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RESEARCH ARTICLE

Biodegradation of Allura Red AC (ARAC) by *Ochrobactrum anthropi* HAR08, isolated from textile dye contaminated soil.

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Abstract

A dye degrading bacterial strain HAR08 was isolated from soil contaminated with textile dyes. This isolate was identified as *Ochrobactrum anthropi* on the basis of biochemical characteristics and phylogenetic analysis based on 16s rRNA gene sequencing. The strain HAR08 was able to decolorize the azo dye Allura Red AC (ARAC) up to 95% in the nutrient medium within 24 h with the dye concentrations of 10 g $l^{-1}$and was confirmed by spectrophotometry. A remarkable reduction in COD (95.7% after 24 h) of the dye ARAC was observed after the action of the isolated strain *Ochrobactrumanthropi*HAR08. Slight decrease in decolorizing ability (91%) of the strain was observed when the nutrient medium was half diluted, but in the presence of external co-substrate, the strain showed enhancement in the ability to decolorize the dye (99%). The cell-free extract showed the remarkable decolorization of the dye (92.1%) proving the involvement of intracellular enzymes for decolorizing the dye. The degradation of the ARAC was confirmed by FTIR and GCMS techniques and was found to be completely mineralized. Phytotoxicity analysis was carried out on seeds of *Sorghum bicolor* plant and the dye degradation product was found to be non-phytotoxic. The isolate HAR08 was able to tolerate, decolorize and degrade the azo dye ARAC at high concentrations (10 g L^{-1}) within 24 h. This biodegradation and detoxification potential of the bacterial strain HAR08 makes it better candidate in treatment of dye effluents.

Introduction

Azo dyes represent a major group of dyes causing environmental concern because of their color, bio-recalcitrance and potential toxicity to animal and human (Levine, 1991). Natural and artificial azo dyes are extensively used as coloring agents for textile, foodstuffs, drugs and cosmetics. With the development of storage and manufacturing methods, processed foods constitute 60% of total foods and are increasing annually along with food additives (Sasaki, *et al.*, 2002). The largest group of dyes (sulfonated azo dyes) has great structural differences due to which they offer the range of different of colors (Bhoosreddy, 2014). Food dyes are used for coloring cosmetics, pills as well as foods. Erythrosine is used as a staining dye for dead *Schizosaccharomyces pombe* (Mutoh, *et al.*, 2005) and to investigate dead bacteria in human dental caries. During re-evaluation of the safety of these additives, some materials have disappeared. For example, permission to use butter yellow, an azo-dye, was withdrawn due to carcinogenicity within a year after it was granted. Twelve chemical food dyes are permitted by the Japanese Government, while European Food Safety Authority is currently undertaking a series of re-evaluations on the safety of food additives, including colors (Poul, *et al.*, 2009). The majority of azo dyes (food and textile) have LD50 values between 250-2,000 mg/kg body weight, which shows that, for a lethal dose many grams of azo dyes have to be consumed in a single dose. As azo dyes are very strong colors and become hyperactive at a level to cause allergies.

ARAC is one of the most widely used synthetic diazo colorants. Joint FAO/WHO Expert Committee on Food Additives and European Union's Scientific Committee for Food assessed the toxicity of ARAC, and it showed a very low acute toxicity as measured in different species of animals (LD50: 10,000 mg kg^{-1} body weight for rats and rabbits and 5000 mg kg^{-1} body weight for dogs) (Fallico, *et al.,* 2011).

Currently the wastewater generated by the textile, food and other industry is a severe problem. The removal of color from the industrial wastewater is currently an issue of discussion and regulation all over the world (Kolekar and Kodam, 2012). Many physico-chemical methods are proposed for treatment of dyes containing textile effluents. These methods include, adsorption on different materials, oxidation and precipitation by Fenton's reagent, bleaching with chloride or ozone photo degradation or membrane filtration (Robinson, *et al.,* 2001). Also many studies are focused on the potential viability of oxidation of various organic pollutants by using physical methods like coagulation/flocculation, membrane filtration and ion exchange. Disadvantage of all these methods is the transfer of waste components from one phase to another, causing secondary loading of environment (Salem, *et al.*, 2009).

The decolorization of the dyes is a challenging process in front of dye industry, where we can harness the great potential of natural microbial flora and fauna as an effective tool to help decolorization of the dyes (Sahastrabudhe, M. and Pathade, G., 2013). Biological treatment of dyes is environment friendly, cost competitive and reproducible. The effectiveness of microbial treatment depends on the survival, adaptability and activity of the selected target organism (Cripps, *et al.,* 1990; PastiGrigsby, *et al.,* 1992) and oxidation potential of the azo dyes. In present study, the degradation of ARAC by isolated strain HAR08 was investigated at very high concentration of dye compared to previous reports. The optimization of different physicochemical and media conditions supports its versatility and adaptability in surviving at high concentration of dye.

Material and Methods

Microorganism and culture medium

The bacterial strain HAR08 was isolated from textile dye contaminated soil, Solapur, Maharashtra, India. The strain was isolated based on its decolorization potential. The strain HAR08 was grown in nutrient medium containing (g l^{-1}): NaCl, 5.0; beef extract, 3.0 and peptone, 10.0 (pH 7.0) at 37^0C. This composition of the media ensured optimum growth of strain HAR08. The culture was routinely maintained on nutrient agar and the glycerol stocks (20%) were also prepared and maintained at 4^0C.

Chemicals

The dye Allura Red AC used for the experiments was obtained from Sigma-Aldrich (St. Louis, USA). All the chemicals used were of highest purity available and of analytical grade.

Identification of bacterial isolate

The biochemical tests were performed by API20NE system (Bio Mérieux Inc., USA). The PCR amplification and DNA sequencing of the 16s rRNA gene was carried out as described earlier (Bachate, *et al.,* 2012).

Decolorization studies

The strain HAR08 was cultivated in an Erlenmeyer flask containing 100 ml nutrient broth and amended with the dye ARAC (10 g L^{-1}). After different time intervals 2 ml aliquot of the culture media was withdrawn and centrifuged at 10,000 x g for 15 min in a refrigerated centrifuge (Biolabs, BL165R) to separate the bacterial cell mass. The supernatant was measured at various time intervals at the maximum absorption wavelength (λmax – 552 nm) using UV-visible spectrophotometer (Systronics Model-106). The percentage of decolorization was calculated from the difference between initial and final absorbance values.

Optimization of conditions

To study the effect of static anoxic and shaking (100 rpm) conditions on decolorization of ARAC, strain HAR08 was cultivated in nutrient medium containing 10 g L^{-1}ARAC dye. To determine the effect of pH on decolorization the isolate HAR08 was cultivated for 24 h in conical flasks containing 100 ml nutrient broth of varying pH (5.0–11.0) containing 10 g L^{-1}ARAC dye. Similarly, effect of temperature on dye decolorization was determined by measuring the decolorization of dye at different temperatures (viz., 25, 30, 37 and 45^0C). After different time intervals, cell free supernatant was used for analysis of decolorization and all the experiments were performed in triplicates.

Effect of media composition on decolorization of ARAC dye

To study the effect of media composition on decolorization, the nutrient media was half diluted and dye decolorization was studied. To study the effect of co-substrate on decolorization, additional 1% glucose was added in nutrient media and the strain HAR08 was inoculated in presence of ARAC dye and the decolorization was observed.

Chemical oxygen demand

To study the reduction of organic compounds in the samples after dye decolorization, COD was measured. After complete decolorization, the COD of the supernatant was measured as per standard APHA (1998) protocol.

Analysis of biodegraded products

After complete dye decolorization, the supernatant was extracted with dichloromethane and dried over anhydrous sodium sulfate. The solvent was evaporated and the samples were analyzed by Gas chromatography and Mass spectroscopy (Shimadzu 2010 MS) using an 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 ºC with oven conditions as: 80ºC kept constant for 2 min and increased up to 200ºC with 10ºC min-1 and raised up to 280ºC with 20ºC min^{-1}. The same sample was used to analyze the infrared spectrum on FT-IR (Shimadzu FT-IR spectrophotometer 8400).

Phytotoxicity

The phytotoxicity of ARAC dye and its degradation products were tested on the seeds of plant *Sorghum bicolor* (Jowar). The seeds (n=30) were sowed into a plastic sand pot. The sand pot was prepared by adding 50 g of washed sand into plastic pot. The dye, ARAC and its degradation metabolites were dissolved separately in distilled water and the final concentration made was of 10,000 ppm. Toxicity study was done by watering (5 ml) the seeds of each plant with ARAC dye sample and extracted metabolites. The control was run by watering the seeds with distilled water. The watering was done two times per day. Germination (%), root and shoot length were recorded after 10 days. The experiments were carried out at 25°C.

Statistical analysis

The data were expressed as mean ± SEM and analyzed by one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparisons test. Readings were considered significant when P was ≤ 0.05.

Results and discussion

Isolation and identification of the isolated strain

The isolate HAR08 was identified as *Ochrobactrum anthropi* on the basis of its 16S rDNA sequence. The 16s rDNA sequence of HAR08 was a continuous stretch of 694 bp. The 16S rDNA sequence of the strain HAR08 is available under the EMBL GenBank accession number FR873648. The phylogenetic tree was constructed by using MEGA 5.0 (Figure 7). The sequence analysis of 16S rDNA showed that strain HAR08 had highest similarity with the species *Ochrobactrum anthropi* (99%) which has been proved to have dye decolorizing ability against the moniliformin or structurally related to mycotoxins (Duvick and Rood, 2000). *Ochrobactrum anthropi* was also reported for removal of chromium, cadmium, copper, toxic metals (Ozdemir, *et al.*, 2003) and phenol degradation (El-Sayed, *et al.*, 2003).

Optimization of physicochemical parameters

Optimization of physicochemical parameters using strain HAR08 was tested for decolorization at static anoxic and shaking conditions. The strain HAR08 was able to decolorize 95% of ARAC dye (10 g L^{-1}) within 24 h at static anoxic conditions, whereas the decolorization was significantly decreased up to 56% at shaking conditions (Figure1). The results suggests that the static anoxic condition was needed for bacterial dye decolorization which is in good agreement with earlier reports on pure bacterial strains such as *Proteus mirabilis* (Chen, *et al.*, 1999), *Pseudomonas luteola* (Chang, *et al.*, 2001) and *Serratia marcescens* (Verma and Madamwar, 2003). Therefore, static conditions were adopted to investigate bacterial dye decolorization in further experiments.

The effect of different temperature and pH studies was carried out on ARAC (10 g L^{-1}) decolorization. The strain HAR08 showed the decolorization above 44% at all pH range (pH 5.0-11.0), 90.00% decolorization was observed at pH 8.0 (Figure3 and 4). The strain HAR08 showed 75% decolorization at pH 7.0. The decolorization of ARAC dye by strain HAR08 was found to be efficient at all pH values tested. In previous reports it has been suggested that the pH has a major effect on the efficiency of dye decolorization, and the optimal pH for color removal was often between 6.0 and 9.0 (Guo, *et al.*, 2007; Kilic, *et al.*, 2007; Wang, *et al.*, 2009). Similarly bacterial mixed cultures *Bacillus sp.* V1DMK, *Lysinibacillus sp.* V3DMK, *Bacillus sp.* V5DMK, *Bacillus sp.* V7DMK, *Ochrobacterium sp.* V10DMK, *Bacillus sp.* V12DMK exhibited more than 85% decolorization over a broad range of pH 5.0-8.5 and maximum decolorization was obtained at neutral pH 7.0 (Jain, *et al.*, 2012).

The strain HAR08 showed decolorization ability in the temperature range of 25 ºC to 45^0C (Figure 4). Although, the decolorization at 25^0C was 65%, it gradually increased with increase in temperature from 79% at 30^0C and optimum decolorization was 97% at 37^0C. The increase in temperature up to 45^0C decreased the decolorization to 60%, this is may be due to the loss of cell viability or deactivation of the enzymes responsible for decolorization (Panswad and Luangdilok, 2000; Cetin and Donmez, 2006). Similar observation were obtained with *Citrobacter sp.*

CK3 (Wang, *et al.,* 2009), where strong decolorizing activity was observed between 27 to 37ºC, whereas it significantly decreased at 42ºC. The important finding of the strain HAR08 was the ability to utilize 10 g L^{-1} of ARAC dye within 24 h, which has not been reported earlier as per our knowledge.

Effect of nutrient media and co-substrate on dye decolorization

The effect of concentration of nutrient media on the decolorization of ARAC dye was studied. The half diluted nutrient medium showed 91.25% decolorization within 24 h with similar dye concentration(Figure 2). The results suggest that the strain HAR08 can grow and degrade the dye in presence of low nutrient indicating the ability of the strain in the bioremediation of dye effluent.

The addition of co-substrate (1% glucose) in the nutrient medium showed 99% of dye decolorization in 24 h (Figure 2). The observed decolorization was slightly increased with addition of co-substrate. The decolorization of reactive red 180 by *Citrobacter sp.* CK3 was dependent on glucose supplementation, where the presence of glucose significantly increased the decolorization ability of the strain (Wang,*et al.,* 2009). It has been reported earlier that the bacteria obtain energy from glucose instead of dyes and glucose can enhance the decolorizing performance of biological systems (Sarioglu and Bisgin, 2007). The progressive increase in glucose concentration increased rate of dye decolorization (Jain,*et al.,* 2012).

COD reduction

The COD removal was observed to be 92.1% after decolorization of ARAC dye (10 g L^{-1}) within 24 h at static anoxic conditions (Figure 2). The decrease in COD to a certain noticeable level within the short span of time compared to the biodegradation suggested that the degradation by strain HAR08 may be a better candidate for bioremediation. Similarly, *Bacillus sp.* showed maximum COD removal of 93-97% after 24-27 h for Congo red where the concentration was only 50 mg L^{-1} (Gopinath, *et al.,* 2009). It was recently reported that *Alishewanella sp.* KMK6 was able to reduce 28% COD immediately after decolorization at static anoxic conditions. Further incubation of decolorized media up to 24 h reduced the COD by 66% at static anoxic, whereas it reduced by 90% under shaking (aerobic) conditions (Kolekar, *et al.,* 2012). In the view of the potential of the strain HAR08 in COD reduction, it is important for the treatment of dye effluent under static conditions.

ARAC dye degradation and proposed mechanism

The FTIR spectrum of ARAC dye (Figure 5) showed peaks at 3400-3468 cm^{-1} (-NH stretch), 2928-2828 cm^{-1} (CH2), 2158 cm^{-1} (nitriles), 1620 cm^{-1} (C=C stretch), 1500-1620 cm^{-1} (N-H), 1068-1186 cm^{-1} (O-C), 979 cm^{-1} (alkenes) and 665 cm^{-1} (secondary amines). After ARAC degradation, significant difference in FTIR spectrum was observed. The peaks at 3400-3468 cm^{-1} reduced in degradation product and peak appeared at 3200-3400 cm^{-1} which was because of (O–H stretch). The vibration range from 2800-1700 cm^{-1} was completely disappeared which was of aldehyde C-H. The peaks from 1700-500 cm^{-1} were decreased and the appearance of new peaks at 1728 cm^{-1} (C=O stretch), 1400-1500 cm^{-1} (C-C stretch), 1273 cm^{-1} (C-N stretch), 704-798 cm^{-1} (C-Cl stretch). The peaks observed after decolorization were for O–H, C-H, C=O, C-C, C-N and C-Cl clearly indicating the removal of amine from the degradation product (Kolekar and Kodam, 2012). Similar results have been reported for the degradation of synthetic dye by *Agaricus bisporus* (Pandey, *et al.,* 2012).

The gas chromatograph of degradation products of ARAC dye showed the presence of several peaks. The structures of the detected compounds were assigned from the fragmentation pattern and m/z values obtained by GC–MS analysis (Table 1). The cleavage of azo bond in ARAC leads to the formation of sodium 6-hydroxy naphthalene 2-sulfonate (m/z 246) and sodium 4-amino 5-methoxy 2-methyl benzene sulfonate (m/z 239), which may be due to the action of *azoreductase* (Kolekar,*et al.,* 2012). Further it give rise to aniline (m/z 93), which was completely mineralized. Based on the intermediates identified by GC–MS, plausible pathway for biodegradation of ARAC dye has been proposed (Figure6).

Phytotoxicity

After 10 days of incubation, the dye ARAC (10 g L^{-1}) showed 30% germination inhibition in Sor*ghum bicolor* (Jowar), whereas metabolites formed after complete decolorization (applied at the same concentration) showed only 5% germination inhibition. The growth observed in presence of metabolites was normal and the root and shoot lengths were decreased by 8% in presence of metabolites, while it decreased by 25% in presence of dye (Table 2). These results suggest that the degradation products of ARAC dye were non-toxic to the common crop *Sorghum bicolor*.

Table 1 - Mass spectral data, retention times, and proposed identities of metabolites formed after degradation of Allura Red AC by *Ochrobactrumanthropi*HAR08.

Proposed intermediates	Abbreviation[a]	R.T. in min	ESI-MS peaks
Sodium 4-amino-5-methoxy-2-methylbenzenesulfonate	A	12.88	239
Sodium 6-hydroxy naphthalene-2-sulfonate	B	12.45	246
4-Amino-5-methoxy-2-methylphenol	C	16.62	153
Aniline	D	4.9	93

[a]Structures of series A-D are in Figure 6

Table 2 - Phytotoxicity study of ARAC (10,000 ppm) and its degradation product extracted after 24 h (10,000 ppm) for *Sorghum bicolor.*

Dye concentration (ppm)	Germination inhibition (%)	Root length (cm)	Shoot length (cm)
Distilled water	0.00	17.8 ± 1.0	14.6 ± 0.9
Dye (10,000 ppm)	40.00**	13.6 ± 0.5**	11.3 ± 0.4**
Metabolites (10,000 ppm)	0.00	16.3 ± 0.7*	13.3 ± 0.5*

Values are mean of germinated seeds of three experiments ± SEM.
Significantly different from the control at * P < 0.05, ** P < 0.001 by one-way ANOVA with Tukey-Kramer multiple comparison test.

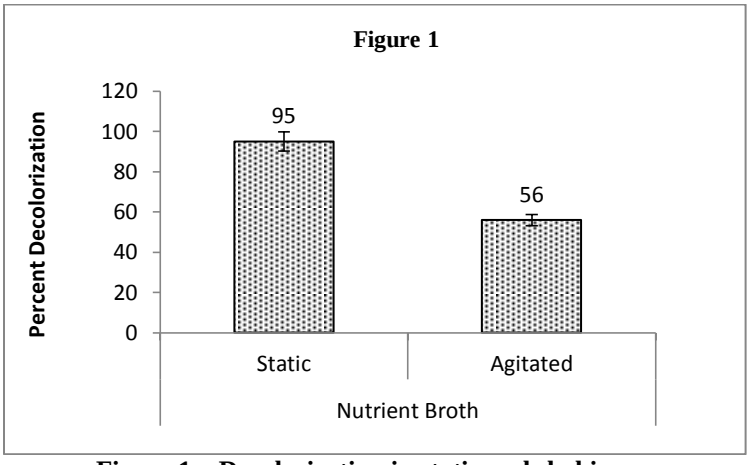

Figure 1 – Decolorization in static and shaking.

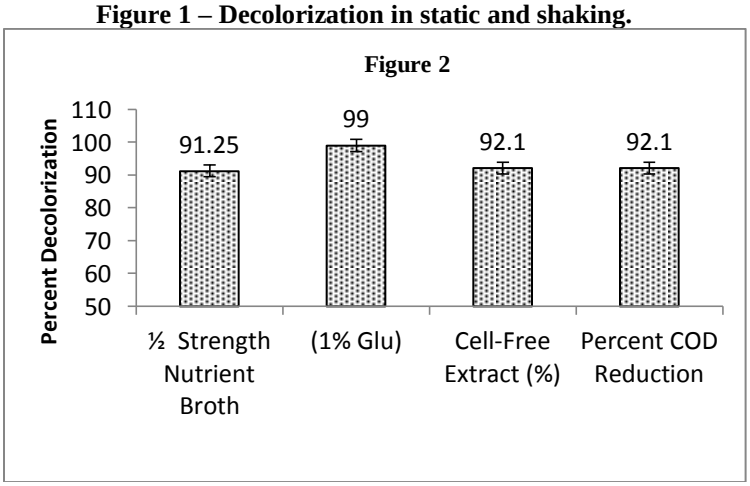

Figure 2– Decolorization in different conditions.

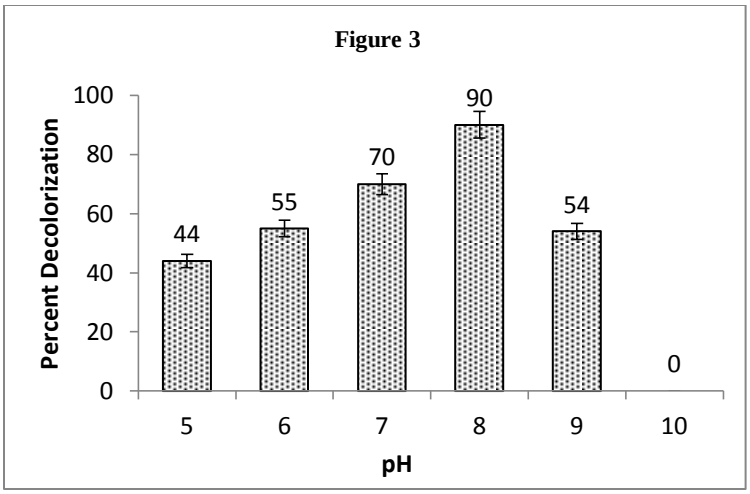

Figure 3 –Decolorization in different pH

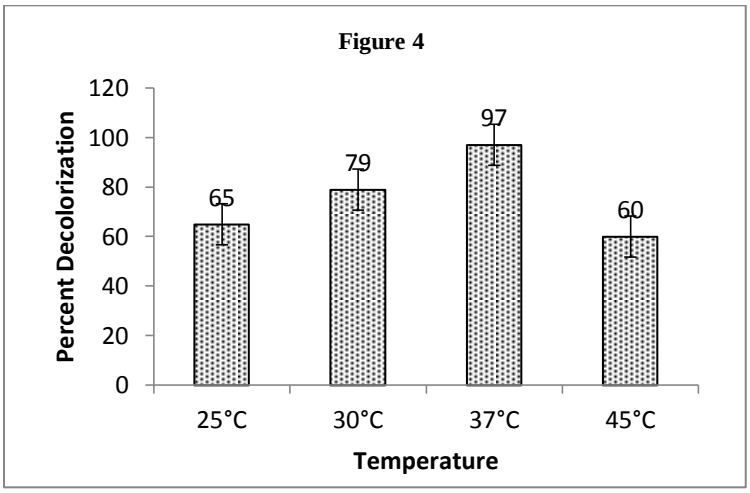

Figure 4 – Decolorization in different Temperature.

Figure 5

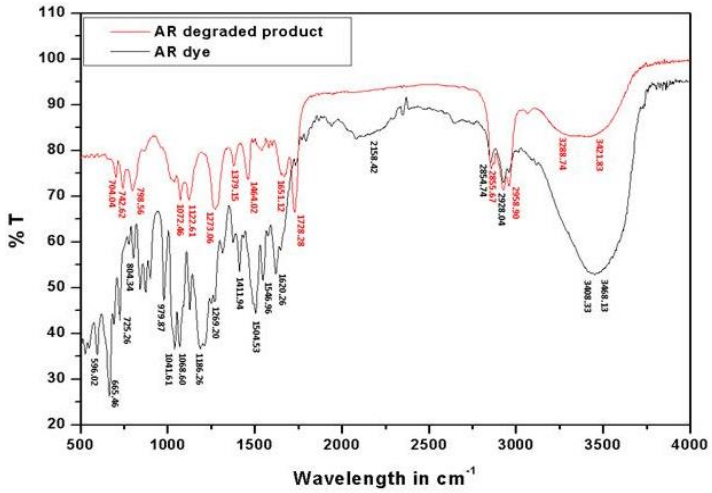

Figure 5 – Fourier transform infrared spectroscopy analysis of ARAC and its degradation metabolites.

Figure6

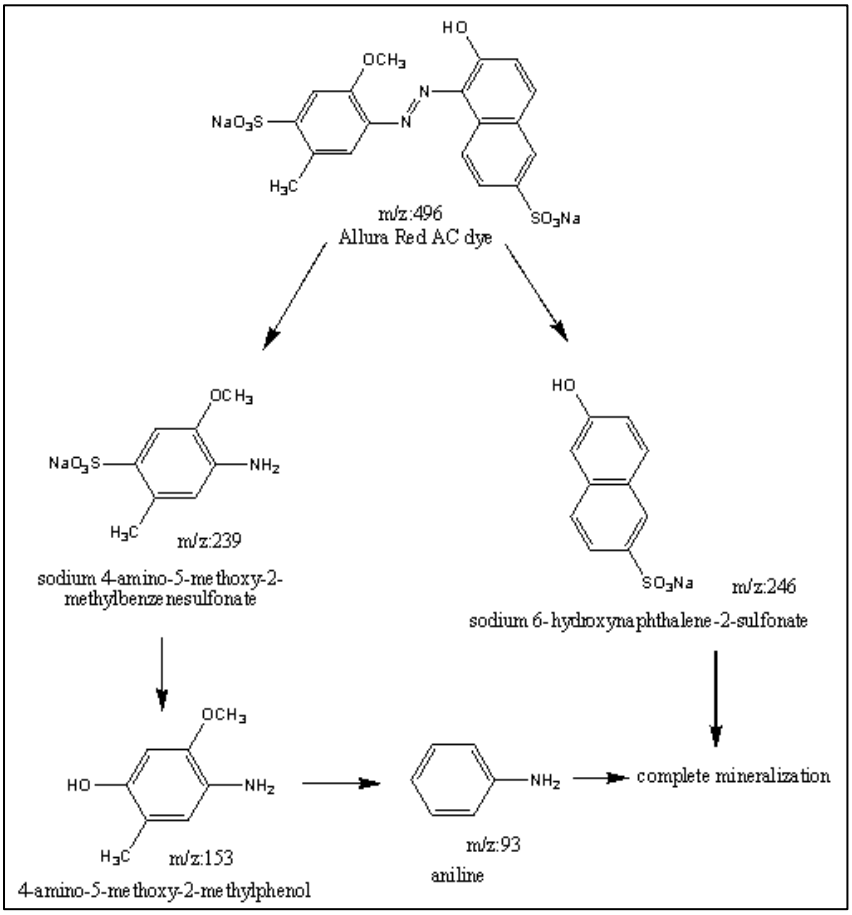

Figure 6 – Proposed mechanism of ARAC by *Ochrobactrum anthropi* HAR08.

Figure 7

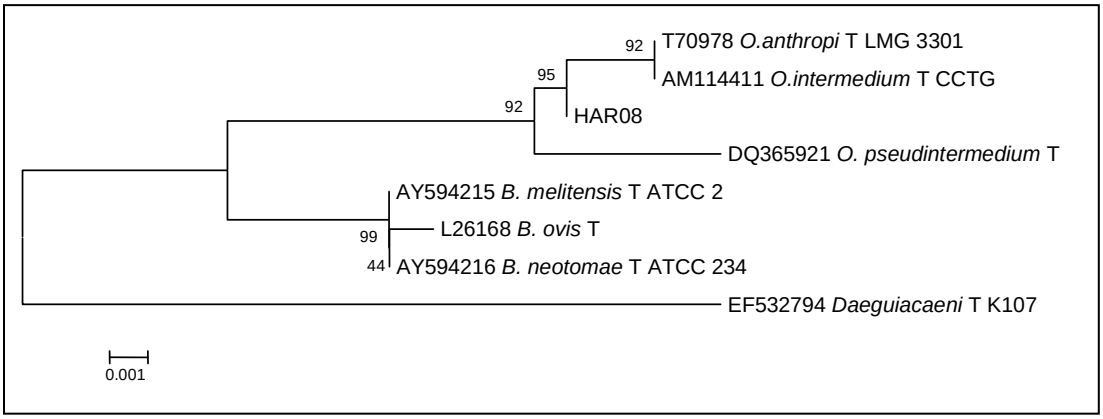

Figure 7 – Phylogenetic analysis of isolates by 16s rRNA gene sequence. The percent numbers at the nodes indicate the levels of bootstrap analysis support based on neighbor-joining analysis of 1000 replicates. The scale bars indicate the genetic distances.

Figure 8

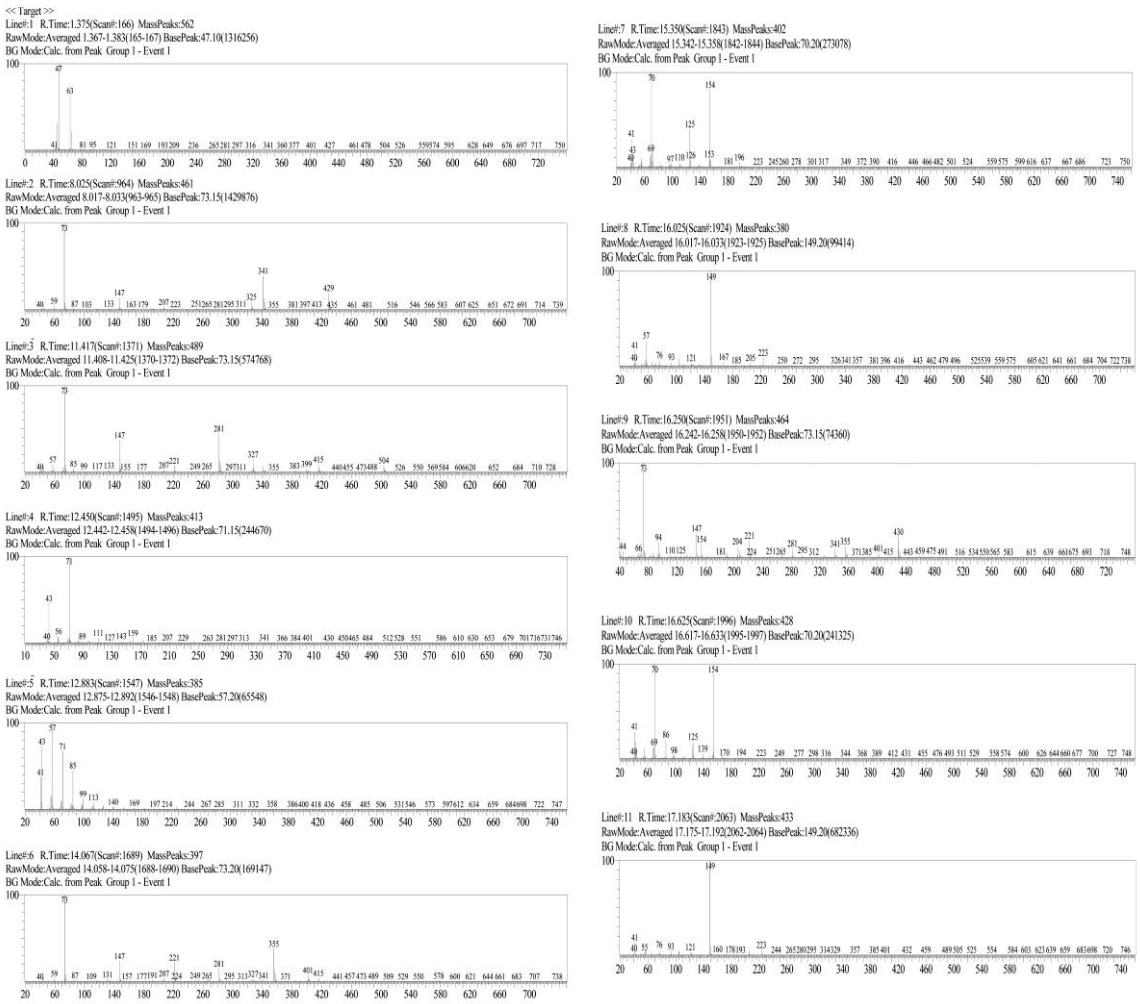

Figure 8 – GC-MS Report of HAR 08.

Conclusion

The results, thus obtained, have characterized the dye degrading bacterial strain *Ochrobactrum anthropi* HAR08 efficient in dye degradation with significant reduction in COD. The strain HAR08 was able to tolerate, decolorize, and degrade the azo dye ARAC at high concentration (10 g L^{-1}) within 24 h, which is very high concentration till now reported. The biodegradation and detoxification potential of the bacterial strain HAR08 makes it better candidate in treatment of dye effluents.

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