Indian J.Sci.Res. 5(1) : 139-152, 2014

ISSN : 0976-2876 (Print)
ISSN : 2250-0138 (Online)

BIODEGRADATION STUDY OF REACTIVE BLUE 172 BY *Shewanella haliotis* DW01 ISOLATED FROM LAKE SEDIMENT

RADHIKA BIRMOLE[a], SWAMINI PATADE[b], VISHWAS SIRWAIYA[c], FIRDAUS BARGIR[d] AND ARUNA K[e1]

[abcde]Department of Microbiology, Wilson College, Mumbai, India

ABSTRACT

Reactive Blue 172 (RB-172) dyeis a reactive sulphonateddiazo dye commerciallyused in the textile industry. Anovel bacterialstrain which degrades RB-172 was isolated from lake watersediment and identified as *Shewanella haliotis* DW01based on physiological and biochemical tests together with 16S rRNAgene sequence analysis. The 93% dye decolorizing activity of 50 ppm RB-172 by *Shewanella haliotis* DW01 was obtained with an inoculum size of 0.8 $O.D_{530nm}$ inoptimized Luria Bertani medium (Tryptone 0.5%, yeast extract 0.5%, NaCl 1%) pH9.5 at 35^0C kept under static condition for 12hwith high COD reduction.The isolate demonstrated significant decolorization over a wide range of NaCl concentration (1-7%).The addition of inorganic nitrogenous compounds to the medium significantly decreased the decolorization process. Lactate and pyruvateweresupporting RB-172 decolorization. Maximum dye concentration decolorized by the isolate after12hwas 550 ppm. An improved decolorization performance was obtained in the presence of higher biomass concentration. Decolorization assay using pre-grown cell mass of *Shewanella haliotis* DW01 exhibited 87% decolorization after 6h. The bacterium was immobilized and the decolorization process was monitored using UV-Vis Spectrophotometry. The degradation of dye was confirmed by HPTL Cand HPL Canalysis. Toxicity results suggested that degradation products of RB-172 werenontoxic to the common crop such as *Phaseolus mungo* and *Triticum aestivum*. It also showed ability to decolorize seven different dyes and mixtures of few dyes.

KEYWORDS : *Shewanella haliotis* DW01, Reactive Blue 172 (RB-172), decolorization, biodegradation

Azo dyes are the largest and most versatile class of synthetic dyes because their synthesis is simple and cost effective and havethe greatestvariety of colors. So they are widely used in textiles, leather, plastics and cosmetic industries (Selvam et al., 2003). Azo dyesare xenobiotic compounds, which are characterized by containing one or more azo groups (-N= N-).A variety of synthetic dyestuffs released in open waters by the textile industries not only pose an aesthetic problem(Allenand Koumanova, 2005) but also have a toxic impact on aquatic life and eventually affect human health (Brown and Devito, 1993). Since azo dyes are highly persistent and ubiquitously distributed in the environment they represent a threat to thesafety of the environment (Selvam et al., 2003; Pearce et al., 2003; Zollinger, 1991).

The treatment of azo dye waste water has become a matter of great concern, and several advanced physical and chemical treatment methods have been suggested and studied. However, they are not widely applied because of the high cost and the inadvertent production of more toxic intermediates. Compared with these methods, biological methods are environment-friendly and more economical(Tan et al., 2009).

In conventional activated sludge treatment most of the azo dyes are resistant to aerobic microbial attack (Ganesh et al., 1994). Hence decolorization with anaerobic microbes is an important step for the biotreatment of wastewater containingdyes. A variety of microorganisms such as obligateand facultative anaerobes can reduce azo dyes (Hu, 1994; Brown, 1981; Chung et al., 1978). Dyeing and printing waste water always has an alkaline pH and contains high amounts of inorganic salts, which usually inhibit the activities of most microorganisms (Mannu and Chaudhari, 2003. Therefore, exploitation of the salt-tolerant as well as alkaliphilic bacteria would be an alternative to the conventional biological treatment systems for azo dye waste water. Dhanve et al., 2008 have earlier reported 91.2% decolorization of the sulphonateddiazo dyeReactive blue 172by *Exiguobacterium* sp. RD3 in 48h. Fixed-bed decolorization of Reactive Blue 172 by *Proteus vulgaris* NCIM-2027 immobilized on Luffa cylindrical sponge has been studied by Saratale et al., 2011; Bhatt et al., 2005)has also studied decolorization of this dye using *Pseudomonas aeruginosa* NBAR12.

Shewanella strains are found to possess the ability to degrade a variety of azo dye (Hau and Gralnick, 2007), triphenylmethane (Cai et al., 2011) and anthraquinone dyes (Chen et al., 2010). Considering their potential marine origins, they might be ideal candidate for treatment of saline wastewaters (Xu et al., 2006).They are the most diverse

[1]**Corresponding author**

respiratory organisms which besides oxygen can respire with approximately 20 organic and inorganic compounds some of which are environmental pollutants. *Shewanella* species have been used for making microbial fuel cells by other researchers (Watson and Logan, 2010)

The present study focuses on the use of anovel salt-tolerant and alkaliphilic bacterium *Shewanella haliotis* DW01 isolated from the lake water sediment, to degrade one of the frequently used reactive textiledyes Reactive Blue 172.To our knowledge, this is the first study on efficient RB-172 decolorization using *Shewanella haliotis* DW01 whichmight help thedevelopment of biological process for treatment of saline and alkalinedye-polluted wastewaters. Optimization of cultural conditions for degradation of RB-172 by *Shewanella haliotis* DW01 is also discussed in this paper.

MATERIALS AND METHODS

Sample

Lake water sediment from Vasai Lake, Thane district was collected in a sterile flask for screening of dye decolorizing and degrading bacteria.

Dyes and Chemicals

Textile dye Reactive blue-172 (λmax568.4nm) and other dyes Reactive green 19 A (λmax650nm), Reactive Red 2 (λmax534.8nm), Reactive orange 94 (λmax491.26nm), Reactive red120 (λmax515.6nm) and Reactive orange 13 (λmax486.2nm) used for the study were purchased from Atul dyes, Mumbai, India. Other dyes such as methylene blue, malachite green, crystal violet, toluidine blue and congo red of analytical grade were purchased from E. Merck AG. Darmstadt. All other chemicals were obtained from Hi-media Pvt. Ltd., Mumbai, India.

Enrichment, Isolation and Identification

One gm of lake water-sediment was suspended in 10ml of sterile phosphate buffered saline which was allowed to settle for 30mins and 5ml of supernatant was inoculated into two flasks, each containing 95ml of nutrient broth with 50ppm of dye RB-172. Dye stock solution of 10mg/ml was prepared in distilled water, autoclaved separately and was used to make a final concentration of 50ppm in the nutrient medium. One of the inoculated media was kept on rotary shaker at 150 rev min^{-1} while other was kept under static condition and boththe flasks were incubated at 30^0C for 7 days for enrichment of dye decolorizers. Isolation of bacterial species was carried out on Nutrient agar containing 50 ppm RB-172 dye from the flask showing decolorization. The morphologically distinct bacterial colonies were picked up for the dye decolorization study. The promising isolate was selected by carrying out decolorization assay using RB-172 and was maintained on nutrient agar medium having composition : peptone 0.5%, NaCl 0.5%, meat extract 0.3% and agar 2.5% at 4^0C. Identification of the isolate was done on the basis of morphological, cultural and biochemical tests (Kim et al., 2007) and the strain was confirmed by 16SrRNAgene sequence analysis.16S rRNA sequencing of isolated bacterium was carried out at Sci Genom Labs Pvt Ltd, Kerala, India. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.

Decolorization Assay

The selected isolate was inoculated ($0.8O.D_{530nm}$) in 20 ml nutrient medium with RB-172 (50ppm), incubated for 12h at 35^0C under static condition. The experiment was carriedout in triplicate with an abiotic control and a blank. Abiotic control without bacterial culture was kept to analyze abioticloss of the dye. The aliquots (4 ml) were withdrawn, centrifuged (10,000 rpm/10min) to separate bacterial biomass and absorbance of the supernatant was measured at568.4nm (λmax of RB-172 dye). The cell pellet was resuspended in an equal volume of methanol to extract the dye adsorbed to cell surface. The methanol sample was centrifuged and supernatant was read atλmax of dye used (Ozdemir et al., 2008). Decolorization was measured by UV-visible spectrophotometer (Systronics2203, India) to calculate the percentdecolorization.

The percent decolorization was calculated using the formula;

$$\% \text{ Decolorization} = (A-B)/A \times 100;$$

where A is initial absorbance of control dye and B is observed absorbance of degraded dye (Dhanve et al., 2008).

Screening of Media for Decolorization

Optimization of decolorization was studied by using various culture media such as LBM (Ozdemir et al., 2008), Mineral salt medium (MSM) (Khalid et al., 2008b), NM9 medium (Wu et al., 2009), M9 medium (0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.5 % NaCl, 0.1 % NH_4Cl, pH-7.2), Nutrient broth (1 % peptone, 0.5 % NaCl, 0.3%, meat extractand pH-7) and synthetic medium (0.25 % $(NH_4)_2SO_4$, 1.33 % of KH_2PO_4 and 2.16 % Na_2HPO_4 with 0.1% yeast extract, pH-7).

Effect of Physicochemical Factors

Various physicochemical parameters such as OD_{530nm} (0.2-1.2), pH (4-10.5), temperature (5-55^{0}C) were studied to optimize decolorization assay using medium LBM. RB-172 decolorization was studied under static and shaker conditions.

Effect of Saline Conditions on Decolorization of RB-172

To study the effect of NaCl concentration, decolorization of RB-172 was investigated in presence of 1-7% NaCl. Effects of other factors on decolorization were studied keeping theNaCl concentration constant at 1%.

Effect of Addition and Substitution of Organic Nutrient Sources

The effect of organic nutrient sources was studied by substituting, yeast extract and tryptone in the LBM with meat extract (0.5%) and Peptone (0.5%) respectively.

Effect of Inorganic Nitrogenous Compounds as Electron Acceptors

The influence of various inorganic nitrogenous compounds (0.5% of each to LBM) such as Ammonium nitrate, Sodium nitrate, Sodium nitrite, Potassium nitrate, and Potassium nitriteas alternative electron acceptor on RB-172 decolorization was checked under static condition.

Effect of Various Electron Donors on Growth of the Isolateand Decolorization of RB-172

The suitability of various carbohydrates and organic acids as growth supportive nutrient for the isolate and its potential to decolorize RB-172 under anaerobic condition was determined. To study this effect, different sugars(1gm%) like fructose, lactose, glucose, sucrose, maltose, xylose, mannitol, arabinose, starch and various organic acids (1gm%) like Sodium lactate, Sodium pyruvate, Sodium formate, Sodium acetate and serine were added to M9 medium.

Effect of Biomass Concentration on Dye Decolorization

To investigate the effect of biomass concentration, at different sampling times (3h to 24h with an interval of 3h), the cells were separated by centrifugation and the cell pellet was dried at 35^{0}C till constant dry weight of the cells was obtained (Ren et al., 2006).To evaluate the effect of pregrown cell mass on RB-172 decolorization, the cells were inoculated (0.2O.D_{530nm}) in LBM, and dye (50ppm) was added 6h after incubation at 35^{0}C.

Effect of Initial Dye Concentration

The effect of initial dye concentration was studied using the concentration range from 50ppm to 800ppm.The results for optimization assays were recorded in the shortest time during which maximum decolorization of RB-172 was observed. Results are presented as the average of triplicate experiments.

Immobilization Technique

Immobilization of the isolate was carried out using 4% sodium alginate and 0.2M $CaCl_2$ solutions (Usha et al., 2010) and decolorization assay for RB-172 was performed.

COD Measurement

COD of the decolorized medium was determined after 24h. The diluted supernatant was refluxed with potassium dichromate, in presence of silver sulfate and mercury sulfate at 100^{0}C. The refluxed solution was then titrated with ferrous ammonium sulfate (FAS) using ferroinindicator (Guivarch et al., 2003).

Toxicity Studies

The toxicity tests were performed in order to assess the toxicity of the dye and its degradation products at the concentration of 500ppm on germination of seeds.The degradation products of the dye RB-172, extracted with equal volume of ethyl acetate were dried and dissolved in distilled water. The toxicity study was carried out at room temperature (30±2^{0}C) for *Phaseolus mungo* and *Triticum aestivum* seeds (10 seeds each per petriplate bedded with filter paper). The seeds were sprayed with 5ml solutions of RB-172 dye (500ppm in distilled water) and its degradation products on each day. Control set was carried out using distilled water. Length of plumule (shoot), radicle (root)

was recorded after seven days (Gomare et al., 2009).

Analytical Methods

Decolorization of RB-172 was monitored using UV-Vis spectrophotometer. The supernatant medium of decolorization at different time intervals was used for wavelength scan from 200 to 800nm (Dhanve et al., 2008).

Metabolites produced after biodegradation of the RB-172 wereextracted with equal volumes of ethyl acetate and concentrated using rotary evaporator. The concentrated dry residue was dissolved in HPLC-grade methanol and then used for analysis by HPTLC and HPLC.The extracted metabolites and dye (control) were subjected to HPTLC analysis by spotting on pre-coated silica gel platesand the solvent system used was methanol : ethylacetate : n-propanol : water : acetic acid (1:2:3:1:0.2 v/v).HPTLC analysis was carried using Camag TLC scanner and detection done at 254nm(Dhanve et al., 2008) and 366nm.HPLC analysis was performed using 410 Prostar Binary LC using C18column (4.6×250mm) and HPLCgrade methanol as mobile phase.

Decolorization of Various Dyes and Mixture of Dyes

The percentage decolorization of the different dyes (50ppm) such asReactive red 2, Reactive orange 94, Reactive red 120,Reactive green 19 A, Reactive orange 13, Methylene blue, Malachite green, Crystal violet, Toluidine blueand mixture of dyes was determined in LBM. The dye mixtures were prepared by using combination of three different dyes (50ppm of each dye).

RESULTS AND DISCUSSION

Screening and Identification of the Dye Decolorizing Organisms

Screening of the dye decolorizing organisms from the lake water sediment resulted in nine isolates. The promising isolate showed highest decolorization performance under static condition and was selected for present study.The cultural, morphological, biochemical tests and 16S rRNA gene sequence analysis identified this promising isolate as *Shewanella haliotis* DW01.The nucleotide analysis of the sequence was performed using at BlastN site from the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST) and the isolated pure bacterial species was here by identified as *Shewanella haliotis* DW01.

Effect of Physicochemical Factors

Ecosystems are dynamic environments with variable abiotic conditions like pH, temperature, presence of oxygen, metals and salts. Microorganisms are affected by changes in these parameters and consequently their decomposing activities are also affected. Thus, while evaluating the potential of different microorganisms for degrading particular organic xenobiotics, the effects of these parameters are to be taken into account. Optimization of such physicochemical factors was performed, and results of these would greatly help in the development of industrial scale bioreactors for bioremediation (Ali, 2010).

Screening of Media for Decolorization

Shewanella haliotis DW01 decolorized RB-172 in various liquid media as shown in figure 1. LBM supported 87% decolorization of RB-172 in 12h. It can be presumed that yeast extract and tryptone in LBM supplied the growth promoting nutrients such as carbon and nitrogen sources which can be easily assimilated by the cells(Dhanve et al., 2008).

Effect of Optical Density of the Culture Suspension on Dye Decolorization

Increase in cell number using optical density as a parameter did not influence decolorization of RB-172 by *Shewanella haliotis* DW01 extensively. Maximum decolorization of 83% was obtained with 0.8 $O.D_{530nm}$ which was used for all decolorization assays.

Effect of pH on Decolorization

The optimumpH range for the decolorization of the dye RB-172 by *Shewanella haliotis* DW01 was found to be 5.0 to 10 with maximum decolorization at pH 9.5 in 12h

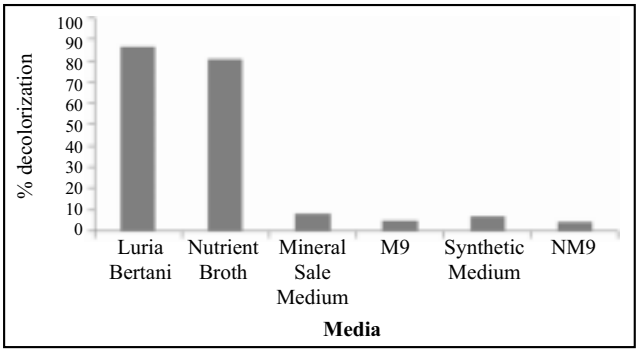

Figure 1 : Effect of Medium Composition on Decolorization of RB-172 Under Static Condition by *Shewanella haliotis* DW01 Observed in 12h

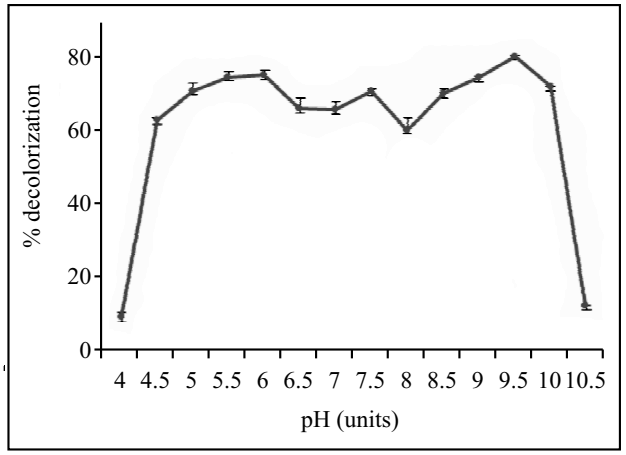

Figure 2 : Effect of pH on Decolorization of RB-172 Under Static Condition by *Shewanella haliotis* DW01 Observed in 12h

(figure 2). Though similar results were reported by Li and Guthrie, 2010 for the free resting cells of *Shewanella* strain J18 143 which exhibited dye reduction at a pH range of 5.6-9.2 but time required was 18 to 24h. To investigate the pH of the colour removal system involving an enzymatic process is important as denaturation of the relevant enzyme could occur if the required pH range was not maintained. The optimum pH for dye reduction was determined as it gives an indication of the extent to which the colored waste water, which is often strongly alkaline, must be diluted and /or buffered prior to treatment (Pearce, 2004; Ali, 2010). Initial buffering step in biological treatmentof colored waste water can be avoided usingalkaliphilic *Shewanewlla haliotis* DW01.

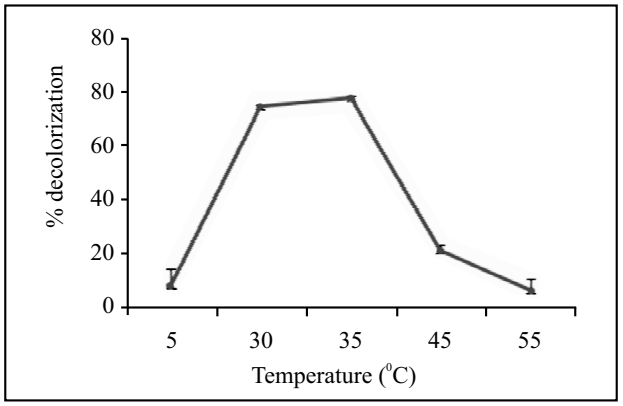

Figure 3 : Effect of Temperature on Decolorization of RB-172 Under Static Condition by *Shewanella haliotis* DW01 Observed in 12h

Effect of Temperature on Decolorization

The decolorization of RB-172 by *Shewanella haliotis* DW01 was found to be significant at 30^{0}C and 35^{0}C with maximum decolorization 78% at 35^{0}C in 12h but decreased drastically at extreme upper and lower limits of temperature (figure 3). These results are more promising thanthe reported decolorization of RB-172 by *Exiguobacterium* sp. RD3 which was 91.2% at 30^{0}C in 48h (Dhanve et al., 2008).Temperature is an important environmental factor, and the biodegradation activities of microorganisms are affected by changes in temperature. Beyond the optimum temperature, the degradation activities of the microorganisms decrease because of slower growth and denaturation of enzymes (Ali, 2010).

Effect of Aeration on Decolorization

Under static condition,82% decolorization of RB-172 was achieved by *Shewanella haliotis* DW01 while53% color removal efficiency was obtained under shaker conditionin 12h. However, the decolorization of RB-172 was demonstrated only under static condition by *Exiguobacterium* sp. RD3 and *Proteus vulgaris* NCIM-2027 (Dhanve et al., 2008; Saratale et al.,2001).Though significant 98% decolorization of Amaranth by *Shewanella decolorationis* S12 was reported under static condition but was taken 36 h and almost no reduction was observed under shaker condition (Hong et al., 2007b).Effect of oxygen on cell growth and dye decolorization is one of the most critical factors to be considered. Oxygen is a very effective electron acceptor for energy conservation due to its high redox potential (Hong et al., 2007b). Earlier reports have revealed that aeration might enhance the competition between the azo compounds and oxygen for reduced electron carriers under aerobic conditions and thus decrease the decolorization process (Khalid et al., 2008a; Chang et al., 2001; Kalme et al., 2007).

Effect of Saline Conditions on Decolorization of RB-172

Shewanella haliotis DW01 exhibited 81-88% decolorization of RB-172 in LBM containing various concentrations ofNaCl (1-6%) in 12h (figure 4). Complete decolorization of Reactive black-5 by *Shewanella* putrefaciens AS96 was observed in medium containing 6% NaCl in 24h (Khalid et al., 2008b). There are other studies showing salinity effects on the growth of bacteria and

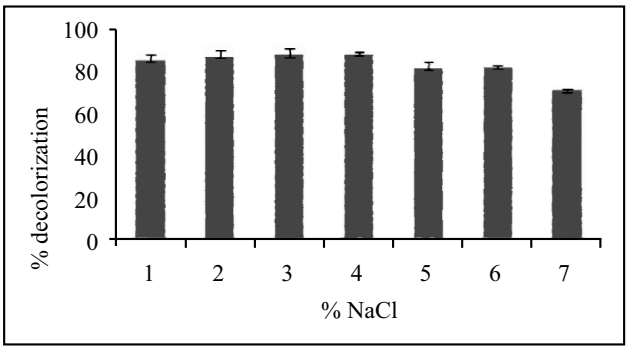

Figure 4 : Effect of Varying Concentrations of NaCl on Decolorization of RB-172 Under Static Condition by *Shewanella haliotis* DW01 Observed in 12h

decolorization in highly saline waste waters (Kairigi and Dincer, 1998; Panswad and Anan, 1999). Salt concentrations up to 15 to 20% have been measured in waste waters from dye stuff industries. Sodium levels are also elevated when sodium hydroxide is used in dye bath to increase the pH. These high concentrations of sodium generally suppress microbial growth at levels above 3gml-1. Among the various azo dye degrading bacteria, members of the genus *Shewanella* are potential candidates, being widely distributed in aquatic environments (Ivanova et al., 2001), that are purportedly able to thrive at salt concentrations up to 6%(w/v) (Yoon et al., 2004).The identification of this salt tolerant bacteriummay facilitate the development of biological treatment of saline azo dye solutions using bioreactor (Khalid et al., 2008b).

Effect of Addition and Substitution of Organic Nutrient Sources

Substitution of nutrient sources like yeast extract (0.5 %) and tryptone(0.5%) from LBM with 0.5% of meat extract and peptone respectively showed decrease in percent decolorization of RB-172 by *Shewanella haliotis* DW01 while addition of extra nutrient sources like meat extract(0.5%) and peptone(0.5%) to LBM, did not speed up decolorization process(figure 5). Therefore it can be inferred that yeast extract and tryptone provide essential nutrients which play a significant role in favouringdye decolorization. Hence, none of the additional organic nutrient sources were incorporated in the medium for further decolorization assay.Dyes are deficient in carbon content and biodegradation without any extra carbon source is found to be very difficult (Padmavathy et al., 2003).Many investigators have reported yeastextract (50 gm l-1) to be the most effective carbon and nitrogensource for the decolorization of azo dye(Kapdan et al., 2000; Hu, 1996).

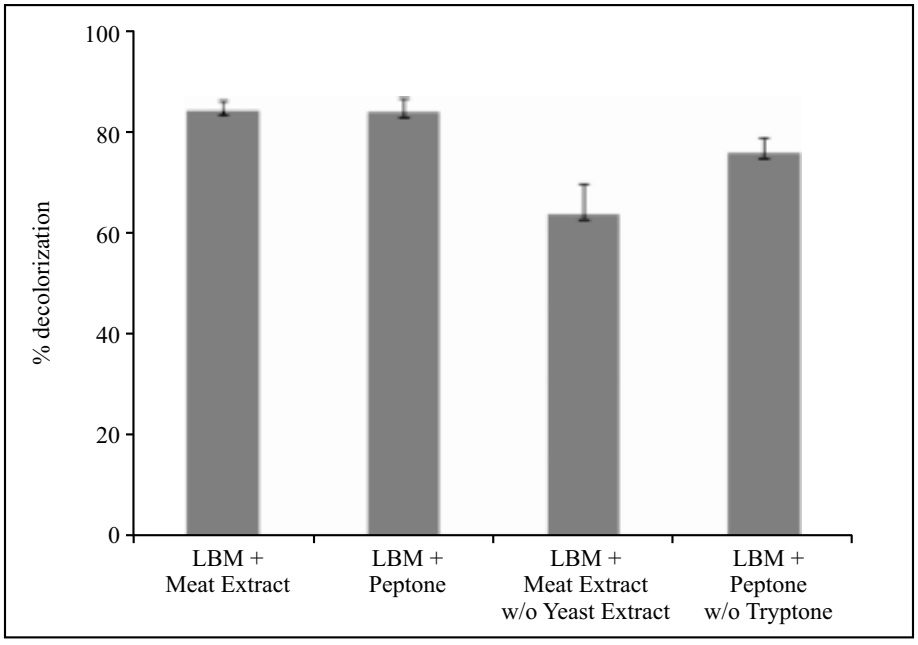

Figure 5 : Effect of Addition and Substitution of Organic Nutrient Sources on Decolorization of RB-172 Under Static Condition by *Shewanella haliotis* DW01 Observed in 12h.

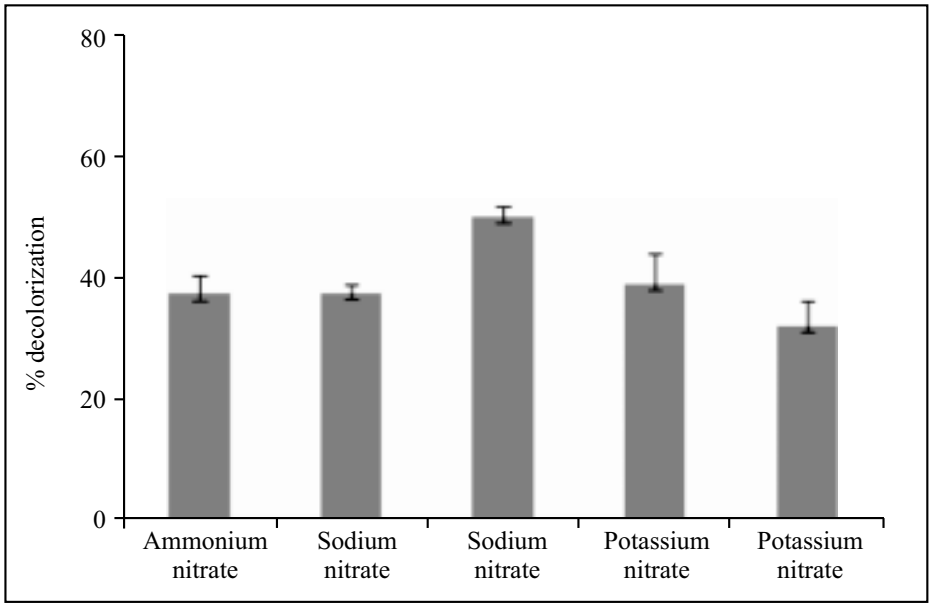

Figure 6 : Effect of Electron Acceptors on Decolorization of RB-172 Under Static Condition by *Shewanella haliotis* DW01 Observed in 12h

Effect of Inorganic Nitrogenous Compounds as Electron Acceptors

Almost 30-50% reduction in decolorization potential of RB-172 by *Shewanella haliotis* DW01was observed in presence of various nitrates and nitrites added to LBM as shown in figure 6. The results are in agreement with those published by other researchers who have evaluated the interference of nitrate with azodye biodecolorization Carliell et al., 1998; Wuhrmann et al., 1980. Xiao et al., 2012 have reported significant inhibition of decolorizationof NGB by *Shewanella* one idensis MR-1 in presence of nitrite and nitrate. The initiation of azo dye decolorization by Shewanellaputrefaciens AS96 and *Shewanella decolorationis* S12 was also delayed by the presence of nitrate (Khalid et al., 2008b; Hong et al., 2007a). Surprisingly, nitrate was not found to inhibit azo dye decolorization by *Shewanella* strain J18 143 (Pearce et al., 2006). Nitrate is used as an electron acceptor for anaerobic growth of *Shewanella* species, and is a potent regulator of the enzymes required for respiration using other electron acceptors. Decolorizaion is an oxidation reduction reaction in which azo dyes serve as electron acceptor. In presence of nitrate as an electron acceptor, the process of color removalslows down (Carliell et al., 1995; Panswad and Laungdilik, 2000).Sodium nitrate is one of the typical salts included in dye baths for improvement of dye fixation to the textile fibres and concentration can reach 40-100g/dm3 (Carliell et al., 1998). Thus, nitrate is also a co-contaminant that can be present in dye waste water, and may therefore play a role in reducing the effectiveness of dye reduction by *Shewanella*, potentially down-regulating azo reduction activity or acting as an electron sink in preference to azobonds(Pearce et al., 2006).Hence, to decrease the concentration of NO_3^- and NO_2^- is a very important strategy for effective decolorization of azo dyes under anaerobic condition (Hong et al., 2007b).

Effect of Various Electron Donorson Growth of *Shewanella haliotis* DW01and Decolorization of RB-172

Growth of *Shewanella haliotis* DW01 was found to cease in presence of various sugars(1gm% of fructose, lactose,glucose, sucrose, maltose, xylose, mannitol, arabinose, starch) in M9 medium while significant growth was observed in presence of amino acid serine and other organic acids (1gm%of Sodium lactate, Sodium pyruvate, Sodiumformate, Sodiumacetate) (data not shown). The results are in accordance with Nealson and Scott, 2006 who screened many isolates of *Shewanella* for carbon utilization and showed their inability to use complex carbon sources. *Shewanella* speciesrarely utilise glucose, and are non-fermentative (Ringo et al., 1984).These strains prefer

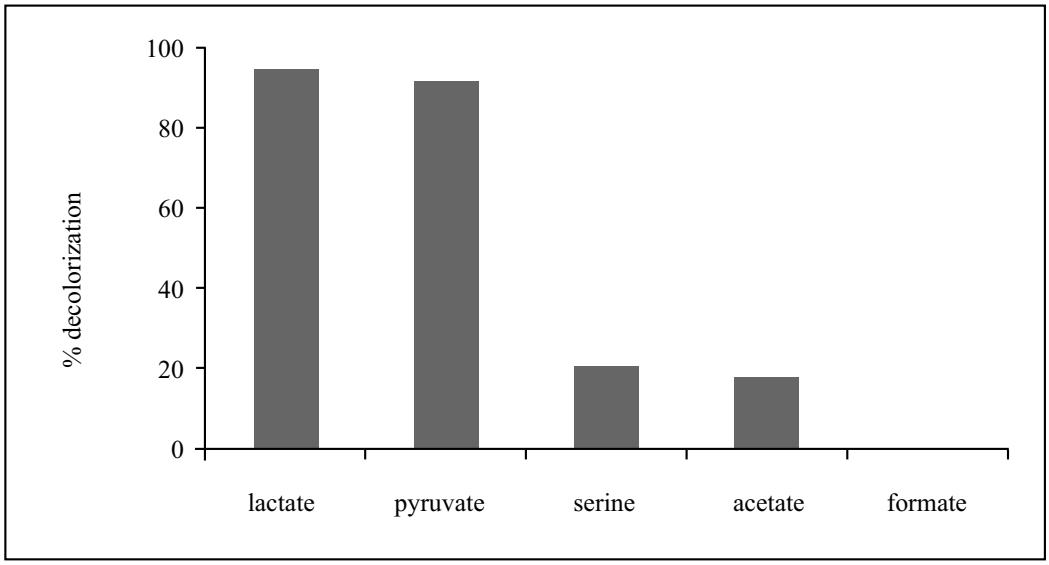

Figure 7 : Effect of Electron Donors on Decolorization of RB-172 Under Static Condition by *Shewanella haliotis* DW01 Observed in 24h

lactate, pyruvate or simple amino acids(Myers and Nealson, 1988).

Various organic acids like 1gm % of Sodium lactate, Sodium pyruvate, Sodium formate, Sodium acetate and amino acid serine in M9 medium were also checked for their potential to act as electron donor for RB-172 decolorization by *Shewanella haliotis* DW01. The two most efficient decolorization performances were observed with lactate and pyruvate, resulting in 95% and 92% decolorization of RB-172 respectively in 24h, but acetate, formate and serine were not effective electron donors for azoreduction (figure 7). Meng et al., 2012, have also reported lactate and pyruvate to be good electron donors forazo dye AR27 decolorizationby *Shewanella decolorationis* S12. Xu et al., 2007 reported lower decolorization of Acid red GR by *S. decolorationis* S12in presence of formate as electron donor.

Effect of Biomass Concentration on Dye Decolorization

Maximum percent decolorization of RB-172 was observed in presence of higher cell concentration of *Shewanella haliotis* DW01. The percent decolorization was in direct proportion to biomass concentration ranging from 0.32 to 6.3 g l-1. About 70.69% and 82.36% of RB-172 was removed in 3 and 6h when the biomass concentration was 0.325 g l -1 and 0.375 g l -1, respectively. Over 82 to 95% decolorization was achieved in 9, 12, 15, 18, 21, 24h for systems containing 0.4, 0.5, 2.95, 4, 4.825, 6.3 g l -1 *Shewanella haliotis* cells, respectively. Similar results were obtained for decolorization of AR27 by *Shewanella aquimarina* (Meng et al., 2012). The time required for 93% decolorization of 50ppm of RB-172 decreased from 12h to 6h when pre grown cells of *Shewanella haliotis* DW01 were used in place of actively growing cells in optimised LBM. After 6h of cultivation, the biomass produced in aerobic condition was around tenfold more than that produced in anaerobic condition. When the aerobic culture was switched to anoxic static condition, color disappeared in 6h due to fast consumption of the dissolved oxygen by the large biomass previously built up under aerobic condition, indicating that anaerobic condition would be essential to achieve an effective azo reduction. Similar results were observed in decolorization of amaranth by *Shewanella decolorationis* S12 (Hong et al., 2007b).

Effect of Initial Dye Concentration

Decolorization percentages decreased from 88% to 18%with increase in initial dye concentrations from 100ppm to 550ppm of the RB-172 by *Shewanella haliotis* DW01. Maximum percentdecolorization (88%) was achieved at 100ppm of RB-172 in 12h and minimum 18% decolorization observed at 550ppm RB-172 in 12h (figure 8). This decrease in decolorization with increase in initial

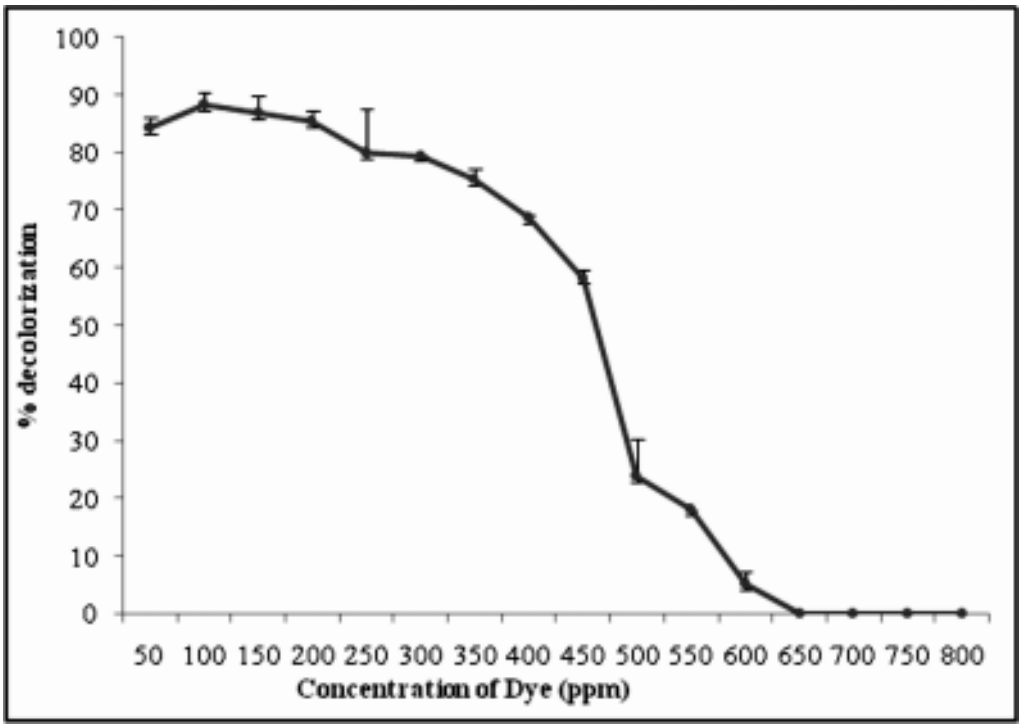

Figure 8 : Effect of Initial Dye Concentration on Decolorization of RB-172 Under Static Condition by *Shewanella haliotis* DW01 Observed in 12h

dye concentration is attributed to the toxicity of the dyes to the growing microbial cells at higher dye concentrations. In the previous study of RB-172, similar results were observed with maximum 91.2% decolorization and minimum 11.11% decolorization of 50ppm and 500ppm of dye respectively in 48h (Dhanve et al., 2008; Kapdan et al., 2000). However, *Pseudomonas aeruginosa* NBAR12 decolorized RB-172 dye within crease in initial dye concentration from 50 to 400ppm in 24h and was inhibited above 500ppm of dye (Bhatt et al., 2005). Dyes at higher concentration can inhibit the microbial cell growth due to interference with synthesis of nucleic acids (Ogawa et al., 1986). It was also observed that reactive group of azo dyes having sulphonic acid (SO_3H) groups on aromatic rings greatly inhibits the growth of microorganisms at high concentration (Dhanve et al., 2008). Concentrations of reactive dyes in dye house effluents have been reported to range from 60-250ppm (Pierce, 1994). Thus our bacterial culture, which could decolorize dyes much above the reported dye concentration in waste waters, can be successfully employed for treatment of textile effluent.

Immobilization Technique

Three repeated additions of dye RB-172 (50ppm) in LBM were decolorized by immobilized cells in the alginate beads with a retention time of 90min for each addition of dye. The time required for complete decolorization of RB-172 decreased from 12h to 90mins when immobilized cells were used in place of non-immobilizedcells.This could be due to conditions achieved inside the beads, where partition effects led to a modified microenvironment (Brodelius and Vandamme, 1987). Entrapment is the most widely used technique for immobilization of whole cells, and alginate is a suitable matrix material because it is nontoxic and the method used for its gelation is mild towards the microorganisms (Sriamornsak, 1998).

COD Measurement

The decolorized medium showed 80.68% reduction in COD as compared to that of control medium with Reactive blue 172. Reduction in COD to 80.68% within 24h indicates the biodegradation from complex dye to simple oxidizable products. This COD reduction

Table 1 : Toxicity Study of RB-172 and its Degraded Metabolites by *Shewanella haliotis* DW01

Plant name	Average length in centimetre					
	Water		RB-172 dye[a]		Metabolites[b]	
	Radicle	Plumule	Radicle	Plumule	Radicle	Plumule
P. mungo	3.94	0.87	1.80.63		2.07	1
T. aestivum	1.17	0.76	0.30.41		1.38	0.64

[a]500ppm concentration of RB-172
[b]500ppm concentration of metabolites resulted from degradation of RB-172 dye

performance is much higher than the COD reduction (56% within 48h) of Reactive blue 172 by isolated *Exiguobacterium* sp RD3 reported earlier (Dhanve et al., 2008).

Thus, from the economic as well as environmental point of view, implementing *Shewanella haliotis* DW01to current biological methods for dye decolorization could be good alternative because it serves the purpose of decolorization and also reduces COD efficiently.

Toxicity Studies

Toxicity studies were performed to confirm the nontoxic nature of the degradation product of RB-172. The results of toxicity studies of RB-172 degradation products revealed neither inhibitory nor harmful effects. Besides it supported shoot and root growth of both *Phaseolus mungo* and *Triticum aestivum* (table 1).

Biodecolorization and Biodegradation Analysis

The UV-Vis spectral analysis showed (figure 9) decrease in absorbance from 0.882 to 0.095at 568.4 nm and appearance of new peak at 375.2nm that confirmed decolorization. If dye removal is attributed to biodegradation, either the major visible light absorbance peak would completely disappear or a new peak will appear indicating that the color removal by the isolated bacterial strain is due to the biodegradation (Chen et al., 2003).The cell pellet was also used to extract the adsorbed dye if any, using methanol. Further when the methanol extract was centrifuged and supernatant was subjected to UV- Vis Spectrophotometric analysis it showed no absorbance at the λmax (568.4 nm) of dye.It can be inferred that decolorization mechanism by this isolate was due to degradation and not due to adsorption of the dye by the cells. HPTLC analysis of ethyl acetate extracted metabolites of RB-172 degradation by *Shewanella haliotis* DW01 showed the disappearance of spot corresponding to parent dye with Rfvalue 0.53 and concomitant appearance of other spot with

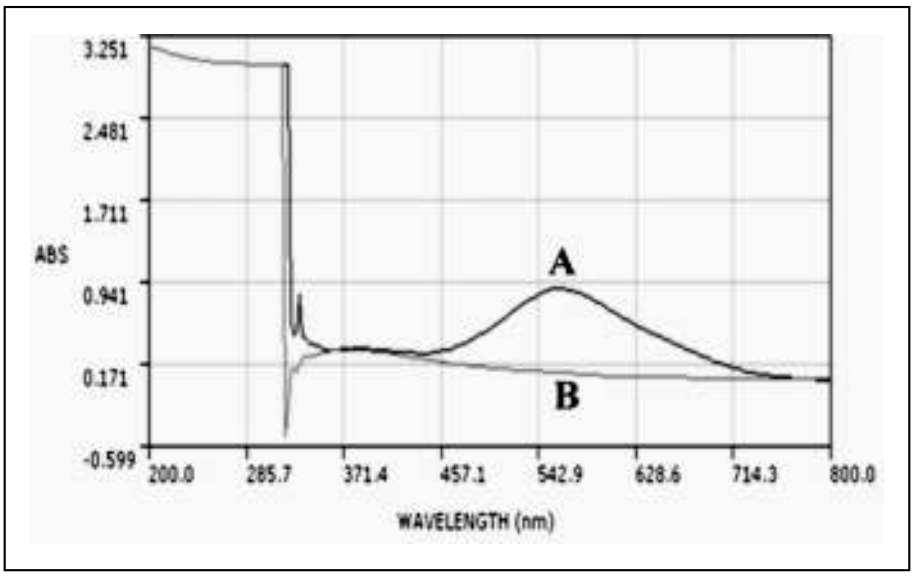

Figure 9 : Variation in UV-Vis Spectra of Reactive Blue-172 Before and After Decolorization by *Shewanella haliotis* DW01 A-0 h B-12h

Table 2 : Decolorization of Different Textile Dyes (15h) by *Shewanella haliotis* DW01

Name of the dye[a]	λ max (nm)	%Decolorization
Reactive Red 2	534.8	77.13
Reactive orange 94	491.26	91.71
Reactive red 120	515.6	80.12
Reactive green 19A	650	93.71
Reactive orange 13	486.2	81.20
Methylene blue	684	77.90
Malachite green	620	-
Crystal violet	690	-
Toluidine blue	629.6	-
Congo red	483	84.66

[a]LBM containing 50ppm of dye

Rfvalue 0.71.Also, analysis at 366nm indicated presence of florescence bands in the degradation product which were absent in the control dye solution, thus confirming biodegradation of RB-172 by *Shewanella haliotis* DW01.

HPLC elution profile of RB-172 and metabolites extracted after its degradation showed different retention times. RB-172 showed major peak at retention time 9.283min, whereas degradation products showed major peaks at retention time 4.379min and 6.065min that confirmed the degradation of RB-172.

Decolorization of various textile dyes and mixture of dyes

Shewanella haliotis DW01 could decolorize five azo dyes in addition to RB-172 and also methylene blue and congo red in LBM within 15h (table 2). Amongstazo dyes, the organism showed highest decolorization (93.71%) of Reactive green 19A and 84.66% decolorization of Congo red in 15h. Yatome et al., 1981 and A. N. et al., 2002 have reported decolorization of boththriphenylmethane andazo dyes by a single species of bacteriumlike *Pseudomonas pseudomallei* 13NA and *Citrobacter* sp.

Shewanella haliotis DW01 also demonstrated decolorization of four dye mixtures, viz., mixture no.1,2,3 and 4 respectively in 24h as shown in table3. Utilization of *Shewanella haliotis* DW01 for the treatment of the textile waste water would prove its worthiness as the textile effluent contains mixture of various dyes with varying concentrations (Patil et al., 2010).

CONCLUSION

Shewanella haliotis DW01 isolatedfrom lake watersediment resulted in 93% decolorization of 50ppm of azo dye Reactive blue-172in optimized LBM pH 9.5 within12h at 35^{0}C under static condition. Biodegradation of this dye was confirmed by UV-Vis spectrophotometry, HPTLC and HPLC analysis. The isolate could tolerate upto 550ppm concentration of RB-172 and no decolorization was observed due to inhibition of growth when the initial concentration was more than 600ppm.The organism being salt tolerant can be used for the treatment of saline textile effluent. Higher biomass concentration and addition of lactate and pyruvate were conducive for decolorization. Almost 30-50% reduction in decolorization potential was observed in presence of nitrates and nitrites. In addition to excellent decolorization activity, *S. haliotis* DW01 was also able to degrade and mineralize the dye leading to a good COD reduction in the decolorized medium. Toxicity studies suggested the nontoxic nature of degradation products. The bacterium was immobilized and its results revealed its potential application to be used in a permanent bed for bioremediation of textile waste water. The isolate also has an ability to decolorize a variety and mixture of textile dyes.Overall findings suggest need to exploit this strain for bioremediation of dye-polluted saline waste waters of

Table 3 : Decolorization of Different Dye Mixtures (24h) by *Shewanella haliotis* DW01

Dye Mixture	Mixture Components	λ max(nm)	% Decolorization
1	Reactive orange 13 +Reactive blue 172	512	87.42
2	Reactive orange 13+ Reactive green 19A	644	90.63
3	Reactive green 19A+ Reactive blue 172	605.6	93.14
4	Reactive orange 13+ Reactive green 19A+ Reactive blue 172	603.2	90.02

alkaline pH discharge from textile and other dye-stuff industries.

ACKNOWLEDGEMENT

We are grateful to UGC for extending financial assistance in the form of major research project[F.No. 40-191\2011 (SR)].

REFERENCES

AliH., 2010. Biodegradation of synthetic dyes A review. Water, Air, Soil pollut., **213**: 251-273.

Allen S.J. and Koumanova B., 2005.Decolourisation of water/wastewater using Adsorption (review). J. Univ. Chem. Technol. Metallurgy., **40**: 175-192.

AN S. Y., Min S. K., Cha I. H., Choi Y. L., Cho Y. S., Kim C. H. and Lee Y. C., 2002. Decolorization of triphenylmethane and azo dyes by Citrobacter sp. Biotechnol. Lett., **24**: 1037-1040.

Bhatt N., Patel K. C., Keharia H. and Madamwar D., 2005.Decolorization of diazo-dye Reactive Blue 172 by *Pseudomonas aeruginosa* NBAR12. J. Basic Microbiol., **45**: 407-418.

Brodelius P. and Vandamme E.J., 1987.Immobilized cell systems, Biotechnology Enzyme Technology. 7a, Weinheim, Federal Republic of Germany: VCH Verlagsgesellschaft, 405-464.

Brown J.P., 1981.Reduction of polymeric azo and nitro dyes by intestinal bacteria. Appl. Environ. Microbiol., **41**: 1283-1286.

Brown M.A. and DeVito S.C., 1993.Predicting azo dye toxicity, Crit. Rev. Environ. Sci. Technol., **23**:249-324.

Cai P. J., Xiao X., He Y. R., Li W. W., Chu J., Wu C., He M. X., Zhang Z., Sheng G. P. and Lam M. H. W., 2011. Anaerobic biodecolorization mechanism of methyl orange by *Shewanella oneidensis* MR-1. Appl. Microbiol. Biot., **253**:3508-3588.

Carliell C.M., Barclay S.J., Shaw C., Wheatley A.D and Buckley C.A., 1998. The effect of salts used in textile dyeing on microbial decolourisation of a reactive azo dye. Environ. Technol., 19: 1133-1137.

Carliell C.M., Barday S.J., Nadidoo N., Buckley C.A., Mulholland D.A. and Senior E., 1995. Microbial decolorization of a reactive azo dye under anaerobic conditions.Water. S.A., **21**: 61-69.

Chang J., Chou C., Lin Y., Lin P., Ho J. and Hu T.L., 2001. Kinetic characteristics of bacterial azo dye decolorization by *Pseudomonas luteola*. Water Res., **35**: 2841-2850.

Chen C. H., Chang C. F. and Liu S. M., 2010. Partial degradation mechanisms of malachite green and methyl violet B by *Shewanella decolorationis* NTOU1 under anaerobic conditions. J. Hazard. Mater., **177**: 281-289.

Chen K.C., Jane Y.W., Liou D.J and Hwang S.C.J., 2003.Decolorization of the textile dyes by newly isolated bacterial strains. J. Biotechnol., **101**: 57-68.

Chung K. T., Fulk G. E. and Egan M., 1978. Reduction of azo dyes by intestinal anaerobes. Appl. Environ. Microbiol., **35**:558-562.

Dhanve R. S., Shedbalkar U. U. and Jadhav J. P., 2008. Biodegradation of Diazo reactive dye Navy blue HE2R (Reactive blue 172) by an isolated *Exiguobacterium* sp. RD3.Biotechnol. Bioprocess Eng., 13: 53-60.

Ganesh R., Boardman G.D. and Michetsen D., 1994.Fate of azo dyes in sludges. Water Res. **28**: 1367-1376.

Gomare S., Tamboli D., Kagalkar A. and Gowindwar S., 2009. Eco friendly biodegradation of a reactive textile dye Golden Yellow HER by *Brevibacilluslaterosporus* MTCC 2298. Int. Biodeterior. Biodegrad., **63**: 582-586.

Guivarch E., Oturan N. and Oturan M.A., 2003.Removal of organo phosphorus pesticides from water by electrogenerated Fenton's reagent. Environ. Chem. Lett. **1**: 165-168.

Hau H. H. and Gralnick J. A., 2007. Ecology and biotechnology of the genus *Shewanella*. Annu. Rev. Microbiol., **61**: 237258.

Hong Y. G., Guo J., Xu Z. C., Xu M. Y., Sun G. P., 2007a. Respiration and growth of *Shewanella decolorationis* S12 with an azo compound as the sole electron acceptor. Appl. Environ. Microbiol.,**73**: 64-72.

Hong Y. C., Chen X., Guo J., Xu Z., Xu M. and Sun G., 2007b. Effects of electron donors and acceptors on anaerobic reduction of azo dyes by *Shewanella decolorationi*s S12. Appl. Microbiol. Biotechnol., **74**: 230-238.

Hu T. L., 1994. Decolourization of reactive azo dyes by transformation with *Pseudomonas luteola*. Bioresour. Technol., **49**:47-51.

Hu T.L., 1996. Removal of reactive dyes from aqueous solutions by different bacterial genera. Water Sci. Technol., **34**: 89-95.

Ivanova E. P., Sawabe T., Gorshkova N. M., Svetashev V. I., Mikhailov V. V., Nicolau D. V. and Christen R., 2001. *Shewanella japonica* sp. nov. Int. J. Syst. Evol. Microbiol., **51**: 1027-1033.

Kalme S., Ghodake G. and Govindwar S., 2007. Red HE7B Degradation Using Desulfonation by *Pseudomonas desmolyticum* NCIM 2112. Int. Biodeterior. Biodegrad., **60**: 327-333.

Kapdan K. I., Kargi F., Mullan G. and Marchant R., 2000. Decolorization of textile dyestuff by a mixed bacterial consortium. Biotechnol Lett., **22**: 1179-1181.

Karigi F. and Dincer A.R., 1998. Saline wastewater treatment by halophile supplemented activated sludge culture in an aerated rotating biodisc contactor. Enzyme Microb. Technol., **122**: 427-433.

Khalid A., Arshad M. and Crowley D. E., 2008a. Accelerated decolorization of structurally different azo dyes by newly isolated bacterial strains. Appl. Microbiol. Biotechnol., **78**: 361-369.

Khalid A., Arshad M. and Crowley D. E., 2008b. Decolorization of azo dyes by *Shewanella* sp. under saline conditions. Appl. Microbiol. Biotechnol.,**79**: 1053-1059.

Kim D., Baik K., Kim M., Jung B., Tai-Sun-Shin, Chung G., Rhee M. and Seong C., 2007. *Shewanella haliotis* sp. nov., isolated from the gut microflora of abalone, *Haliotis discus hannai*. Int. J. Syst. Evol. Microbiol.,**57**: 2926-2931.

Li T. and Guthrie J., 2010. Colour removal from aqueous solutions of metal-complex azo dyes using bacterial cells of *Shewanella* strain J18 143. Bioresour. Technol., **101**: 4291-4295.

Mannu B. and Chauhari S., 2003.Decolorization of indigo and azo dyes in semicontinous reactors with long hydraulic retention time. Process Biochem., **38**:1213-1221.

Meng X., Liu G., Zhou, Fu S. and Wang G., 2012.Azo dye decolorization by *Shewanella aquimarina* under saline conditions. Bioresour. Technol., **114**: 95-101.

Myers C. R. and Nealson K. H., 1988. Bacterial manganese reduction and growth with manganese oxide as the sole electron acceptor. Science.,240: 13191321.

Nealson and Scott, Prokaryotes 3rd Edition. 6 (2006) 1133-1151.

Ogawa T., Yatome C., Idaka E. and Kamiya H., 1986.Biodegradation of azo dyes by continuous cultivation of *Pseudomonas cepacia* 13NA. J. Soc. Dyes Colour.,**102**: 12-14.

Ozdemir G., Pazarbasi B., Kocyigit A., Omeroglu E., Yasa I. and Karaboz I., 2008. Decolorization of Acid Black 210 by *Vibrio harveyi* TEMS1, a newly isolated bioluminescent bacterium from Izmir Bay, Turkey. World J. Microbiol. Biotechnol, **24**: 1375-1381.

Padmavathy S., Sandhyak., SwaminathanY., Subramanium T., Chakrabarti S., Kaul, 2003. Aerobic decolorization of reactive azo dyes in presence of various co substrates. Chem. Biochem. Eng. 17: 147-151.

Panswad T. and Anan C., 1999. Impact of high chloride wastewater on an anaerobic/anoxic/aerobic process with and without inoculation of chloride acclimated seeds. Water Res., 33: 11651172.

Panswad T. and Luangdilik W., 2000.Decolourisation of reactive dyes with different molecular structure under different environmental conditions. Water Res., 34: 4177-4184.

Patil P.S., Phugare S.S., Jadhav S.B. and Jadhav J.P., 2010.Communal action of microbial cultures for Red HE3B degradation.J. Hazard. Mater.,**181**: 263-270.

Pearce C.I., 2004. The reduction of coloured compounds using whole bacterial cells (Shewanella strain J18 143). PhD thesis, University of Leeds, UK.

Pearce C. I., Christie R., Boothman C., Canstein H. V., Guthrie J. T. and Lloyd J.R., 2006. Reactive azo dye reduction by *Shewanella* strain J18 143. Biotechnol.Bioeng.,**95**: 692-703.

Pearce C. I., Lloyd J. R. and Guthrie J.T., 2003.The removal of colour from textile wastewater using whole bacterial cells- A review. Dyes Pigm.,**58**:179-196.

Pierce J., 1994. Colour in textile effluents-the origin of the problem. J. Soc. Dyers Colourists.,**110**: 131-134.

Ren S., Guo J., Zeng G. and Sun G., 2006.Decolorization of triphenylmethane, azo, and anthraquinone dye by a newly isolated *Aeromona shydrophila* strain. Appl. Microbiol. Biotechnol.,**72**: 1316-1321.

Ringo E., Stenberg E. and Strom A. R., 1984. Amino acid and lactate catabolism in trimethylamine oxide respiration of *Alteromonas putrefaciens* NCMB 1735. Appl. Environ. Microbiol., **47**: 1084-1089.

Saratale G. D., Saratale R.G., Chang J. S. and Govindwar S.P., 2011. Fixed-bed decolorization of Reactive Blue 172 by *Proteus vulgaris* NCIM-2027 immobilized on *Luffa cylindrica* sponge. Int. Biodeterior. Biodegrad., **65**:494-503.

Selvam K., Swaminathan K. and Keo-Sang C., 2003.Microbial decolorization of azo dyes and dye industry effluent by Fomeslividus. World J. Microbiol. Biotechnol.,**19**: 591-593.

Sriamornsak P., 1998. Preliminary investigation of some polysaccharides as a carrier for cell entrapment. Eur. J. Pharm. Biopharm., **46**: 233-236.

Tan L., Qu Y., Zhou J., Li A. and Gou M., 2009. Identification and characterization of a novel salt-tolerant *Exiguabacterium* sp. for azo dyes decolorization. Appl. Biochem. Biotechnol., **159**:728-738.

Usha M. S., Sanjay M. K., Gaddad S. M. and Shivannavar C. T., 2010. Degradation of h-acid by free and immobilized cells of *Alcaligenes latus*. Braz. J. Microbiol., **41**: 931-945

Watson V. J., and Logan B.E., 2010. Power production in MFCs inoculated with *Shewanell aoneidensis* MR-1 or mixed cultures. Biotechnol. Bioengin., **105**:489-498.

Wu J., Kim K., Sung N., Kim C. and Lee Y., 2009. Isolation and characterization of *Shewanell aoneidensis* WL-7 capable of decolorizing azo dye Reactive Black 5. J. Gen. Appl. Microbial., 55: 51-55.

Wuhrmann K., Menscher K. and Kappeler T., 1980. Investigation on rate determining factors in microbial reduction of azo dyes. Eur. J. Appl. Microbiol. Biotechnol.,**9**: 325-338.

Xiao X., Xu C., Wu Y., Cai P., Li W., Du D. and Yu H., 2012. Biodecolorization of Naphthol Green B dye by *Shewanella oneidensis* MR-1 under anaerobic conditions. Bioresour. Technol., **110**: 86-90.

Xu M., Guo J. and Sun G., 2007.Biodegradation of textile azo dye by *Shewanella decolorationis* S12 under microaerophilic conditions. Appl. Microbiol. Biotechnol.,76: 719-726.

Xu M.Y., Guo J., Zeng G.Q., Zhong X.Y. and Sun G.P., 2006. Decolorization of anthraquinone dye by *Shewanella decolorationis* S12. Appl. Microbiol. Biotechnol.,71:246251.

Yatome C., Ogawa T., Koga D. and Idaka E., 1981.Biodegradibility of Azo and triphenylmethane dyes by *Pseudomanas pseudomallei* 13NA. J. Soc. Dyers Colour.,**97**: 166-168

Yoon J.H., Yeo S.H., Kim I.G. and Oh T.K., 2004. *Shewanella marisflavi* sp. nov. and *Shewanella aquimarina* sp. nov., slightly halophilic organisms isolated from sea water of the Yellow Sea in Korea. Int. J. Syst. Evol. Microbiol.,**54**: 2347-2352.

Zollinger H., 1991. Color chemistry: syntheses, properties and applications of organic dyes and pigments, 2nd edn., Wiley, New York,.