extract as co substrate for decolorization efficiency of bacterial strain (Fig 14). With increase concentration of azo dye (0-500mg/lit) and decrease concentration of yeast extract (500-0mg/lit) the time taken for decolorization was found to be 72hrs. At 500mg/L dye concentration and in absence of yeast extract the decolorization after 70 h was found to be 78.91% suggesting that the organism could utilize azo dye as a sole C-source (fig.4).

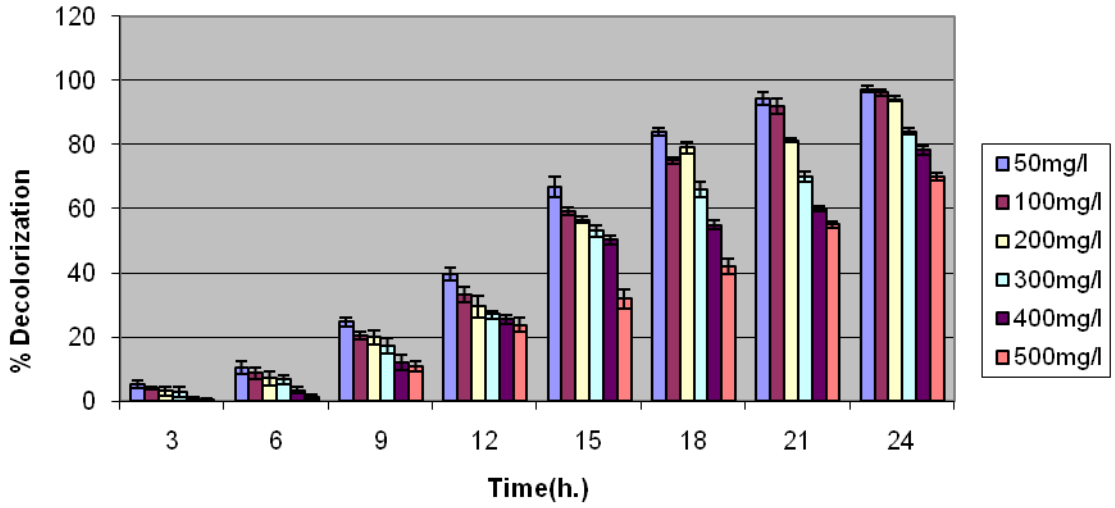

Figure.4. Utilization of Azo Dye Reactive Red BL as Sole C-source

Effect of pH on decolorization indicated that the bacterial culture generally exhibited maximum decolorization rate at pH values near 7 (Fig 5). An increase in pH from 5.0 to 9.0 did not show any marked changes in the values of percentage decolorization indicating that the organism could be used in treatment of effluents which are either acidic or alkaline. The optimum pH for decolorization of azo dye Reactive Red BL however remains 7.0.

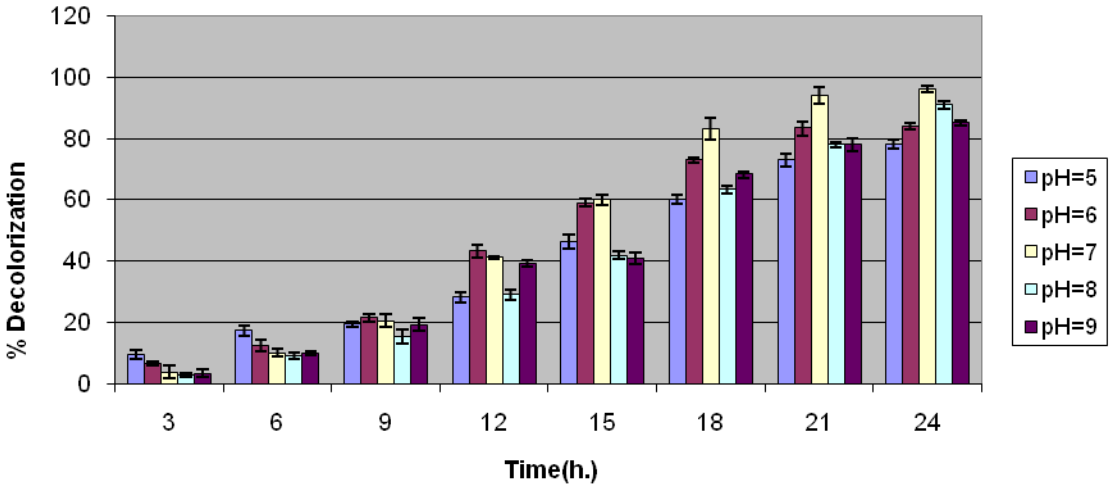

Figure. 5. Effect of pH on the decolorization of azo dye Reactive Red BL

3.2. Identification of dye decolorizing bacterial strain

The dye decolorizing bacterial strain was identified by 16S rDNA gene sequence analysis of 387 base pair and it was designated as *Alcaligenes* sp. AA09.

3.3. Determination of azoreductase activity

Crude protein extract obtained from *Alcaligenes* sp. AA09 cells was found to decolorize Reactive Red BL dye using NADH as electron donor. The absorbance of the enzyme assay mixture was taken at 518nm by using spectrophotometer at the 5 min of interval. The absorbance was found to be decrease, which indicates that the NADH is working as coenzyme for Azoreductase. By using Lowry method the protein concentration was found to be 180µg/ml in crude extract and the enzyme activity was found to be 0.025 U/mg. Azoreductase enzyme was visualized from the crude protein extract on a zymogram. The development of zymogram was based on the decolorization of Reactive Red BL stained gel by azoreductase in presence of NADH, which could be seen as a clear band against a red background. The enzyme involved in dye decolorization was partially purified by a combination of purification methods by using ammonium sulphate (70% concentration) precipitation (0.067 U/mg; 140µg/ml), dialysis (0.072 U/mg; 120µg/mL), anion exchange column chromatography (0.081 U/mg; 90µg/mL), and gel filtration chromatography (0.086 U/mg; 85µg/mL). The molecular weight of purified azoreductase was determined by gel electrophoresis using standard marker. The partially purified azo reductase revealed band on 10% SDS-PAGE with an estimated molecular weight of 60 KDa approximately.

3.4. Analysis of dye degraded products

The TLC analysis of dye degraded products shown Rf value of the dye sample 0.42, and the Rf value of degraded product was found to be 0.02. Therefore, the TLC analysis has confirmed that there was no starting material, present in the degraded sample and two new degraded products were formed For confirmation of the dye degradation Gas chromatography-mass spectrometry was carried out with Jeol GC Mate-II fitted with HP5 column. The dye and degraded samples were injected into a GC/MS (Fig. 24). First the blue dye sample, handpicked from aquous solution, showed the elution of the dye's expected mass 984.208 at retention time 1.78 min. Then the degraded sample was injected in normal temperature gradient to find out the residual amount of compound remains. The GC did not show any peak at 1.78 min. The following GC have shown three major elution at 10.4 and 20.58 min. The respective mass spectra does not show any mass with 984.208. So, it is concluded that no starting material, i.e. Reactive Red BL dye, remains in the culture media Despite the fact that untreated dyeing effluents might cause serious environmental and health hazards, they are being disposed off in water bodies and this water is being used for an agriculture purpose. Use of untreated and treated dyeing effluents in the agriculture has direct impact on fertility of soil. Thus, it was of concern to assess the phytotoxicity of the dye before and after degradation.

IV. Conclusion

Toxic azo dye Reacive Red BL was completely biodegraded by *Alcaligenes* sp. AA09 bacterial strain, isolated from textile dye wastewater, it required less incubation time for decolorization under static condition. Enzymatic studies indicated the involvement of NADH/NADPH-dependent enzyme Azoreductase in the biotransformation at pH 7.0 and temperature 25^0C with 50-200mg/l dye concentration, within 24 h, under aerobic condition. TLC and GC-MS analysis confirmed the complete biodegradation transformation of Reactive Red BL. The forerunning results suggest the potential of utilizing *Alcaligenes* sp. AA09 to decolorize textile effluent containing textile azodye Reactive Red BL via appropriate bioreactor operations. The prospect plan of work focused on the pilot scale treatment of textile tannery effluent, based on laboratory scale research by using free and immobilized cells of *Alcaligenes* sp. AA09.

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