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BIODECOLORIZATION AND DEGRADATION OF TEXTILE DIAZO DYE REACTIVE BLUE 171 BY MARINOBACTER SP. NB-6 – A BIOREMEDIAL ASPECT

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ABSTRACT

The discharge of highly coloured effluents containing dyes can be damaging to the receiving marine water bodies and can result in serious environmental pollution problems. Hence, considerable attention has been given in determining the ability of marine microorganism in decolorization and degradation of textile dyes. Decolorization and Degradation of Reactive Blue 171 was carried out using the acclimatized Marinobacter sp. NB-6 (Accession No. HF568873) isolated from soil. The decolorization of dye Reactive Blue 171 in 24 hours was up to 95.00 % in nutrient broth having 8.0% NaCl and also it showed 93.11% decolorization in half strength nutrient broth having the same NaCl concentration. The percent decolorization of the dye was also studied by cell-free extract and was observed that the isolate can decolorize the dye 90.00 % in 24 hours. The percent decolorization of the dye was determined spectrophotometrically at 590nm. The percent COD reduction of the dye by the isolate was 86.00%. The degradation products formed after degradation were analyzed by GC-MS technique and it was found that this culture degraded Reactive Blue 171 to the products having molecular weights 98, 99, 149, 150, 223, 70, 86, 125, 154, 155, 149, 150, 223, 149, 150, 223, 57, 113, 149, 167 and 279. The microbial toxicity study revealed the degradation of Reactive Blue 171 into non-toxic products by Marinobacter sp. NB-6. From the study performed, we conclude that, this acclimatized species can prove better option for bioremediation of textile dye in wastes containing high salts and in marine environment.

KEY WORDS

COD Reduction, Decolorization, Degradation, GCMS, Marine Bacteria,Reactive Blue 171

INTRODUCTION

Synthetic dyes and pigments are extensively used in the textile industries. So, synthetic dyes are release in textile effluent. Dye effluents are among the major pollutants discharged into the environment. Coloured wastewater from textile industry is rated as the most polluted in all industrial sectors. The colour of synthetic dyes in effluent is one of the most obvious indicators of water pollution. The discharge of highly coloured effluents containing dyes can be damaging to the receiving bodies [1] and can result in serious environmental pollution problems. These effluents have common characteristics as their high coloration since a small amount of residual dye (of the order of mg l/1) can be sufficient to cause a significant visual effect and affects the aesthetic merit, water transparency and gas solubility in lakes, rivers and other water bodies. Different methods are available for the remediation of dye wastewaters. These include physicochemical methods, like adsorption, chemical oxidation, precipitation, coagulation, filtration, electrolysis, photo degradation, and

biological, and microbiological methods. Adsorption on activated carbon is an effective method for the removal of colour, but it is too expensive [2]. The major disadvantage of physicochemical methods has been largely due to the high cost, low efficiency, limited versatility, interference by other wastewater constituents, and the handling of the 252 Water Air Soil Pollut (2010) waste generated [3,4]. Copper complexed direct dyes were found to be highly toxic to fishes like Daphnia magna than unmetallized new dyes [5].Traditional wastewater treatment technologies have proven to be markedly ineffective for handling wastewater of synthetic textile dyes because of the chemical stability of these pollutants [6]. The development of efficient and environmentally friendly technologies to decrease dye content in wastewater to acceptable levels at affordable cost is of utmost importance [7]. Biological methods are generally considered environmentally friendly as they can lead to complete mineralization of organic pollutants at low cost [8]. Bioremediation may be the most effective method of treating industrial dyes wastewater [9].

Large number of textile industries are located on the coastal areas due to ease of transport to the various places in world and help in building nations economy, but on the contrary the effluents released from these industries are proving a great problem for the marine life. Therefore, industrial effluents containing dyes must be treated before their safe discharge into the environment. Reactive dyes have been identified as the most problematic compounds in textile dye effluents [10,11].

In the present study, a bacterium was isolated from marine environment capable of decolorizing and degrading Reactive blue 171 textile dye. This species was studied for decolorization of the dye Reactive blue 171 in various different conditions like in complete nutrient medium, in half strength nutrient medium, cell-free extract and in presence of different co-substrates. The decolorization of the dye was monitored spectrophotometrically (Systronics-106) at its specific absorbance maxima (λmax) 590nm. Percent COD reduction of the dye was calculated.

MATERIALS AND METHODS

- Samples – Soil samples were collected from salterns (Saltpan), areas nearby waste disposal sites of the textile industry, sewage, sludge, effluent treatment plants and compost as the source of microorganisms.
- Dye– Reactive Blue 171 (λmax-590nm)

Preparation of the dye solutions and isolation of microorganisms

Dye solutions of 1% are prepared in distilled water, which are stored as stock solutions and used for further study.

After acclimatization process of the dye for 2 months isolation was carried out by adding the different NaCl concentrations and dye solution in soil collected from salterns (Saltpan), areas nearby waste disposal sites of the textile industry, sewage, sludge, effluent treatment plants and compost in increasing concentration. Isolation of microorganisms was done from the acclimatized soil. For isolation of microorganisms, the soil was mixed with nutrient broth having different NaCl concentrations and incubated for 24 hours at ambient temperatures for the growth of microorganisms. This nutrient broth was then streaked on the nutrient agar plates containing the dye and different NaCl concentrations. After incubation the colonies showing decolorization of the dye were selected for further study.

Acclimatization of microorganisms-

Soil samples from salterns (Saltpan), area nearby waste disposal site of textile industries, sewage,

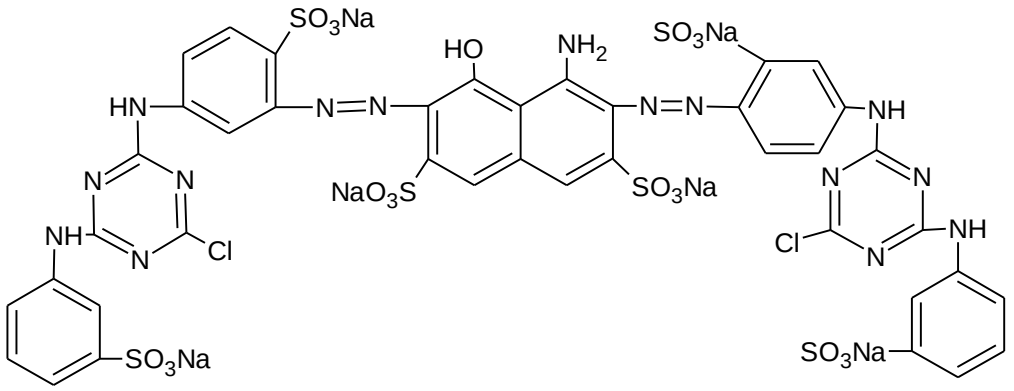
sludge, ETP along with compost were collected and homogenized properly. The micro-flora from the samples were acclimatized in the dye Reactive Blue 171 (1%) and 8.0% NaCl concentrations for the period of one month. One gram of acclimatized soil was inoculated in the nutrient broth with 8.0% NaCl concentrations, after incubation isolation was carried-out on nutrient agar incorporated with same NaCl and dye concentration. The colonies showing decolorization were selected for the further studies.

Determination of Biodegradation Assay

Acclimatized, 24 hours old culture of the isolate NB-6 was inoculated in nutrient medium containing dye Reactive Blue 171 **(Figure 1)** and 8.0% NaCl concentration at a concentration 10ml of 1% dye from stock and incubated at 37°C. An aliquot of 5 ml was removed after different time intervals. The aliquot was centrifuged at 10,000 rpm for 20 min to remove the cell mass. The supernatant was then used to investigate the decolorization of the dye by observing the change in the absorbance at maximum absorption wavelength (λ max) 590nm nm on spectrophotometer (Systronics-106 model).

Figure 1- Structure and properties of Reactive Blue 171

Structure



Properties:

Molecular Formula = $C_{40}H_{23}Cl_2N_{15}Na_6O_{19}S_6$

Molecular weight =1418.93

Composition =C (33.86%) H (1.63%) Cl(5.00%) N (14.81%) Na (9.72%) O (21.42%) S (13.56%)

Decolorization of Dye in Nutrient Broth

The selected microorganism was inoculated in 20 ml of nutrient broth containing 8.0% NaCl and 1000µg/ml concentration of dye. The tube was then incubated at ambient temperature for 24 hrs and observed for decolorization of the dye.

Decolorization of dye in Half Strength Nutrient Broth

The selected culture was then inoculated in 20 ml of half strength nutrient broth containing 8.0% NaCl and 1000µg/ml concentration of dye. The tube was then incubated at ambient temperature for 24 hrs and decolorization pattern was studied.

Cell-free extract studies on Decolorization of Dye

The cells grown in nutrient broth with 8.0% NaCl concentrations were lysed by using Sonicator (Vibra Cell System) and centrifuged in cooling centrifuge (BIO-LABS 165-R) at 10000 rpm for 15 min. The supernatant was added with 1000µg/ml concentration of dye solution in nutrient broth

with same NaCl concentrations and incubated at ambient temperature. The percent decolorization studies were done by using spectrophotometer (Systronics – 106 models).

Decolorization of dye in presence of different Co-substrates

The promising isolate was inoculated in 20ml nutrient broth containing 8.0% NaCl concentrations, 1000µg/ml concentration of dye and 1% Glucose. Tube was then incubated at ambient temperature for 24 hours and observed for decolorization of the dye. Additionally, nutrient medium containing 1% Starch and 1% Yeast Extract with the same dye and 8.0% NaCl concentrations were also used to test the ability of *Marinobacter sp. NB-6* to decolorize the dye Reactive Blue 171.

Determination of Chemical Oxygen Demand (COD)

Percent COD reduction value of the dye decolorized in nutrient medium having 8.0% NaCl were calculated by using strong oxidizing agent potassium dichromate ($K_2Cr_2O_7$).

GCMS analysis

To study the products formed after degradation of dye Reactive Blue 171, decolorized samples were analysed by GCMS. The isolate was inoculated in 100mL of sterile nutrient broth containing 1000 µg/mL of dye Reactive Blue 171 and 8.0% NaCl. The broth was then incubated at ambient temperature for 24 hrs in separate flasks. The decolorized broth was then centrifuged at 10,000 rpm for 15 minutes in cooling centrifuge. Centrifugate was mixed with equal amount of dichloromethane in separating funnel. Samples were shaken vigorously for 15 minutes and kept for 10-15 minutes to separate solvent and aqueous phases. After separation, aqueous phase was discarded and solvent phase allowed for partial evaporation. Partially evaporated samples were analysed by GCMS technique.

Microbial Toxicity of Dyes and their Biodegradation Products Testing

It is very important to know whether biodegradation of a dye leads to detoxification of the dye or not. Agar well bioassay is the most common technique used to evaluate the microbial toxicity. This can be achieved by microbial toxicity tests with the original dyes and their biodegradation products. The microbial toxicity was tested on three test organisms *viz. Azotobacter sp., Pseudomonas sp. Rhizobium sp.* The 24 hours old culture of the test organisms were used for the toxicity testing. After the confirmation of dye degradation, the degraded solution (decolorized broth) was poured in the wells prepared in nutrient agar previously spreaded with the test organisms. These plates were incubated at ambient temperatures for 24 hours. The zone of inhibition around the wells proved the toxicity of the dye and their degraded products.

Identification of the isolate and sequencing of 16S rRNA gene

The 16S rRNA was determined in National Center for Cell Sciences, University of Pune Campus, Pune. Genomic DNA isolation of isolate was carried using Qiagen DNA isolation kit as per manufacturer's instruction. Its presence was checked by running in agarose gel (0.8%) stained with ethidium bromide. The sequence was deposited to European Bioinformatics Institute (EBI).

Sequence was analyzed at the Ribosomal Database Project (RDP-II)

(http://rdp.cme.msu.edu/) for closed homology. The sequences downloaded from the RDP II database were aligned by using CLUSTAL X2 multiple sequence alignment tools. The Phylogenetic tree was constructed by the neighbor joining method using Kimura-2-parameter distances in MEGA 4.0 [12].

RESULTS

Isolation and Identification

The organism was isolated from the soil on nutrient agar containing 8.0% NaCl and was identified by using biochemical observations and 16s rRNA analysis technique. From the analysis the isolate was identified as *Marinobacter sp. NB-6*. Biochemical results showed that the isolate was unable to hydrolyze Starch and Gelatin, able to reduce nitrate, whereas fermentation of glucose, sucrose, mannose, maltose, lactose showed acid and gas production. The isolate was Oxidase and Catalase positive whereas Urease negative. Biochemical results are given in (**Table 1**). The phylogenetic tree was developed by using Neighbor joining method by Kimura-2-parameter with 1000 replicates in MEGA 4.0. [13] (**Figure 2**).

Table 1 Biochemicals of the isolate

Characteristics of isolate NB-6	***Marinobacter sp. NB-6***
Gram nature and Motility	Gram negative motile rods
Optimal growth temperature (^{0}C)	37^{0}C
Optimal NaCl Concentration (%)	8.0%
Utilization of	
D-Glucose	+
Sucrose	+
Mannose	+
Maltose	+
Lactose	+
Hydrolysis of	
Starch	+
Gelatin	+
Casein	-
Enzyme activity	
Amylase	+
Oxidase	+
Catalase	+
Urease	-
Nitrate reduction	+

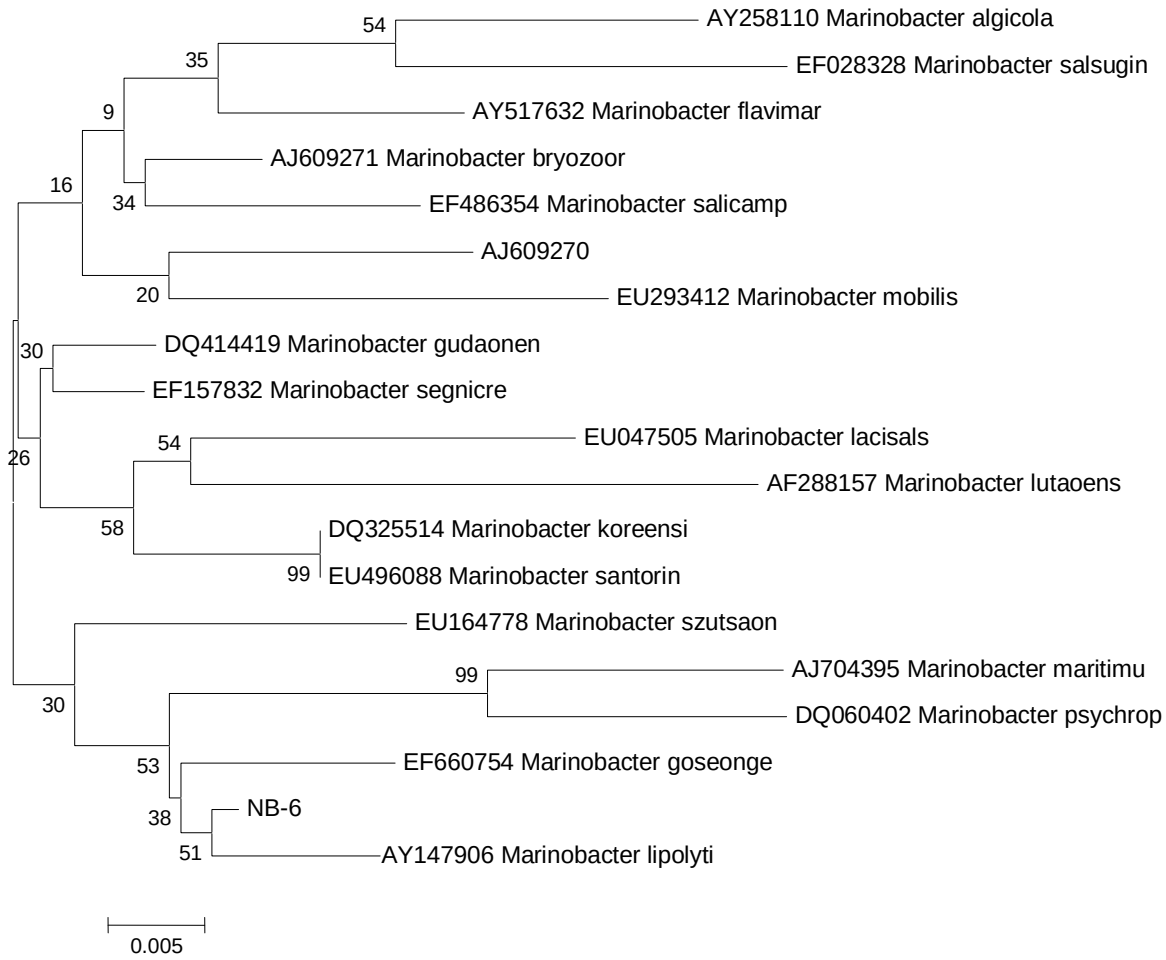


Figure 2: Phylogenic tree of *Marinobacter sp. NB-6*. Phylogenetic analysis of 16s rRNA gene sequence of *Marinobacter sp. NB-6*. The percent numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analyses of 1,000 replicates. The scale bar (0.005) indicates the genetic distance.

Percent Decolorization of the Isolate

Marinobacter sp. NB-6 was studied for its percent decolorization capacity in nutrient broth with 8.0% NaCl concentration and Dye 1000µg/ml concentration. The results of percent decolorization of dye Reactive Blue 171 in nutrient broth is given in (**Table 2 and Figure 3**).

Table 2: Percent Decolorization in Nutrient Broth, ½ Strength Nutrient Broth and Cell-Free Extract in 24 hrs at λmax-590nm and percent COD reduction value

Culture Code	Identified As	% Decolorization in			COD Reduction Value
		Nutrient Broth	½ strength Nutrient Broth	Cell-Free	
NB-6	*Marinobacter NB-6*	95.00	93.11	90.00	86.00

Figure 3: Percent Decolorization of Reactive Blue 171 in Nutrient medium having different NaCl concentrations at 37^{0}C in 24 hours

Percent Decolorization in Half (½) Strength Nutrient Broth

Marinobacter sp. NB-6 was studied for its ability to decolorize the dye in half (½) strength nutrient broth with 8.0% NaCl concentration and the dye in same concentration 1000µg/ml as in complete nutrient broth. The results of percent decolorization of dye Reactive Blue 171 in half strength nutrient broth is given in (**Table 2** and **Figure 4)**.

Percent Decolorization in Cell-Free Extract

Marinobacter sp. NB-6 was studied for its ability to decolorize the dye in Cell-Free extract. The results of percent decolorization of dye Reactive Blue 171 in cell-free extract is given in (**Table 2** and **Figure 4)**.

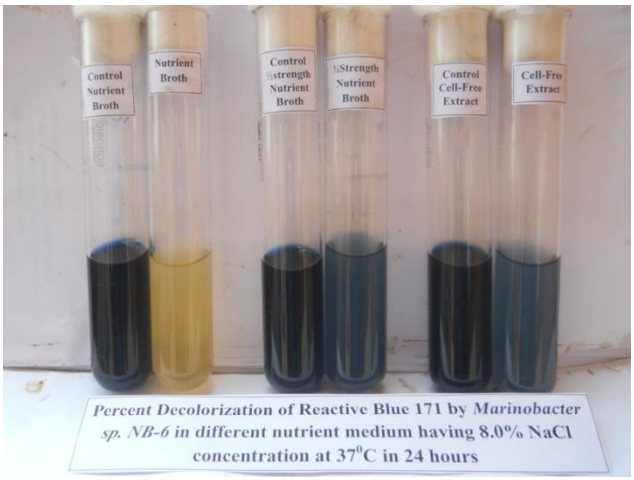


Figure 4: Percent Decolorization of Reactive Blue 171 by *Marinobacter sp. NB-6* in different nutrient medium having 8.0% NaCl concentration at 37^{0}C in 24 hours

Percent COD reduction

The Percent COD reduction of the dye after decolorization of the dye by the *Marinobacter sp. NB-6* is given in (**Table 2)**.

Percent Decolorization of Dye in presence of different Co-substrates

Marinobacter sp. NB-6 was further studied for its ability to decolorize the dye Reactive Blue 171 in nutrient medium containing 8.0% NaCl

concentration and 1% Glucose, 1% Starch and 1% Yeast extract as Co-substrates and 1000µg/ml concentration of dye. The results of percent decolorization of dye Reactive Blue 171 in presence of different Co-substrates is given in (**Table 3 and Figure 5).**

Table 3 Percent Decolorization in presence of Different Co-Substrates *viz* 1% Glucose, 1% Yeast Extract and 1% Starch in 24 hrs at λ max-590nm

Culture Code	Identified As	% Decolorization in		
		1% Glucose	1% Yeast Extract	1% Starch
NB-6	*Marinobacter NB-6*	95.22	96.00	95.11

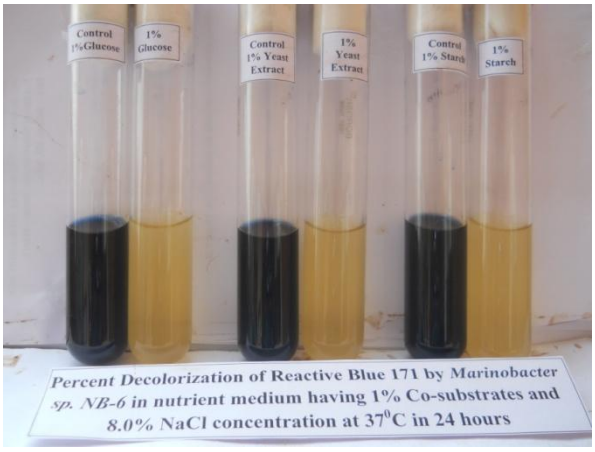


Figure 5: Percent Decolorization of Reactive Blue 171 by *Marinobacter sp. NB-6* in nutrient medium having 1% Co-substrates and 8.0% NaCl concentration at 37^{0}C in 24 hours

GC-MS analysis

The GC-MS analysis reports of the dye are shown in **Figure 6, 7,** and **8** respectively. The reports showed that the dye was degraded by the isolate having different molecular weights (**Table 4**). The results showed that the isolate from the acclimatized soil have good decolorization and degradation of the dye Reactive Blue 171. Confirmation of the biodegradation of the dye Reactive Blue 171 was done by analysing the samples with GC-MS. The degradation products of the dye were of much lower mass than the original compounds.

Table 4 Molecular weights of the degraded products

Culture Code	Identified As	Molecular weights of the degraded products
NB-6	*Marinobacter sp. NB-6*	98, 99, 149, 150, 223, 70, 86, 125, 154, 155, 149, 150, 223, 149, 150, 223, 57, 113, 149, 167 and 279 respectively

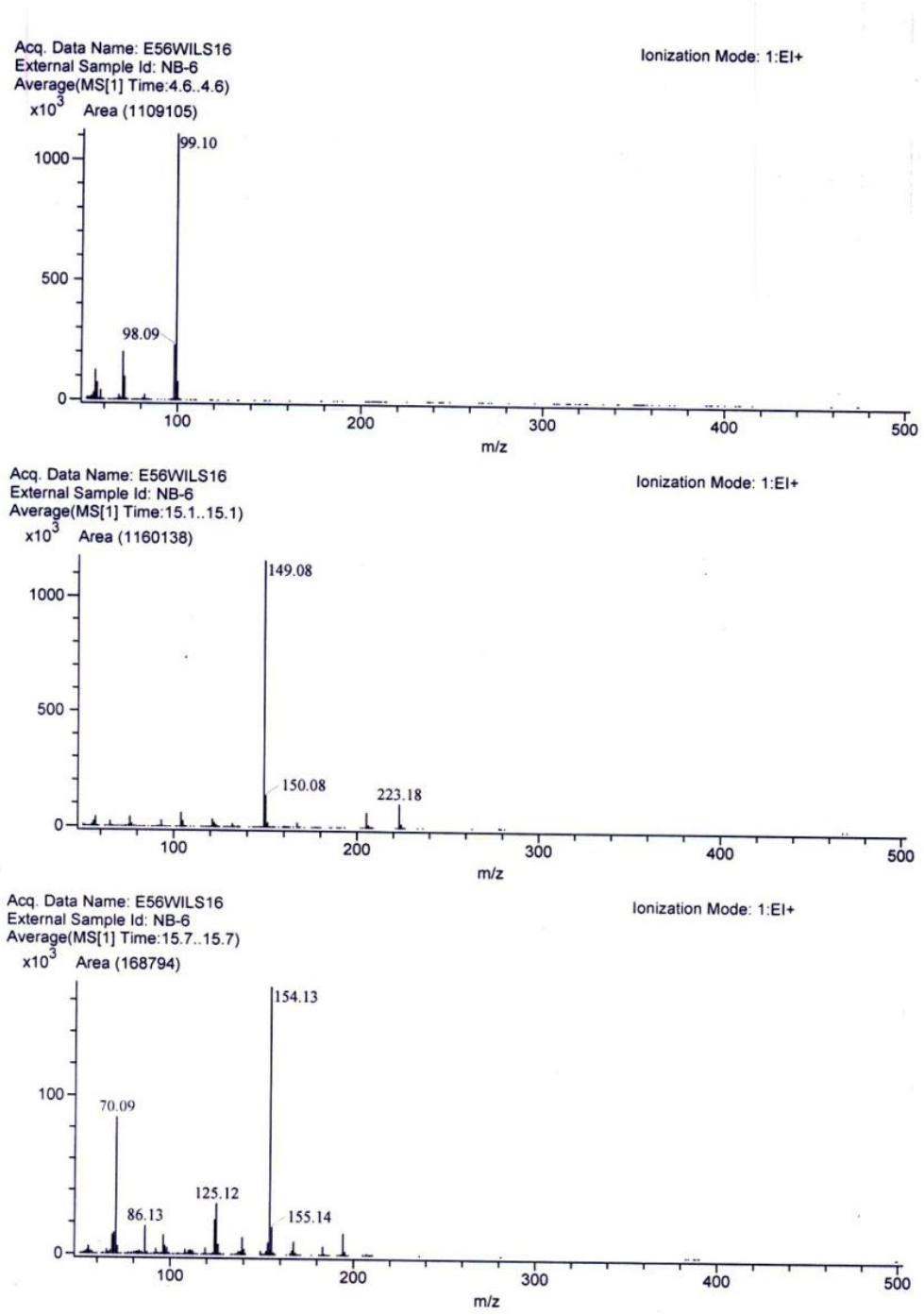


Figure 6: GCMS analysis report of degraded products of Reactive Blue 171 dye by *Marinobacter sp. NB-6.*

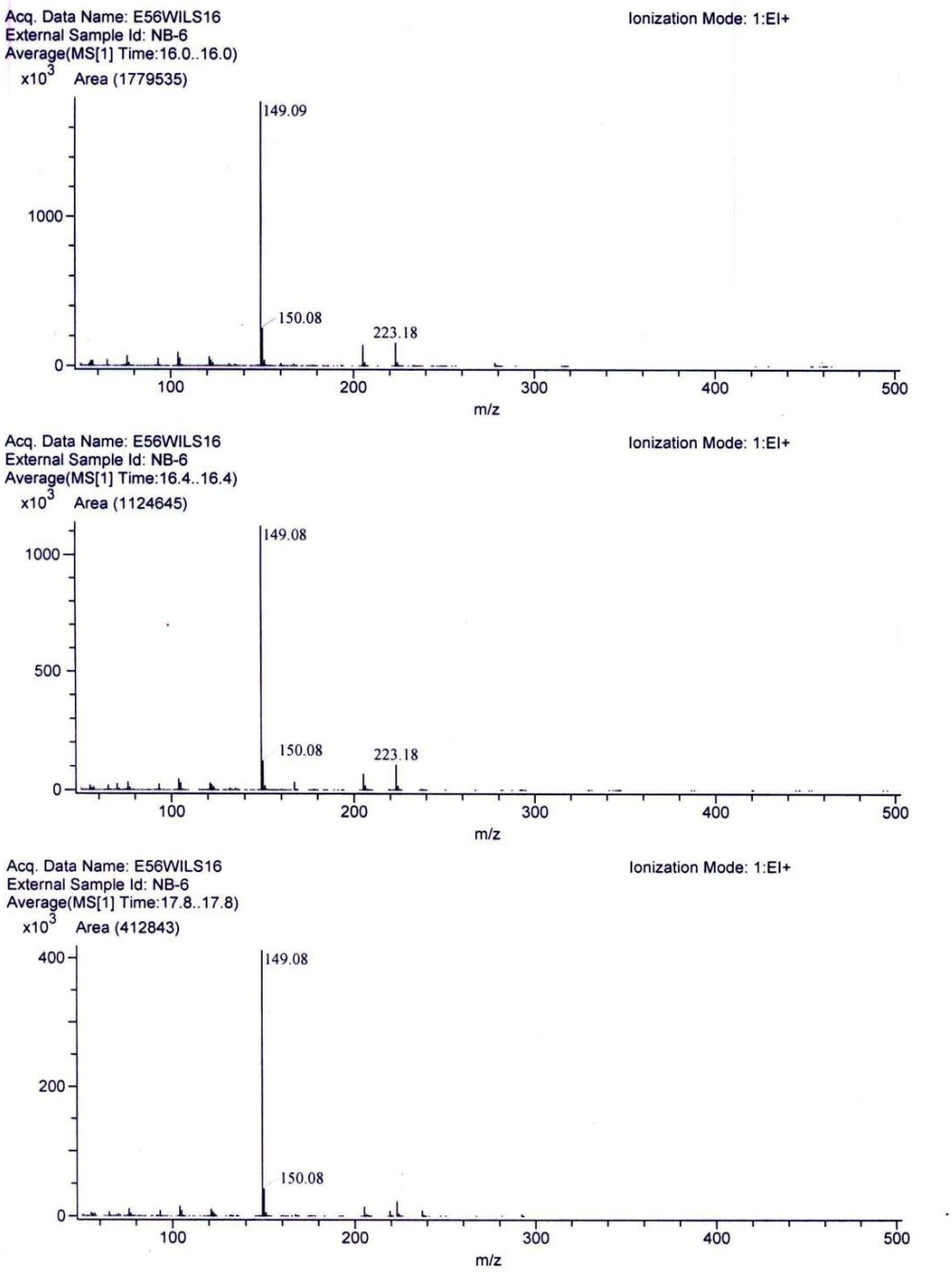


Figure 7: GCMS analysis report of degraded products of Reactive Blue 171 dye by *arinobacter sp. NB-6.*

Molecular weights of the degraded products of dye

The GC-MS analysis report showed that the dye Reactive Blue 171 was degraded and not only decolorized. The molecular weights of the degraded products are given in (**Table 4)**

Microbial Toxicity Studies

Microbial toxicity of the dye Reactive Blue 171 was studied on microorganisms *viz. Azotobacter*

sp., *Pseudomonas sp.* and *Rhizobium sp.* The toxicity of the dye and its degradation products was studied by the agar well assay. The results showed that the wells which were poured with decolorized broth had no zone of inhibition and wells with original dye solution had zone of inhibition. This confirmed that the original dye solution 1000 µg/ml was toxic to the bacteria but its degradation products were non toxic to the bacteria (**Figure 9**)

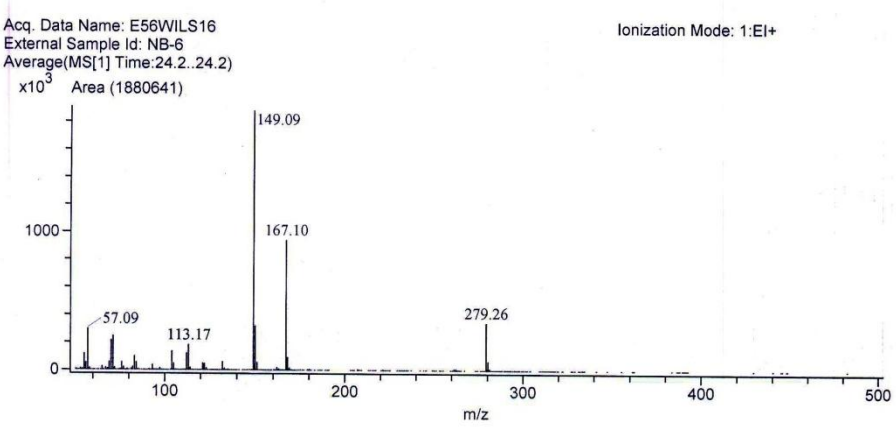


Figure 8: GCMS analysis report of degraded products of Reactive Blue 171 dye

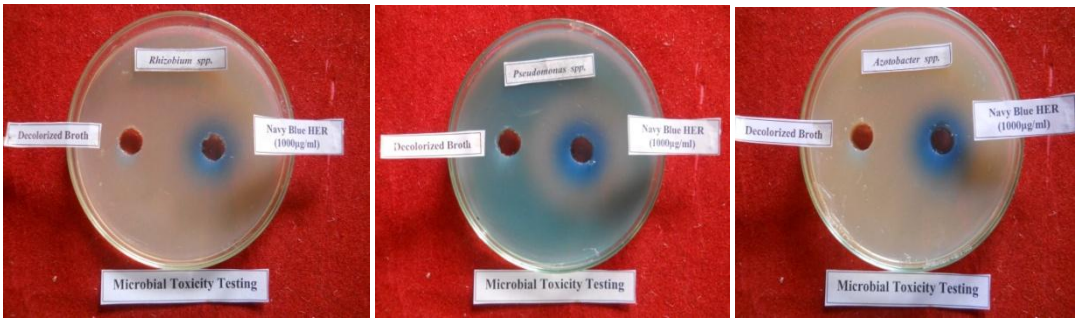


Figure 9: Microbial toxicity testing against Azotobacter sp., Pseudomonas sp. and Rhizobium sp.

DISCUSSION

In present study, the decolorization of Reactive Blue 171took place in nutrient medium containing 8.0% NaCl, this suggests that the presence and availability of a co-substrate is necessary, because it acts as an electron donor for the azo dye reduction (Nigam *et al.,* 2006). Effect of co-substrates *viz.* Glucose, Starch and Yeast Extract was studied which showed slight increase in the percent decolorization of the dye was observed in 24 hours. From the percent COD reduction values it can be concluded that dye was degraded by the isolate. The isolated marine organism carried out biotransformation of Reactive Blue 171, which was confirmed by GC-MS analysis. These results were similar to Khalid, *et al.,* (2008b). Khalid, *et al*., (2008b) reported enhanced decolorization of azo dyes by *Shewanella putrefaciens strain AS96* in presence of yeast extract as co-substrate under hypersaline condition. Rania, M.A., (2008) used Glucose, Sucrose, Starch and Sodium citrate as carbon sources among which Starch was best for decolorization of Crystal violet up to 96 % .

Gondaliya and Parikh, (2012) reported the highest percentage decolourization 97.04% of Reactive Orange–16 was obtained by *Serratia marcescens* when additional supplement of glucose (1 g/l) was added in Nutrient broth. These study shows that additional supplement of

carbon source give rise in percentage of decolourization but among them addition of yeast extract give highest percentage decolourization in Reactive Blue 171.

In the present study, the halophilic *Marinobacter sp. NB-6* isolated from acclimatized soil decolorized the azo dye Reactive Blue 171 to an extent of 85.20% in nutrient medium, 84.25% in half strength nutrient medium. This observation was found to be in accordance with the previous studies of Shertate and Thorat, (2012) who reported the decolorization of azo dye Mordant Orange-1 by *Marinobacter algicola MO-17* at 15% salinity. This result was also very much similar to the decolorization of azo dye Reactive Blue 171by *Marinobacter sp. NB-6.* This rate of decolorization may be due to the high metabolic diversity being observed in the halophiles due to their extremophilic nature (Oren *et al*., 1992; Ventosa *et al*., 1998). These results were similar to Jeremy Martin *et al*., (2011) who reported three marine-derived fungal strains, *Phialophora* sp. (MF 6), *Penicillium* sp. (MF 49) and *Cladosporium* sp. (EMF 14) which showed complete decolorization of 0.01% Congo red and up to 91 % decolorization of 0.01% crystal violet at 33g/L marine salts. Similar result was obtained by Bumpus and Brock (1998) in their experiment on the degradation of crystal violet by *P. chyrosporium*.

The results of microbial toxicity indicate that the azo dye degradation products formed after biodegradation by *Marinobacter sp. NB-6* were less toxic compounds compared to the original azo dye. These results are in agreement with result of Kalyani *et al*., (2009) and Mane *et al*., (2008) who found that the metabolites products after biodegradation of Reactive Red 2 and Reactive Blue 59 were less toxic compared to the original dye.

The use of such marine microorganism able to degrade azo dye in presence of salt could help prevent costly dilution to lower the salinity, or the removal of salt by physico-chemical methods before biological treatment and can be used for the treatment of effluents containing high salt content.

ACKNOWLEDGMENT

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