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BIODECOLORIZATION AND DEGRADATION OF A TEXTILE AZO DYE MORDANT ORANGE 1 BY HALOMONAS SP. MO-11

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Abstract: *Presence of huge amount of salts in the wastewater of textile dyeing industry is one of the major limiting factors in the development of an effective biotreatment system for the removal of azo dyes from textile effluents. Bacterial spp. capable of thriving under high salt conditions could be employed for the treatment of saline dye-contaminated textile wastewaters. The present study was aimed at isolating the most efficient bacterial species capable of decolorizing azo dyes under high saline conditions. Decolorization and Degradation of Mordant Orange 1 was carried out using the acclimatized Halomonas sp. MO-11 (Accession No.HE964773) isolated from soil. The decolorization of the dye Mordant Orange 1 in 24 hours was up to 91.00 % and also it showed 89.00% decolorization in half strength nutrient broth. The percent decolorization of the dye was studied by cell-free extract and was observed that the isolate can decolorize the dye by 80.11% in 24 hours. The percent decolorization of the dye was determined by spectrophotometrically at 385nm. The percent decolorization of the dye was also studied in presence of 1% co-substrates like glucose, yeast extract and starch and was found to be upto 92.22%, 93.00% and 91.11% respectively. The percent COD reduction of the dye by the isolate was 75.55%. The degradation products formed after degradation were analyzed by GC-MS technique and it was found that this culture degraded Mordant Orange 1 to the products having molecular weight 70, 99, 70, 97, 112, 125, 140, 168, 128, 169, 83, 98, 111, 154, 72 and 154 respectively. The microbial toxicity study revealed the degradation of Reactive Blue 171 into non-toxic product by Halomonas sp. MO-11. The use of such bacterium would be very cost effective and eco-friendly technology for the treatment of pollutant like dyes.*

Keywords: *Azo dye, Marine Bacteria, Degradation, Decolorization, GC-MS, COD reduction*

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INTRODUCTION

Azo dyes are the largest group of synthetic dyes used in textile, paper, plastic and leather manufacturing units (Gurulakshmi *et al*., 2008, Saharan and Ranga, 2011). These colorants are environmental pollutants. Azo dyes are synthetic organic colorants characterized by great structural variety. They have the same chromophore N=N and different auxochromes such as NH_2, NR_2 and OH groups (Meyer, 1981). Azo dyes were found to be difficult to degrade because of their complicated structure (Kim and Shoda, 1999). Thus, it becomes very essential to treat the textile effluent before their release into the environment.

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 (Anjaneyulu *et al*., 2005). Bioremediation is a process in which the natural capacity of microbes is enhanced to degrade toxic chemicals and waste (Senan and Abraham 2004). Microbial decolourization and degradation has appeared as an environmentally friendly and cost-competitive alternative to chemical decomposition processes (Whiteley, 2007; Khalid *et al*., 2008).

Saline and hyper-saline environments are frequently contaminated with organic compounds as a result of industrial activities (Margesin and Schinner, 2001a; Oren *et al*., 1992). Contamination of these habitats constitutes a serious environmental problem mainly due to the high toxicity exhibited by aromatic hydrocarbons. In most cases, biodegradation constitutes the primary mechanism for contaminant removal. However, biodegradation processes are difficult to perform under saline conditions (Margesin and Schinner, 2001a; Oren *et al*., 1992; Ventosa *et al*., 1998). In addition, it is known that the traditional pollutant biodegradation is less efficient or does not function when salinity increases above that of the sea (Oren *et al*., 1992). Conventional microorganisms do not survive under these saline conditions. One remedy for the removal of these xenobiotic compounds in the saline environment is the use of halophiles which are adapted to live in such saline conditions. The degradation or transformation of organic pollutants by halophilic and halotolerant microorganisms has received little attention.

To the best of our knowledge, this is the first report on biodegradation of Mordant Orange 1. Enzymatic status before and after biodegradation were monitored to access whether any induction of the enzyme systems occurs. Analysis of samples extracted after biodegradation

were performed with GC-MS analysis. Particularly dye wastes are harmful to agricultural and marine habitat; hence to determine the toxic nature of the dye as well as its degraded metabolites, microbial toxicity testing was carried out on three ecologically important organisms *viz. Azotobacter sp, Rhizobium sp.* and *Pseudomonas sp.*

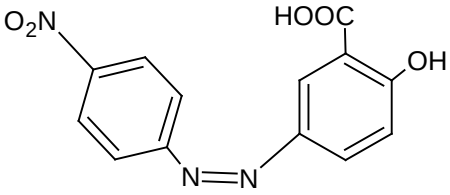
In the present study, a bacterium was isolated from marine environment capable of decolorizing and degrading a textile azo dye Mordant Orange 1. This species was studied for decolorization and degradation of the dye Mordant Orange 1 in various different conditions like in complete nutrient medium, in 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) 385nm. Percent COD reduction of the dye was calculated.

MATERIALS AND METHODS

Materials – 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 –The textile dye Mordant Orange-1 (λmax-385nm) was obtained from Sigma-Aldrich, (USA).

Table 1- Properties of the Dye

Dye Name	Structure	Properties
Mordant Orange-1	 2-hydroxy-5-[(*Z*)-(4-nitrophenyl)diazenyl]benzoic acid	**Molecular Formula** - $C_{13}H_9N_3O_5$ **Formula Weight** - 287.22766 **Composition** – C (54.36%), H (3.16%), N (14.63%), O (27.85%) **λmax –** 385nm.

Methods

Acclimatization and isolation of microorganisms –

Soil was used as a source of microorganisms to isolate morphologically distinct bacterial colonies capable for decolorizing the dye. The soil samples were subjected to enrichment using nutrient broth amended with increasing concentrations of NaCl (0.5% to 20.0%) and

dye (250µg ml^{-1} to 10,000µg ml^{-1}) with incubation time of 24 hours at 37°C. Repeated transfers were carried out to isolate stable dye decolorizing organism and the isolated organism was stored at 5°C for further use. The high decolorizing bacteria were screened by performing a decolorization assay with the dye using UV- VIS spectrophotometer (Systronics-106 model) at its respective λ max 385 nm and designated isolate MO-11 and used for further studies.

Determination of biodegradation activity

Acclimatized, 24 hours old culture of the isolate MO-11 was inoculated 20 ml in nutrient medium containing 5.0% NaCl and dye Mordant Orange 1 **(Table 1)** at a concentration 1500µg $ml^{-1}$and incubated at 37^{o}C with no aeration or agitation. 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) 385 nm on spectrophotometer (Systronics-106 model).

Percent Decolorization in Nutrient Broth, Half (½) Strength Nutrient Broth–

Isolate MO-11 was used to inoculate in 20 ml nutrient broth (peptone – 1.0g, NaCl – 0.5g, Beef Extract – 0.3g, Distilled Water – 100.0ml) containing 1500µg/ml concentration of dye and 5.0% NaCl. These tubes were then incubated at ambient temperature for 24 hrs and observed for decolorization of the dye. In addition, half strength nutrient broth (peptone – 0.5g, NaCl – 0.25g, Beef Extract – 0.15g, Distilled Water – 100.0ml) was also used to test for the ability of isolate MO-11 to decolorize the dye Mordant Orange-1 with same dye concentration and salinity.

Percent Decolorization in Cell Free Extract

Bacterial cells, 24 h cultivated were harvested by centrifugation at 7,000 rpm, for 15 min, at 4°C and suspended in 50 mM phosphate buffer pH 7.4. These cells (100 mg ml^{-1}) were chilled properly and sonicated (Sonics-vibracell ultrasonic processor), keeping sonifier output at 40 amp and giving 7 strokes each of 30 s, with time interval of 2 min at 4°C. The homogenate was then centrifuged at 8,000 rpm for 15 min and the supernatant obtained was used as crude enzyme source. The supernatant containing the crude enzyme was then added with 1500µg/ml concentration of dye solution and observed for dye decolorization. The percent decolorization studies were monitored by using spectrophotometer.

Decolourization rate was expressed as percentage decolourization and calculated using the formula (Jadhav et al., 2010).

$$\% \text{ Decolorization} = \frac{A-B}{A}$$

(Where, A – initial absorbance, B – observed absorbance)

Effect of carbon and nitrogen sources (Co-substrates) on percent Decolorization

During the study, the isolate was supplemented with carbon and nitrogen sources *viz. 1%*Glucose, 1%yeast extract and 1%starch. The effect of these sources on the decolorization were observed.

Effect of physico-chemical parameters

Various physicochemical parameters like temperature and pH were monitored to study their effect on decolorization of Mordant Orange 1. Nutrient medium (100 ml each) with 24 h old culture was inoculated with dye (1500µg/ml) and incubated at different temperatures as 25°C, 30°C, 37°C, 40°C, 50°C. Similarly broths with different pH as 5, 6, 7, 9, and 11 were inoculated with strain and after 24 h of incubation; dye (1500µg ml^{-1}) was added to observe the effect on decolorization.

Determination of Chemical Oxygen Demand (COD)

Percent COD reduction value of the dye decolorized in nutrient broth by isolate MO-11 was calculated by COD analysis using $K_2Cr_2O_7$ as a strong oxidizing agent under reflux conditions.

GCMS Analysis

Degradation of the dye Mordant Orange-1 by the isolate MO-11 was confirmed by GC-MS analysis. The samples for GCMS were prepared by extracting the degraded products in Di-Chloro Methane (DCM). The decolorized broth was centrifuged at 10,000 rpm for 20 min. The supernatant was decanted and was collected in a separating funnel. Equal amount of DCM was added to the separating funnel containing the supernatant. The funnel was shaken vigorously for 20 min to extract the products in DCM. The separating funnel was kept undisturbed for the separation of the solvent phase and the liquid phase. The separated solvent was then taken out from the funnel. The products that are extracted in the solvent were concentrated in the vial by evaporation of the solvent at room temperature. This was then analyzed by Gas chromatography and Mass spectroscopy (GC-

MS). The GC-MS analysis of metabolites was carried out using a Shimadzu 2010 MS Engine, equipped with integrated gas chromatograph with HP1 column (60 m long, 0.25 mm id, non-polar). Helium was used as carrier gas at a flow rate of 1 ml min^{-1}. The injector temperature was maintained at 280^0C with oven conditions as: 80^0C kept constant for 2 min and increased up to 200^0C with 10^0C min^{-1} raised up to 280^0C with 20^0C min^{-1} rate.

Phylogenetic analysis and sequence alignment

Initially the 16S rRNA gene sequence was analyzed at NCBI server (http://www.ncbi.nlm.nih.gov) using BLAST (blastn) tool and corresponding sequences of homologous species were downloaded and used for phylogenetic analysis. The evolutionary history was inferred using neighbor joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) was shown next to the branches (Felsenstein 1985). The phylogenetic tree was linearized assuming equal evolutionary rates in all lineages. The clock calibration to convert distance to time was 0.01 (time/node height). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and were in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007).

RESULTS AND DISCUSSION

Screening and Identification of the isolate

Dye decolorizing isolate were screened form the soil heavily contaminated by azo dyes as a source. The potential isolate was selected and designated as MO-11 showing the zone of decolorization on nutrient agar containing dye and 5.0% NaCl. Due to acclimatization, this isolate was resistant to 10,000ppm of dye concentration. The isolate was gram negative, motile rod. On the dye and 5.0% NaCl containing nutrient agar plates, the colonies were transparent and circular in shape. On the basis of biochemical tests and 16S rRNA analysis, the isolate was identified as *Halomonas sp. MO-11.* The sequence was deposited in EBI with accession no. HE964773. Phylogenetic tree was constructed using MEGA 4.0. **(Figure I).**

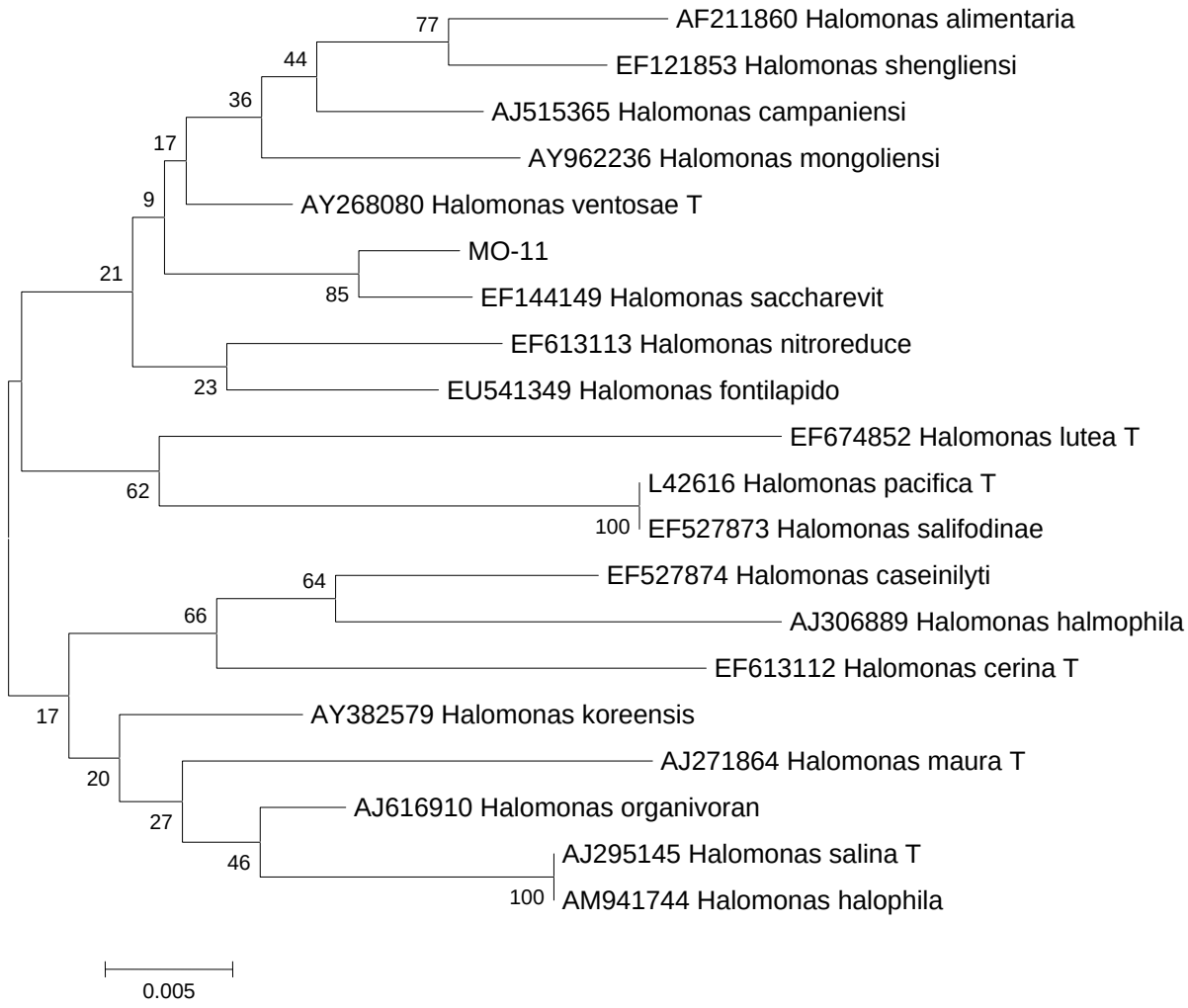


Figure I – Phylogenetic analysis of the 16S rRNA sequence of *Halomonas sp. MO-11.* The percent numbers at the nodes indicate the level of bootstrap support based on Maximum Composite Likelihood analysis of 1000 replicates. The scale bar indicates the base pair substitutions per site.

Decolorization studies

The decolourization experiments were carried out using spectrophotometric method.(Meiyin Xu *et al.,* 2006; Hala *et al;* 2008, Sheth and Dave, 2009, Jadhav *et al;* 2010). The absorbance spectra of dye before and after decolourization were scanned by SL- 160 double beam UV – Vis spectrophotometer (Elico), at maximum absorbance of each individual dye. The effectiveness of microbial decolorization depends on the survival, adaptability of the microorganisms and also the activity of enzymes produced by microorganisms.

Decolorization studies in Nutrient Broth, Half (½) Strength Nutrient Broth

The decolorization was carried with the Mordant Orange-1 dye, supplemented with nutrient broth having 5.0% NaCl at 37^{o}C and was found to be up to 91.00% (**Figure II and Table 2**). This results are in accordance with the Pourbabaee *et al*., (2011) who reported Maximum decolorization of Cibacron Black w-55 by new strain of *Halomonas sp*. was detected in the presence of 1-1.5M NaCl. Sheth and Dave, (2009) reported 91.1% decolourization of Reactive Red BS. C. I–III by *Pseudomonas aeruginosa.* Halophiles have been reported to be involved in the dye decolorization (Khalid *et al*., 2008a). Khalid *et al*., (2008a) has been investigated that the decolorization of azo dyes by a member of the genus Shewanella: *Shewanella putrefaciens strain AS96* under hypersaline conditions. Ammozegar *et al*., (2011) has also been reported that reported halophilic microorganism *Shewanella putrefaciens* to be capable of the complete removal of Reactive Black-5, Direct Red-81, Acid Red-88, and Disperse Orange-3 (all 100 mg/L) within 8 hours in presence of 40g/L NaCl. Also the decolorization of the dye in half (½) strength nutrient broth was slightly less (**Figure II**) but if this method is used it will be cost-effective.

Percent Decolorization in Cell Free Extract

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. A major mechanism behind biodegradation of different recalcitrant compounds in microbial system is driven by the biotransformation enzymes. The action of cell free extract of the *Halomonas sp. MO-11* to decolorize the dye Mordant Orange-1 was observed to be 80.11% **(Figure II and Table 2)**. The role of *oxidoreductive enzymes* in the decolorization of sulfonated reactive azo dyes have been characterized in *Rhizobium radiobacter MTCC 8161* (on Reactive Red 141), and *Pseudomonas sp. SUK1* on Reactive Red 2 (Kalyani et al. 2009).

Table 2: Percent Decolorization in Nutrient Broth, Half (½) Strength Nutrient Broth and Cell Free Extract by *Halomonas sp. MO-11* in 24 hrs at λ max 385nm.

Culture code	Dye	% Decolorization (after 24 hrs)		
MO-11	Mordant orange-1 (λmax 385nm)	Nutrient Broth	½ Strength Nutrient Broth	Cell-Free Extract
		91.00	89.00	80.11

Effect of carbon and nitrogen sources on percent Decolorization of dye

Gondaliya and Parikh (2012) reported the highest percentage decolorization 97.04% of Reactive Orange–16 was obtained by *Serratia marcescens* when additional supplement of glucose (1 g/l) was added in Nutrient broth. Jadhav *et al., (*2010), Sheth and Dave (2009) studied *Serratia marcescens* with carbon source supplements gives increase percentage decolourization in Nutrient broth, but % decolourization by *Pseudomonas aeruginos* and *Pseudomonas* strain BCH were reduced. Since the dyes are deficient in carbon source, it seems necessary to supplement additional carbon or nitrogen source to assist biodegradation of dyes by the bacterial consortium (Senan and Abraham, 2004). So as depicted in Figure II, *Halomoans sp.* Meiying *et al., (*2006) showed *Shewaness decolorationis* SR reduce percentage decolourization with additional carbon sources. On the contrary, *MO-11* showed the decolorization of the dye Mordant Orange-1 to a greater extent in the presence of 1% yeast extract (co-substrate) was slightly higher than that in its absence (**Figure II and Table 3)**. Jadhav *et al*., (2010) pointed out that the presence of various carbon and nitrogen sources in medium might have stimulatory or inhibitory effect on enzyme systems involved in the decolorization.

Table 3: Percent Decolorization in presence of Co-substrates like 1% glucose, 1% yeast extract and 1% starch by *Halomonas sp. MO-11* in 24 hrs at λ max 385nm.

Culture code	Dye	% Decolorization in different Co-substrates		
		1% Glucose	1% Yeast Extract	1% Starch
MO-11	Mordant orange-1 (λmax 385nm)	92.22	93.00	91.11

Effect of physico-chemical parameters

The selected isolate showed less decolorization at pH 5.0, 6.0, 9.0 and 11.0 and showed maximum decolorization at 7.0. In case of Mordant Orange 1, the isolate MO-11 showed maximum decolorization up to 91% in 24 hours at pH 7.0. The optimum pH for the decolorization of Mordant Orange 1 by *Halomonas sp. MO-11* was 7.0. (**Table 4**) The isolate MO-11 showed comparatively less decolorization at temperatures 25^0C and 50^0C but showed maximum decolorization up to 91% in 24 hours at temperature 37^0C. With rise in

temperature from 25^{0}C to 50^{0}C the decolorization rate was increased but further increase in temperature from 40^{0}C to 50^{0}C drastically affected decolorization activity of isolate. The optimum temperature for the decolorization of Mordant Orange 1 was 37^{0}C. It was observed that the optimum temperature for the decolorization of Mordant Orange 1by *Halomonas sp. MO-11* was 37^{0}C (**Table 4).**

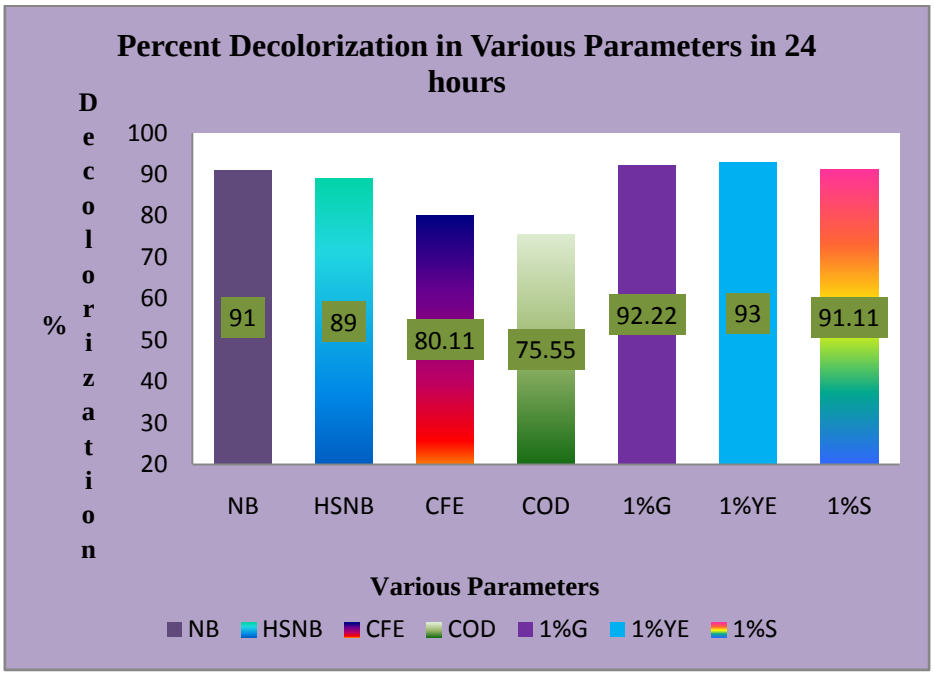
Table 4: Percent decolorization of dye Mordant Orange 1 by *Halomonas sp. MO-11* at different pH and temperatures

Culture code	% Decolorization									
	pH					Temperature (^{0}C)				
	5.0	6.0	7.0	9.0	11.0	25	30	37	40	50
MO-11	60	62	91	70	65	61	64	91	59	55

Percent COD reduction

To evaluate the level of biodegradation of Mordant Orange-1 by *Halomonas sp. MO-11*, we have determined the percentage of mineralization (represented by COD removal) by measuring the initial and final organic content. 75.55% of COD was removed which is significant removal of COD was observed after a period of 24 hours (**Figure II**) Moreover, the COD removal efficiency is better than that reported earlier (Kalyani *et al*., 2009), as a COD reduction of 55.55 and 52% was observed for Reactive Blue 172 by *Exiguobacterium sp. RD3* and Reactive Red 2 by *Pseudomonas sp. SUK1*, respectively.

Figure II: Percent Decolorization of Mordant Orange 1 in various parameters in 24 hours



Note - NB-Nutrient Broth, HSNB-Half Strength Nutrient Broth, CFE-Cell-Free Extract, 1%G-1% Glucose, 1%YE-1% Yeast Extract, 1% S-1% Starch

GC-MS Analysis

The GC-MS analysis reports of the dye is shown in Fig. III. 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 Mordant Orange 1. Confirmation of the biodegradation of the dye Mordant Orange 1was done by analysing the samples with GC-MS. The degradation products of the dye were of much lower mass than the original compounds.

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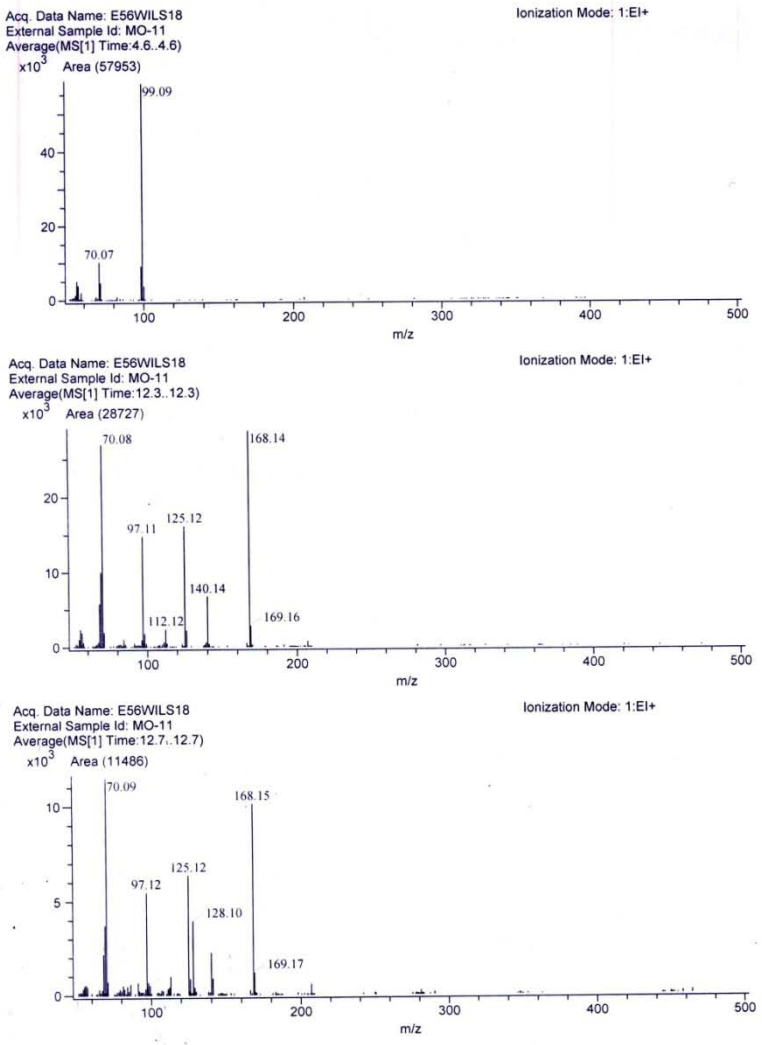
Figure III – GC-MS Analysis Report of the Mordant Orange 1.



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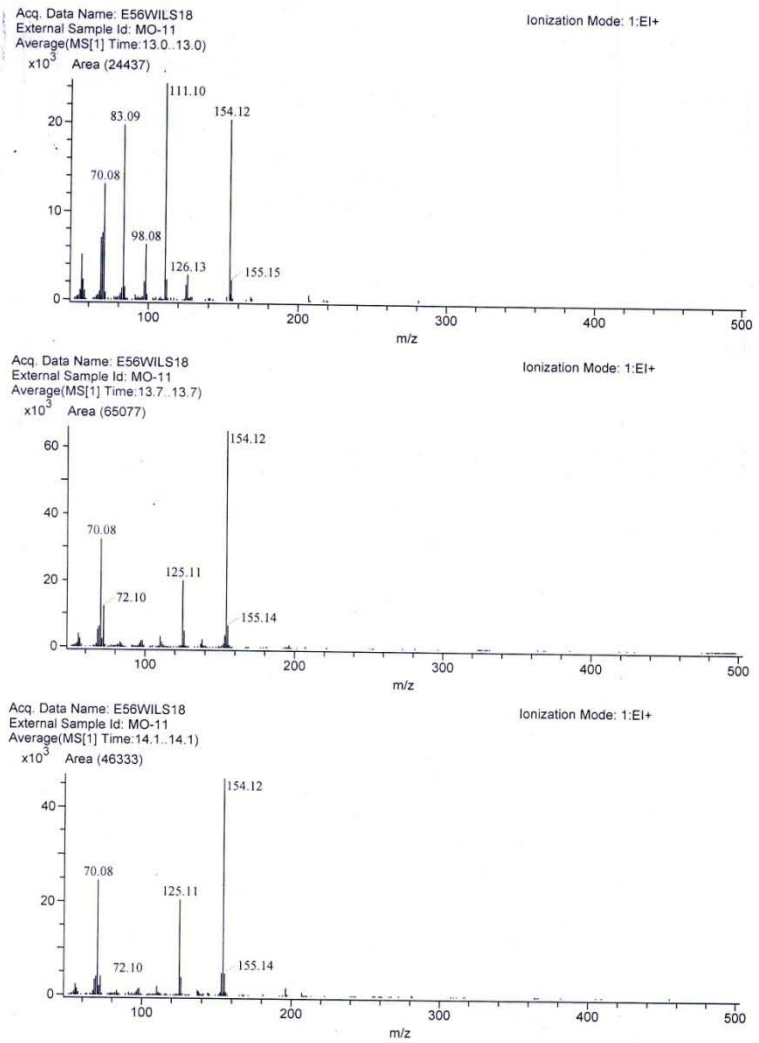

Confirmation of Biodegradation of dye

The GCMS analysis report showed that the dye Mordant Orange-1 was degraded by *Halomonas sp. MO-11* and not decolorized **(Figure III)**. The molecular weights of the degraded products are 70, 99, 70, 97, 112, 125, 140, 168, 128, 169, 83, 98, 111, 154, 72 and 154.

Molecular weights of the degraded products of dye

The GC-MS analysis report showed that the dye Mordant Orange 1 was degraded and not only decolorized. The molecular weights of the degraded products are given in **Table 4.**

Table 4 : Molecular weights of the degraded products

Culture code	Identified as	Molecular weights of the degraded products
MO-11	*Halomonas sp. MO-11*	70, 99, 70, 97, 112, 125, 140, 168, 128, 169, 83, 98, 111, 154, 72 and 154

Toxicity study

The degradation metabolites of Mordant Orange 1 were extracted with Dichloromethane. This sample was then used for toxicity study. The microbial toxicity study was carried out 37°C using *Azotobacter sp., Rhizobium sp*. and *Pseudomonas sp*. separately and Mordant Orange 1 and its degradation metabolites.

CONCLUSION

Bioremediation is the microbial clean-up approach. Microbes can acclimatize themselves to toxic wastes and new resistant species develop naturally, which can transform various toxic chemicals to less harmful forms. The results presented here indicated that, the dye Mordant Orange-1 was biodegraded in 24 hours by the isolate MO-11 which was identified as *Halomonas sp. MO-11*. The degradation of the dye was enhanced when 1% co-substrates like glucose, yeast extract and starch were used as carbon source and nitrogen source. Also the intracellular enzymes showed the considerable amount of degradation of the dye. The isolate reduced the COD of the dye to a much greater extent proving it a good agent for bioremediation. So the isolate might play a possible role in bioremediation of the dye contaminated soil.

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