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Degradation of Drimarene Red, a reactive textile dye by an extremophilic *Bacillus sp.*isolated from fresh water

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Abstract

Azo dyes are known for their application in textile dyeing by virtue of their wide range of colours. During the dyeing process, almost 30% of the dye remains unused and enters the effluent stream. Presence of Glauber's salt (sodium sulphate) or sodium chloride in concentrations 40-100 gl^{-1} and the highly alkaline (pH 9.8-11.8) nature of the effluent makes conventional treatment (activated sludge process) difficult, as the normal microbial consortium cannot carry out degradation in such conditions. An ideal predominant microorganism, an alkaliphilic and halotolerant bacterium, has been isolated and shown to degrade an azo textile dye (Drimarene Red). The isolate was identified as a species of Bacillus (closely resembling Bacillus beveridgei) and designated Bacillus sp. SG2, based on 16S rDNA sequence analysis. Dye degradation assessed primarily by decolourization efficiency was assessed under different conditions of temperature, initial pH, aeration and sodium chloride concentration. 90% decolourization was seen at ambient temperature under stationary conditions at pH 9.5 and sodium chloride concentration of 5% (w/v) at 24 hours of incubation. UV-Vis absorbance spectra showed that the maximum absorption peaks for the coloured solutions (522 nm and 542 nm) disappeared after 24 hours of incubation. The m/z values of the peaks obtained after HPLC-MS analysis of the uninoculated broth control (344.05 and 655.28) and the decolourized samples (226.95) indicate that the decolourization can be attributed to biodegradation of the dye.

Key words: Alkaliphilic bacteria; Bacillus sp.; decolourization; microaerophilic conditions; eactive azo dye; Textile dye house effluent.

I. INTRODUCTION

The most widely used colourants for textile dyeing are azo dyes, for their range of brilliant shades, various application methods and relatively low cost (Asad et al., 2007; Pandey et al., 2007; Przystas et al.,2012; Wong et al., 2007)[1,2,3,4]. These dyes contribute to almost 70% of the total dyes used in textile globally (Wang et al., 2013) [5]. Azo dyes have poor exhaustion properties (use up in the dyeing process) and therefore almost 30 % of the dye applied for dyeing gets washed out into the effluent.

In natural environments, azo dyes (chromophore -N=N-) are recalcitrant, resistant to light treatment and commonly escape conventional treatment processes (Ong et al., 2011) [6]. The dyes are toxic to biota in waterbodies and soil into which they are disposed. Many microorganisms in soil and waterbodies reduce the azo dyes, non-specifically, to their corresponding amines (Wang et al., 2013) [5]. These amines, though colourless, are known to be carcinogenic and pose a greater danger to aquatic life than the dye itself (Soares et al., 2006) [7].

Treatment methods for textile dye house wastewater presently include physical and chemical methods such as adsorption, flocculation, coagulation, precipitation, ozonisation, irradiation etc. (Soares et al., 2006; Wang et al., 2009a; b) [7, 8, 9]. Each of these methods (**Table 1**) (B. Ramesh Babu et al.,2007) [10] though effective in dye removal from the waste waters, is complex and generates dye-containing sludge thereby

limiting their application and cost-effectiveness. Owing to these limitations, biological methods can provide an alternative. Biological anaerobic treatment followed by aerobic process has been employed for dye house effluent treatment[11, 12, 13, 14]. Azo dyes have been shown to be decolourized anaerobically by enzymes (azoreductases) to form a corresponding amine and these amines are further oxidized to safer non-toxic products [15].

Table 1. Possible treatments for cotton textile wastes and their associated advantages and disadvantages (B. Ramesh Babu et al., 2007)

Processes	Advantages	Disadvantages
Biodegradation	Rates of elimination by oxidizablesubstances about 90%	Low biodegradability of dyes
Coagulation– flocculation	Elimination of insoluble dyes	Production of sludge blocking filter
Adsorption on activated carbon	Suspended solids and organic substances well reduced	Cost of activated carbon
Ozone treatment	Good decolorization	No reduction of the COD
Electrochemical processes	Capacity of adaptation to different volumes and pollution loads	Iron hydroxide sludge
Reverse osmosis	Removal of all mineral salts, hydrolyzes reactive dyes and chemical auxiliaries	High pressure
Nanofiltration	Separation of organic compounds of low molecular weight and divalent ions from monovalent salts. Treatment of high concentrations	-----
Ultrafiltration– microfiltration	Low pressure	Insufficient quality of the treated wastewater

Textile dyeing process involves use of harsh chemicals leading to generation of effluents with high salt concentration and highly alkaline pH (**Table 2**) (B. Ramesh Babu et al., 2007) [10]. Hence, design and operation of microbial treatment processes should be centred on microorganisms that can thrive and show metabolic vigour in the extreme conditions existing in dye-house waste waters.

Table 2. Composition of cotton textile mill waste (B. Ramesh Babu et al., 2007)

Characteristics	Values
pH	9.8–11.8
Total alkalinity	17–22 mgl^{-1} as CaCO3
BOD	760–900 mgl^{-1}
COD	1400–1700 mgl^{-1}
Total solids	6000–7000 mgl^{-1}
Total chromium	10–13 mgl^{-1}

In the present study, a bacterium belonging to the genus *Bacillus* has been isolated and characterized to demonstrate its ability to decolourize and degrade Drimarene Red (a commercial azo dye), under different conditions of aeration, temperature, initial pH and sodium chloride concentration.

II. MATERIALS AND METHODS

A. Chemicals and media

Drimarene Red (DR), a reactive textile azo dye was obtained from a textile dyeing industry in Perundurai, India. Nutrient Broth and Agar (Hi-Media, India) were used for decolourization studies. All other chemicals (Thomas Baker, India) used were of laboratory grade.

B. Isolation, screening and identification of cultures

Water samples were collected in sterile containers at the confluence ("Sangam") of rivers Mula and Mutha in Pune, India, in an area where industrial effluents are discharged. The samples were transported to the laboratory and processed immediately for isolation of alkaliphilic and halophilic bacteria by enrichment in Alkaline and Saline Nutrient Broth (ASNB - Nutrient Broth containing 5% sodium chloride and pH adjusted to 9.5). Isolates differentiated on the basis of morphology were selected, checked for purity and screened for

decolourization of azo and tri-phenyl methane dyes. Three bacterial isolates, designated SG2, SG3 and SG4 were used for further study, as they showed distinct decolourization in cultures grown in ASNB. Of these, SG2 was used in these studies.

Identification of SG2 up to genus level was carried out on the basis of phenotypic, biochemical characterization by using the Bergey's Manual of Determinative Bacteriology (9^{th} edition). The 16S rRNA gene was amplified from genomic DNA, purified and sequenced as described previously (Pidiyar et al., 2004) [16]. The sequence was submitted to NCBI database to avail the accession number. To ascertain the phylogenetic affiliation of the strain SG2, its 16S rRNA gene sequences were manually corrected and aligned using CLUSTAL X2 (Larkin et al., 2007) [17]. Phylogenetic trees were constructed based on neighbour-joining (Saitou and Nei, 1987) [18] method using MEGA 5.2 (Tamura et al., 2011) [19]. Evolutionary distances were determined with Kimura's two parameter model (Kimura, 1980) [20]. Bootstrap analysis (Felsenstein,1993) [21] was performed for 1000 replications. Reference sequences of closely related type strains were retrieved from Ribosomal database project (RDP) under the accession numbers indicated on the trees.

C. DyeDecolourization

An aqueous solution of DR (1% w/v) was added to sterile ASNB to give final concentration of $100mgl^{-1}$. The culture was grown in sterile ASNB at ambient temperature overnight to give approximate cell density of 1 x 10^8 cells ml^{-1} and used as inoculum. 0.5 mL of this culture suspension was inoculated in 10 mL of ASNB (in 18mm x 150 mm test tubes) containing 100 mg l^{-1} of DR. The tubes were incubated at ambient temperature ($30^0C \pm 2^0C$) for 24 hours. After incubation the culture was centrifuged at 10,000 rpm for 10 min at ambient temperature. The supernatant obtained was used to determine residual concentration of DR.
'Percent decolourization' was calculated using the standard dose response curve of DR. The un-inoculated control was used to determine 0% decolourization.

$$\textbf{Percent Decolourization} = \frac{(\textbf{Dye concentration of Control} - \textbf{Dye concentration of Sample})}{\textbf{Dye concentration of Control}} \textbf{ x 100}$$

The effects of environmental factors such as initial pH of the medium (pH 6.0, 7.0, 8.0, 9.0, 9.5 and 10.0); temperature (30^0C and 37^0C); stationary and shake flask culture (aeration); sodium chloride concentration (0.5%,1%, 2%, 3%, 4%, 5% and 6%); and inoculum size (200µL, 400 µL, 600 µL, 800 µL and 1000 µL of overnight grown inoculum in 10mL of medium) on efficiency of decolourization of DR were investigated. Since decolourization efficiency was found to be maximum under stationary conditions, volume of the medium was gradually increased (5mL, 10mL, 15mL, 20 mL medium in 18 mm x150 mm test tube and 5mL medium was overlaid with sterile mineral oil) to determine decolourization efficiency under reduced oxygen availability.

D. Analytical Procedure

UV-Vis spectrum analysis.

The concentration of dye in all experiments was estimated using spectrophotometry (UV-Vis spectrophotometer Shimatzu-UV1800; 200-800nm). Estimations of residual/ initial dye concentrations were made using cell free supernatants of samples.

HPLC-MS Analysis.

The biodegradation products of DR produced by *Bacillus* sp. in ASNB were analysed by HPLC-MS. Culture samples were centrifuged at 10,000 x g for 20 minutes and the supernatant obtained was filtered through sterile 0.20 µm pore size filter. Aliquots of 2 µL of the filtrates were injected into a HPLC-MS system (Bruker Daltonik GmbH, Germany). A reverse phase C18 HPLC column (4.6 × 250 mm, 5 µm particle size) from Thermo Scientific ™ (Acclaim ™ 120) was used to separate the biodegradation products. The column temperature was set at 25°C. The mobile phase was composed of 0.1 % formic acid in water and 0.1 % formic acid in acetonitrile.

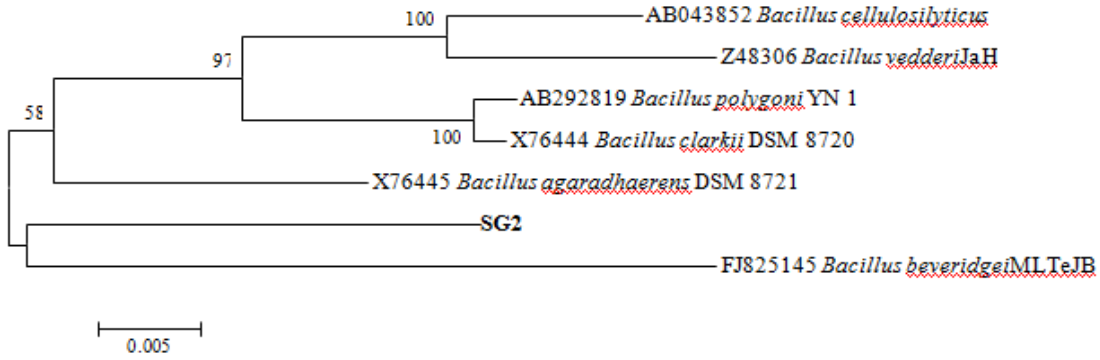
The isocratic elution profile consisted of mobile phase water: Acetonitrile (30:70) for 30 min at a flow rate of 1 mL min^{-1}. The quadrupole analyser was programmed to select ions with m/z in the range 50 to 1200 u. The ionization conditions were: cone gas flow (150 Lh^{-1}), desolvation gas flow (350 L h^{-1}), polarity (ESI+), capillary energy (3000 V), sample cone energy (30 V), extraction cone energy (2.0 V), desolvation temperature (120^0C), ionization energy (2.0 V), collision energy (4 V) and multi-channel plate detector energy (2700 V).

III. RESULTS AND DISCUSSION

Almost all dye degradation studies involving bacteria have focused on parameterization of conditions for maximum removal of dyes, irrespective of the conditions in which it would be applied for pollution control. The present study differs, in which the isolation was directed to obtain a bacterium that would grow and degrade the dye under conditions of application, namely textile dye house effluent. Biological methods have been reported for treatment of such effluents [6, 22, 23] however, their efficacy may be questioned when used for textile dye house effluent conditions. Bibliography [24] have tapped the biodegradation potential of textile effluent-adapted and non-adapted bacteria but only few studies report utilization of halophilic bacteria [25, 26, 27] and alkaliphilic bacteria [28] and halo/alkali stable enzymes for textile wastewater treatment [29].

An alkaliphilic, halotolerant, dye decolourizing bacterium was isolated from river water sample collected from an aquatic receiving body into which the industrial effluent was being disposed. Circular, orange-yellow coloured, raised colonies were obtained on ASNA (ASNB + 2% agar) after 24 hours incubation at ambient temperature (30°C ± 2°C). The isolate was Gram positive, rod-shaped, motile and facultative anaerobe. Phylogenetic analysis using 16S rRNA gene sequence of the strain (1361 bp, NCBI database accession number KT987233) indicated that its closest relative is *Bacillus beveridgei* (**Fig.1**).The isolate was designated as *Bacillus* sp. SG2.

Fig.1: The phylogenetic relationships of Bacillus sp. SG2 and the other species of genus Bacillus



Decolourization of Drimarene Red by *Bacillus* sp. SG2commenced within 6 hours of inoculation of ASNB with 100 mgL^{-1} dye, and 85- 90% decolourization was seen at 24 hours. On continued incubation up to 48 hours, decolourization increased to 95% (**Fig.2**). On testing for decolourization of other azo dyes by this isolate, it was seen to decolourize Congo Red and Drimarene Yellow (**Table 3**).

Fig. 2: Decolourization of DR by Bacillus sp. SG2. a- Test tube assay, b- 2L fermenter batch

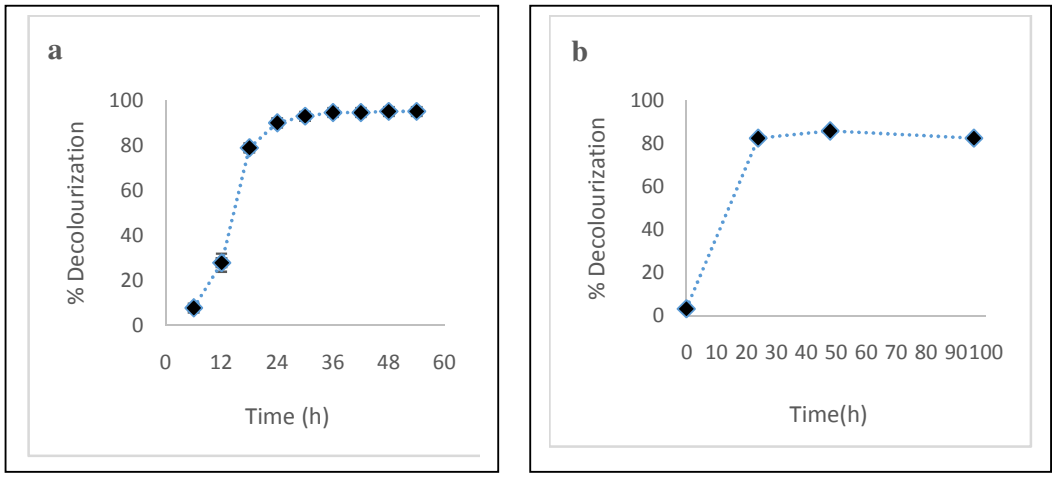


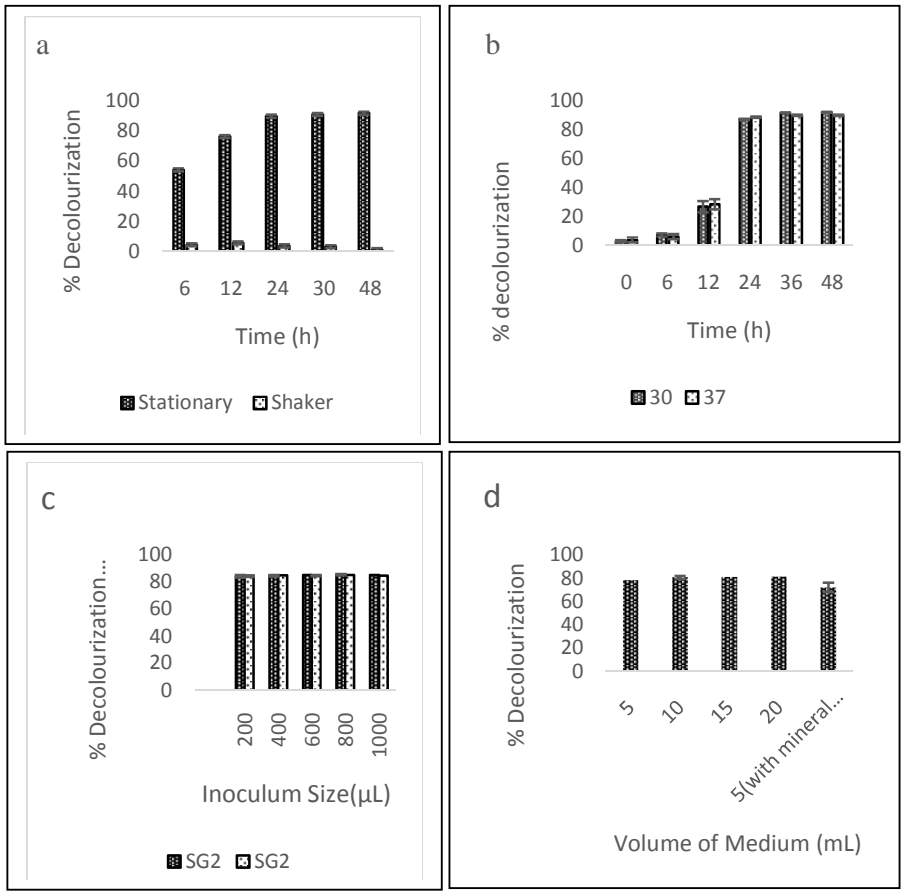
Table 3: Decolourization of textile dyes by SG2

extile Dye*	% Decolourization
Crystal Violet	**1.32**
Congo Red	**20.40**
Drimarene Red CLBL	**80.28**
Drimarene Blue HFRL	**0.75**
Drimarene Yellow CL2R	**7.45**

*Initial dye concentration 100 mgl^{-1}.

Stationary conditions favoured the decolourization process as compared to shake flask conditions (**Fig. 3a**). The decolourization after 24 hours in stationary conditions was 88% as compared to 3% in the shake flask conditions. All studies thereafter were carried out under stationary conditions. Incubation at different temperatures (30^0C and 37^0C), change in the inoculum size (0.2 % to 1.0 % v/v) and decolourization efficiency under reduced oxygen availability did not significantly affect the percent decolourization of DR (**Fig. 3b, 3c and 3d**).

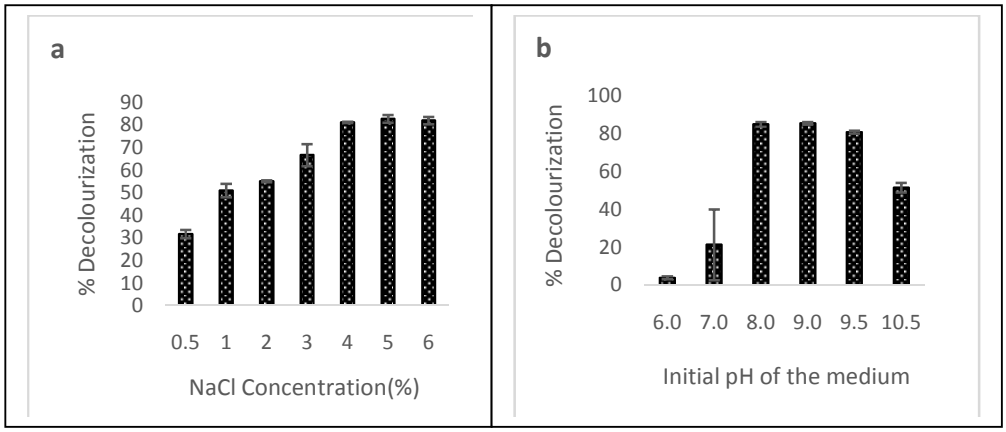
Fig. 3: Effect of a- aeration, b- temperature, c- inoculum size , d- oxygen availability on decolourization of DR by Bacillus sp.SG2



High concentration (40-100 gL^{-1}) of salt (either sodium chloride or Glauber's salt) is reportedly used in textile-dye baths for fixation of the dye to the cotton fibre resulting in highly saline effluent. Sodium chloride concentration significantly affected, wherein decolourization efficiency increased with increase in sodium chloride concentration. At 0.5% (w/v) sodium chloride concentration, 30% decolourization of DR was seen which increased with increasing salt concentration up to 5 %, where 83% decolorization was observed (**Fig. 4 a**).

When tested under different initial pH conditions, maximum decolourization was seen at pH 9.0 (85 % decolourization after 24 hours) (**Fig. 4 b**). It was also seen that significant decolourization of the dye occurred at pH 8.0 and above. Decolourization of the dye at pH values 6.0 and 7.0 was 3.85% and 21.22% respectively.

Fig. 4: Effect of a- sodium chloride concentration, b- initial pH of the medium on decolourization of DR by Bacillus sp.SG2

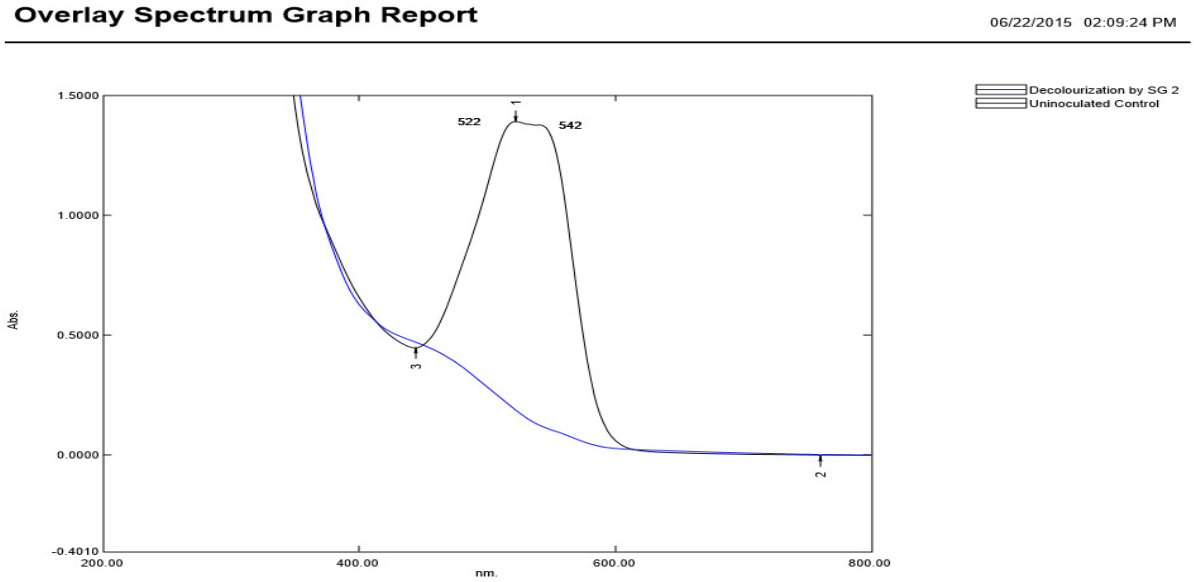


Efficient decolorization under the conditions shown above conclusively prove that the isolate is a good candidate organism for use for textile dye house effluent treatment.

The intended use of such an isolate would be to incorporate it as a primary degrader of the dye as a pollutant. Mere relocation of the dye onto the bacterial cell surface (adsorption) would not achieve the desired result. Hence, decolorization needs to be effected by degradation. To show that a degradative process is involved, UV-Vis spectrum analysis and HPLC-MS was carried out to ascertain the possible degradation process involved.

Decolourization of dyes by bacteria has been attributed to either adsorption or biodegradation. When decolourization is due to adsorption, the UV-VIS peaks diminish in proportion to each other and the cell pellet takes up the dye colour [25, 30]. When decolourization is due to biodegradation, the major visible light absorbance peak disappears completely or a new peak appears [23]. In this study, the UV-VIS spectrum (200-800 nm) of the control samples showed peaks at 522nm and 542nm which disappeared completely in the decolourized sample indicative of biodegradation of DR by the isolate (**Fig.5**).

Fig. 5: UV-VIS spectrum of Drimarene Red decolourization by Bacillus sp.SG2

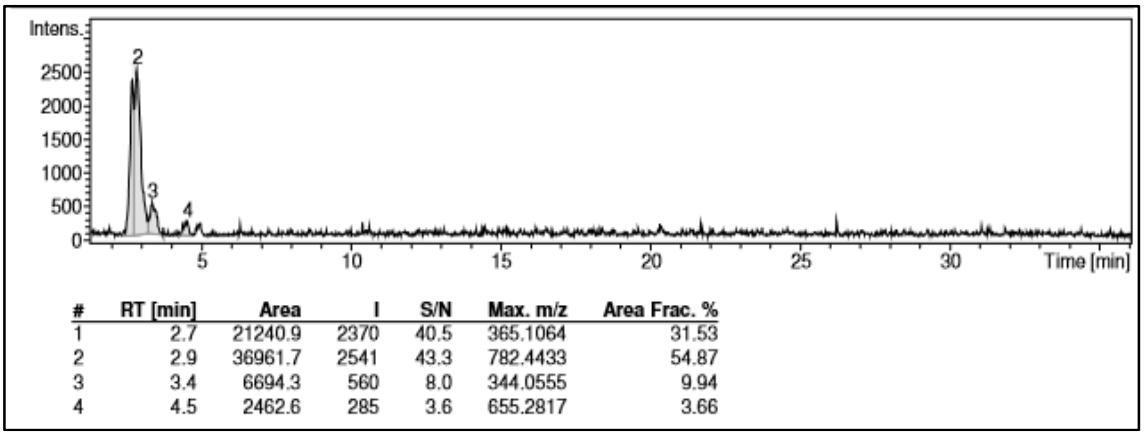


HPLC-MS chromatogram of un-inoculated ASNB broth containing the dye showed two peaks (**Fig.6 a**) corresponding to m/z values of 344.05 and 655.28 respectively, and the other two peaks with m/z values 365.1 and 782.44 seen in the chromatogram correspond to ASNB without dye. Degradation of DR was confirmed by this analysis (**Fig.6 b**), as the chromatogram shows a single major peak (m/z 226.95) which does not correspond to any of the peaks seen in the ASNB with dye.

Decolourization of DR by *Bacillus* sp. SG2 is therefore attributed to degradation of the dye and not adsorption. The conditions under which DR is efficiently degraded (stationary conditions, pH 9.5 and 5% sodium chloride) show that the isolate can be used effectively for reducing pollution loads of textile dye house effluents.

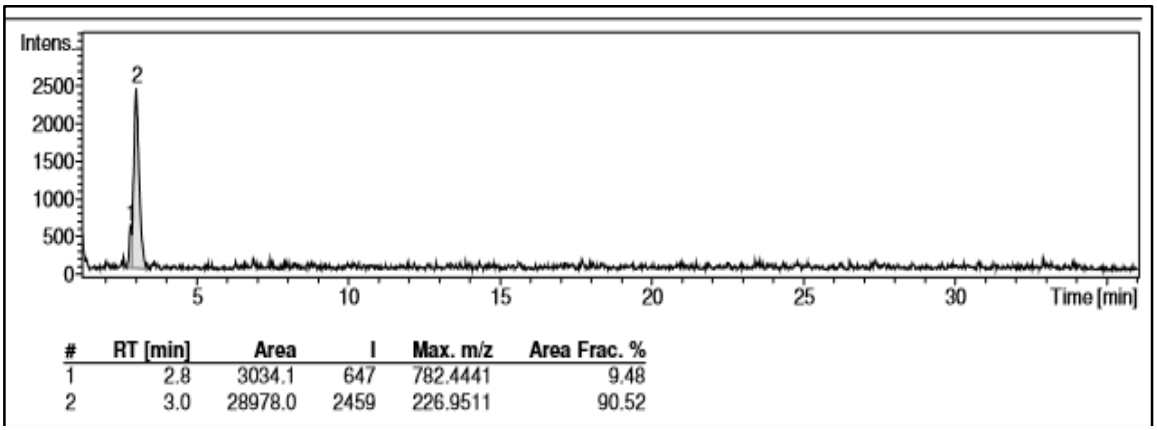
Fig. 6: LC-MS analysis of Bacillus sp.SG2 mediated degradation of azo dye Drimarene Red
a- Control dye b- Decolourized dye

a



#	RT [min]	Area	I	S/N	Max. m/z	Area Frac. %
1	2.7	21240.9	2370	40.5	365.1064	31.53
2	2.9	36961.7	2541	43.3	782.4433	54.87
3	3.4	6694.3	560	8.0	344.0555	9.94
4	4.5	2462.6	285	3.6	655.2817	3.66

b



#	RT [min]	Area	I	Max. m/z	Area Frac. %
1	2.8	3034.1	647	782.4441	9.48
2	3.0	28978.0	2459	226.9511	90.52

IV. CONCLUSION

Bacillus sp. (designated SG2; closely resembling *Bacillus beveridgei*) showing extremophilic characteristics (alkaliphily and halophily) was isolated from a non-extreme environment and gave efficient degradation (90%) of Drimarene Red, a reactive azo textile dye. The culture could degrade Drimarene Red under stationary conditions at pH 9.5 in the presence of 5% sodium chloride; conditions typical for textile dye house effluents. This isolate could be a promising candidate organism to tackle pollution loads of textile dye house effluents.

V. ACKNOWLEDGEMENT

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