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Biodegradation of Diazo Dye, Reactive Blue 160 by Isolate *Microbacterium* sp. B12 Mutant: Identification of Intermediates by LC-MS

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A B S T R A C T

A bacterial strain, B12, proficient in decolourizing commercial dye - Reactive Blue 160 (RB160) was isolated from the soil sample collected from a textile industry. Phenotypic characterization and phylogenetic analysis of the 16S rDNA sequence indicated that the bacterial strain belonged to the genus *Microbacterium*. Bacterial isolate B12 showed 96% decolourization of RB160 at the concentration of 250 mg/l under microaerobic conditions with temperature $35 \pm 2°C$ and pH 5.0 in 24 h. The decolourization efficiency of isolate B12 was improved by using physical and chemical mutagens, the mutant strain offered 12 h reduction in time required for the complete decolourization by wild strain when exposed to 30 sec of UV radiation and 0.5µg/ml of acridine orange. The laccase and reductase enzymes cleaved RB160 and metabolised the degraded product, the different intermediates were identified using HPLC and LC-MS. The phytotoxicity studies confirmed that intact RB160 was more toxic than dye degraded intermediates. *Microbacterium sp.* B12 was found competent to degrade RB160 containing textile industry effluents showing the usefulness of the organism for possible commercial application.

Introduction

The environmental problems created by the textile industry have received increased attention for several decades; the textile industry is one of the largest generations of contaminated effluents (Solis *et al.,* 2012). The discharge of dyes containing effluents in to the environment is of great concerned due to colour, toxicity, mutagenicity and carcinogenicity of dyes. Textile dyes have always been considered in the context of

recalcitrant xenobiotic compounds in water and soil ecology (Daneshwar *et al.,* 2007; Dafale *et al.,* 2010)

Reactive dyes consist of a chromophoric system (e.g azo, anthraquinone, phthalocyanine), the anchor groups and the group which increases the water solubility (mostly $-SO_3^-$). The decolorization of

reactive dyes results in destroying the chromophoric system, and formation of toxic/nontoxic byproduct (Bansal *et al.,* 2012). The toxic byproduct must be properly treated before disposing into the environment. Advanced oxidation methods such as sonolysis, fenton oxidation, ozonation, photochemical oxidation and electrochemical destruction are available for the treatment of dye containing effluents but biodegradation is considered as an economic one compared to the above mentioned processes (Dos Sontos *et al.,* 2007). The use of microbes that possess the ability to decolourize synthetic dyes including bacterial strains and fungi (Liu *et al.,* 2004; Hadibarata *et al.,* 2011a; Hadibarata *et al.,* 2012b).

For decolourization and degradation many techniques are practiced that includes bio-engineering of organisms. The technique, like, random mutagenesis, is used to induce mutation in organisms for better distinctiveness (Sandesh *et al.,* 2008). Mutagens such as UV irradation, Acridine orange are employed for inducing mutation (Chandra *et al.,* 2008 and Chen *et al.,* 2001). Improvement of the microbial strain by subjecting the genetic material to physical and chemical mutagenic agents offers furthermost opportunity for cost reduction without significant capital outlay (Stanbury *et al.,* 1995; Lofty *et al.,*; 2007).

The genus *Microbacterium* was described by Orla-Jensen (1919) and comprises a diverse collection of yellow pigmented gram positive, nonspore forming rods that were isolated from various environmental habitats like soil, water and steep liquor (Young *et al.,* 2010). *Microbacterium amylolyticum* sp. nov., isolated from soil from an industrial waste (Anand *et al.,* 2012). At present, the genus *Microbacterium* comprises 83 species (www.bacterio.cict.fr/m/microbacterium.html).

Reactive dye Blue 160 is a diazo commercial dye, commonly used for dyeing cotton, viscose, flex and jute but not suitable for silk, polyesters and wool. Very few studies on the decolourization and degradation of B160 have been reported.

In the present study, we first time report the use of *Microbacterium* sp. B12 for the decolourization and degradation of a Reactive Blue 160. Effect of physico-chemical parameters such as temperature, pH and agitation has been investigated to optimize the dye decolourization. The mutagenesis approach has been made to improve the efficiency of the decolourization by using UV irradiation and acridine orange mutagenes. Used LC-MS techniques to identify various intermediates. Furthermore the toxicity of the dyes both before and after the treatment with *Microbacterium* sp. B12 was evaluated by utilizing *Phaseolus aureus* seeds as a bioindicator.

Materials and Methods

Dyes, chemical and media

Diazo dye Reactive Blue 160 (RB160) (C.I. No. 137160) was procured from the Ahmadabad dyeing and printing industry, Ahmedabad, Gujarat, India. NADH was purchased from Sigma Chemical Company (USA). Nutrient broth was obtained from HiMedia Laboratory, India. All the chemicals used in the study were of analytical grade.

Strain Isolation and Culture Condition

Microorganisms used in this study were isolated from the soil samples of textile industry in Ahmadabad, Gujarat. The soil sample was added 1% w/v in nutrient broth containing RB160 250 mg/l and incubated at

$32\pm2°C$ temperature under microaerobic as well as aerobic conditions at 150 rpm conditions. After 24 h of incubation, 1.0ml of the culture was serially diluted and 0.1ml was withdrawn from 10^{-5} dilutions and inoculated by spread plate method on the nutrient agar plates containing 250 mg/l RB160. After 24 h of incubation at optimum temperature, the colonies were screened for their ability to form a clear zone around them. The selected colony with highest zone size was selected, isolated and subculture in nutrient broth containing 250mg/l RB160. Morphological and biochemical test were carried out to identify the selected organism.

Identification of Bacterial Isolate

Bacterial cells of strain B12 were collected by centrifugation at 10,000 *g* for 10 min and subjected to sequential digestion by lysozyme of 2.5 mg/l, 37 °C for 1 h and proteinase K was 20 mg/l in 1% SDS, 55 °C for 1 h, followed by incubation in 1% CTAB and 0.7 M NaCl at 65 °C for 15 min. After extraction with phenol/chloroform, the recovered DNA was precipitated with ethanol and then dissolved in ddH2O. The obtained product was amplified using forward primer 27f 5'-AGAGTTTGATCMTGGCTCAG-3' and reverse primer 1492r 5'CGGYTACCTTGTTACGACTT-3'.Sequence data analysis was done using Chromas Pro and Sequencing Analysis software.The sequence was input to NCBI to identify it by BLAST search.

Phylogenetic Tree Construction

All the sequences were compared with 16S rRNA gene sequences available in the GenBank databases by BLASTn search and the multiple sequence alignments of partial 16S rRNA gene sequences from1 to 712 bp were aligned using CLUSTALW, version 1.8 (Tompson *et al.,*1994). Phylogenetic trees were constructed from evolutionary distances using. The Neighbor-Joining method implemented through NEIGHBOR (DNADIST) from the PHYLIP version 3.61packages (Felsenstein JP 1993). The robustness of the phylogeny was tested by bootstrap analysis using 100 iterations. Trees generated were analysed with the TREEVIEW program (Page RDM 1996). Accession numbers of all isolate can be viewed in a respective phylogenetic tree.

Inoculum Preparation

A loop full cell growth from the preserved slant was inoculated in 100 ml nutrient broth and incubated on an orbital shaker agitating at 150 rpm and 32 ± 2 °C temperature for 24 h. For RB160 dye decolourization study, 10% (v/v) of the actively growing culture having 3×10^8 cells/ml was used as inoculum in all the further experiments.

Optimization of Physico-Chemical Parameters

Temperature, pH and agitation were optimized during the experimentation for maximizing decolorizing efficiency of the isolate. Effect of different incubation temperature and pH for dye RB160, were carried out in 250ml Erlenmeyer flasks, containing 80 ml nutrient broth, 10ml dye 2.5mg RB160 and the bacterial culture 10ml having 3×10^8cells/ml contained Optical Density 1 at 604 nm .Flasks were incubated at 25, 35 and 45 °C temperature. Effect of pH was studied at 3, 5, 7 and 9 and the influence of the initial decolourization experiment was also carried out under aerobic and microaerobic conditions. Each experiment was carried out in triplicate.

Mutagenesis Induction

The cells were harvested from the culture medium by centrifugation at 10,000 *g* for 10

min. The cell pellet was washed with distilled water and resuspended in sterile deionised water. The colony counting method was used for bacterial count and the count was adjusted to 2.34×10^7 CFU/ml. For UV mutagenesis, 15 ml of cell suspension was taken and placed under a germicidal lamp in a sterile petri –plate, distance of 30 cm and exposed to UV light upto a 5μ Joule^{-mm2} dose (260 nm) for the induction of a mutant. The time of exposure was varied from 30 sec. to 120 sec. For chemical mutation, the distilled water washed cell pellet was redissolved in 20 ml of 0.5, 10, 30 μg/ml concentration of acridine orange and were shaken well for 30 min in an orbital shaker. Samples were taken at every 300 sec. and studied. The viability of cell count was carried out by the spread plate technique and % survival was calculated using Equation:

$$S = (N_i - N_d / N_i) \times 100$$

Where S is the % survival, N_i is the initial viable cell count and N_d is the viable cell count after mutation.

Preparation of Cell Free Extract

The cells of *Microbacterium* sp. B12 were grown for 24 h under microaerobic condition at 32 ± 2 °C in 100 ml sterile nutrient broth, centrifuged at 10,000 g for 10 min. The culture supernatant was used as an extracellular enzymes. Potassium phosphate buffer 50 mM, pH 7.4 was used to suspended the cells and sonicated (Labsonic®M, Sartrious, Germany) at an output of 60 A with seven strokes each of 30 sec. with a 120 sec. interval at 4 °C. The obtained extracts were used as a source of the intracellular enzyme after centrifugation at 10,000 g for 10 min at 4 °C. All the enzyme assays were run in triplicates and average rates were calculated.

Enzyme Activity

Azo reductase assay and NADH-DCIP (2, 6 dichloro-phenol indophenol) reductase activity were determined using the procedure reported earlier by Lade *et al.,* (2012).One unit of enzyme activity was defined as change in absorbance unit per ml of enzyme (Mate at al. 2012). Laccase assay was carried out by modified procedure of Senan and Abraham, (2004). All enzyme activities were assayed under ambient conditions and performed in triplicates.

Biodegradation Analysis

The decolourization assayed was monitored by measuring the change in absorbance maxima of the dye at λ_{max} 604 nm using the UV-vis spectrophotometer. The colour removal was calculated as percent decolourization and was expressed as:

Decolourization (%) = [(Initial Absorbance – Final Absorbance)/Initial Absorbance] X 100

The decolourized culture medium was centrifuged at 10000 *g* for 10 min and supernatant was collected and extracted with ethyl acetate evaporate it and re-dissolved in HPLC grade methanol. The mobile phase consisted of water: methanol (60:40) with flow rate 1 ml/min. The purity was checked by UV detector at 254 nm, isocratic system and 10 min run time. LC-MS was carried out by LCMS-2010SA, Shimadzu, Japan. Mass spectra was obtained using an ion – trap mass spectrometer fitted with an electronic spray (ESI, a Thermo Finnigan LCQ-DUO, USA) interface operated in a negative ionization mode with a spray voltage of 4.5 KV, at a capillary temperature of 275^{0}C, sheath gas at 40 AU (arbitrary unit) and auxiliary gas at 26 AU.

Phytotoxicity Studies

To assess the toxicity of dye RB160 and its metabolite produced after decolourization by *Microbacterium* sp. phytotoxicity tests were carried out on *Phaseolus aureus*. The ethyl acetate extracted products of dye 250mg/l RB160 was dried and dissolved in sterile distilled water for phytotoxicity studies. Toxicity effect was measured in terms of percent inhibition in germination, lengths of the plumule and radical of the *Phaseolus aureus*.

$$\text{Germination (\%)} = \frac{\text{No. of seeds germinated}}{\text{No. of seeds sowed}} \times 100$$

Statistical Analysis

Data were analysed by one way analysis of variance (ANOVA) with the tukey-Kramer multiple comparisons test.

Results and Discussion

Screening, Isolation and Identification of decolourizing bacteria

Soil and wastewater samples contaminated with dye RB160 from dye manufacturing industry facilitated isolation of twelve morphologically distinct isolates showing decolourization zone of 2–10 mm on nutrient agar plates containing 250 ppm RB160. The isolate 1, which gave the largest ratio of decolourisation zone: colony diameter of 1.82 for a colony size of 3–4 mm was selected for further study. The colony of isolated bacteria was yellow-pigmented, opaque with moist consistency and smooth texture. The isolate was gram-positive, showed positive test for carbohydrate fermentation, lead acetate, H_2S production, casein and lipid hydrolysis, citrate utilization and triple sugar iron and negative test for nitrate reduction, indole production, gelatin/hydrolysis, starch

hydrolysis, decorboxylation, deamination, urea hydrolysis and ammonia production Based on these results the isolate was tentatively identified as *Microbacterium* sp. Sequence analysis of 16S rRNA gene showed that isolated strain had highest similarity of only 97% with the species *Microbacterium paraoxydans* , which has not been reported for decolorizing ability for any dyes. Moreover the species is reported in the blood of leukemia patient (Laffineur *et al.,* 2003). Thus the isolate could be other species of *Microbacterium.* Some species of *Microbacterium* are also isolated from oil polluted soil (Sheng *et al.,* 2009). Levels of less than 97% similarity were found in the type strains of all other recognized *Microbacterium* species. Based on the phenotypic characteristics and phylogenetic analysis, strain B12 was designated as a species of *Microbacterium* (Figure 1). The16S rRNA sequence of *Microbacterium* sp.B12 has been deposited in GeneBank with the accession number JX984606.

Optimization of Physico-Chemical Factors

It is evident from Figure 2a that when temperature raised from 25 to 35°C there was an inconsistent trend in the decolourization by isolate, but when the temperature was raised to 45, it was observed that the decolourization was suddenly declined this might be due to the loss of cell viability or deactivation of the enzymes responsible for decolourization at higher temperature (Ponraj *et al.,* 2011). Maximum decolourization for the dyes RB160 was observed at 35°C ± 2°C. Least decolourization was observed at 45°C was 24% after 96 h of incubation. Temperature is very important parameter for wastewater treatment. Selected isolate was mesophilic bacteria because it showed best decolourization at the temperature 35°C ±

2°C. The mesophilic range is traditionally used (Varel *et al.,* 1980) since it is generally thought that maintaining high temperature would be uneconomical, while degradation within the psychrophilic range is too slow (Mahmood *et al.,* 2011).

The best decolourization for dye RB160 was at pH 5 with 94% in 96 h. Moreover, from the obtained data it can be inferred that *Microbacterium.* sp. exhibited more than 90% decolourization for dye RB160 over a broad range of pH 3 to 9. Decrease in the decolourization for dye RB160 was more towards alkaline pH compare to acidic pH under study. So the study indicates that the acidic pH is less detrimental compare to alkaline pH. Figure 2 b. The microaerobic condition as compared to aerobic conditions, which is an oxygen limiting condition, was found to better for decolourization of RB160 Figure 2 c.

Mutagenesis

The dose–response analysis for the UV mutagenesis and acridine orange of *Microbacterium* sp. was done and the % survival values are shown in Table 1. The dose of UV was assessed by time, 30 sec of UV radiation treated strain decolourized 95% of RB160 in 72 h and 0.5μg/ml of acridine orange showed 94% decolourization in 72 h indicated that the enhancement in decolorization of RB160 was due to the bacteria strain after mutated. The physical mutagens are time dependent and chemical mutagens dose dependent (Mouurti *et al.,* 2010). Hence, here, the UV radiation and acridine orange reduced the time for dye decolourization by 24 h the survival capacity of the organism was decreased drastically with an increase in dosage and this is due to lethal DNA damage by UV irradiation. The frame shift mutation in DNA, due to acridine orange

was responsible for the DNA damage. Since the self DNA repair mechanism fails in certain cases, the mutation caused by the acridine orange was permanent. (Gopinath *et al.,* 2009).

Enzyme Analysis

In the present study, significant induction of laccase, azoreductase and NADH-DCIP reductase activities suggested their involvement in the dye degradation shown in Table 2. In the same contest, the inductive pattern of reductase was reported during the degradation of triazo acid black 210 by *Providencia* sp. SRS82. (Agrawal *et al.,* 2014).

Biodegradation Analysis

Conformation of biodegraded product of RB 160 by LC-MS. During the degradation there was asymmetric cleavage of azo bond in RB160 resulting in the formation of six known metabolites 2-[(4-chloro-1,3,5-triazin-2yl) amino-5-4{(4-choro-6-{(3 sulphophenyl)amino}-1,3,5-triazine-2-yl)amino)] benzene-1,4-disulphonic acid m/z value 667 , 3-[{4-chloro-1,3,5-triazin-2-yl}amino]benzene sulfonic acid m/z 286, 2-amino-5 [(4-chloro-1,3,5-triazine-2-yl) amino] benzene-1,4-disulfonic acid m/z 381, 3,5-diamino-4-hydroxybenzenesulfonic acid m/z 204, 2-amino-5[(4-chloro-1,3,5-triazine-2-yl) amino]benzene sulfonic acid m/z 301, 3-amino-4-hydroxybenzenesulfonic acid m/z 189 and two unknown metabolites 2[{(3 amino-2-hydroxy-5 sulfophenyl) diazenyl(phenyl) methyl} diazenyl]-5-sulfobenzoic acid and 2{amino(phenyl) methyl}diazenyl-5-sulfobenzoic acid (Figure 3). Earlier the LC-MS was used to find different intermediates produced in diazo dye degradation by Soybean Peroxidase by Kalsoom *et al.,* (2013).

Table.1 Effect of Physical and Chemical Mutagen on B160
Decolorization by *Microbacterium* sp. B12

	Decolourization of RB 160(%) at Time (h)							
UV (seconds) Treatment					**Acridine orange (µg/ml)Treatment**			
	0	24	48	72		0	24	48	72
0.0	5.0±1.7	25±2.1	48±0.88	70±1.2	**0.0**	3.0±2	24±2.3	50±1.66	72±2.7
30	4.8±2.8	**95±2.1**	48±1.2	26±1.98	**0.5**	3.3±2.4	**94±3.2**	54±3.1	25±1.15
60	5.2±2.3	25±1.15	44±2.8	57±0.64	**10**	2.9±1.7	22±0.54	32±1.89	65±3.2
120	5.0±2.6	20±0.88	22±2.1	37±1.11	**30**	3.1±2.1	20±0.76	22±1.32	45±1.1

Values are mean of three experiments ± standard deviations

Table.2 Enzyme Activity during Decolourization of RB160 by *Microbacterium* sp.B12

	Enzyme activity	
Enzymes	Control (U/mg/ml/min)	RB 160 (U/mg/ml/min)
Laccase[a]	0.101±0.78	0.172±1.6
NADH-DCIP reductase[b]	5.263±2.3	8.263±1.1
Azo-Reductase[c]	1.95±0.81	3.26±2.2

Enzyme activity:
[a] μM of ABTS Oxidized ml^{-1}min^{-1}
[b] μg of DCIP reduced min^{-1} mg-1
[c] μg of methyl red reduced min^{-1} mg^{-1}
Control= enzyme extracted from culture medium without dye after 24 h; Test = enzyme
extracted from the dye decolourized culture medium after 24 h. Values are mean of three
experiments ± SD. Percent induction calculated from mean values.

Table.3 Phytotoxicity of RB160 and its Metabolites Formed after Degradation by
Microbacterium sp. B12

Parameters	Toxicity effect on *Phaseolus aureus*		
	Distilled water	RB160	RB160 metabolite
Germination (%)	95	25	86
Plumule (cm)	9.8±0.6 [ab]	3.9±0.8[a]	8.99±0.99 [b]
Radicle (cm)	3.67±0.9 [ab]	0.29±0.5[a]	2.32±1.37 [b]

Values are mean of three experiments. Seeds germinated (in both plumule and radicle growth) in Dye RB160 and Dye RB160
metabolite is significantly different from the seeds germinated in distilled water at $P < 0.05$, by one-way analysis of variance
(ANOVA) with the TukeyeKramer comparison test

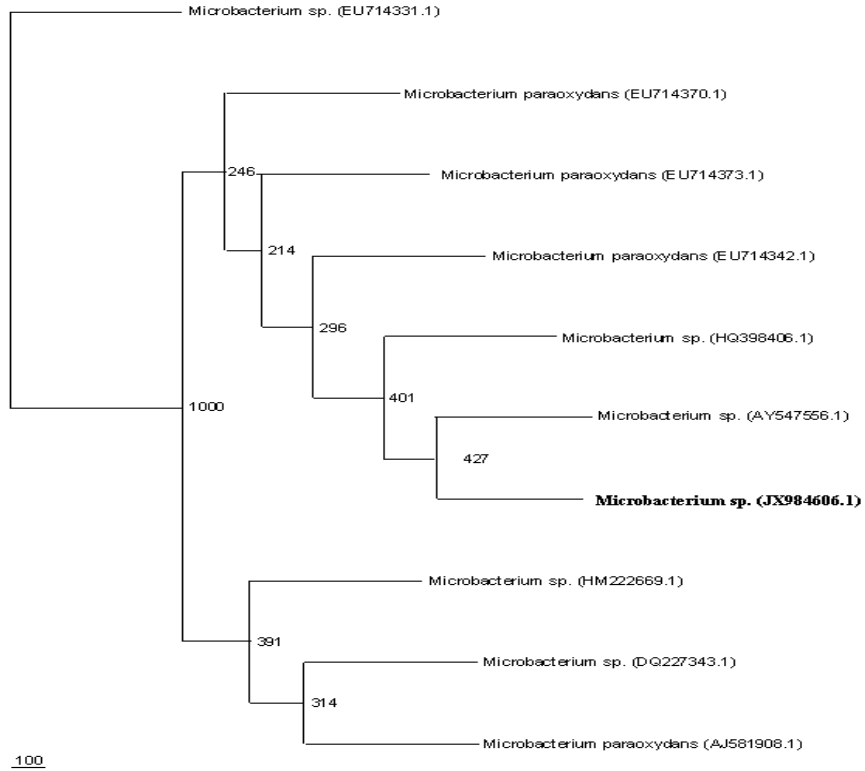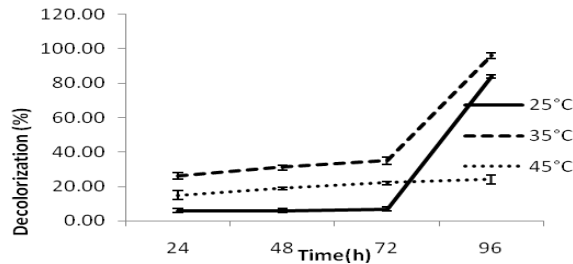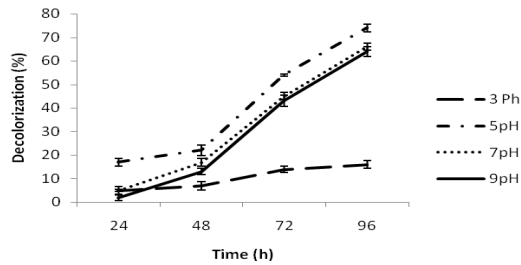**Fig.1** Phylogenetic Tree of the *Microbacterium* Sp.B12 and Related Organisms

Fig.2 Effect of Physico-chemical Factors on Decolourization of B160 by *Microbacterium* sp. B12 (a) Incubation Temperature, (b) Initial pH. (c) Aerobic/Microaerobic
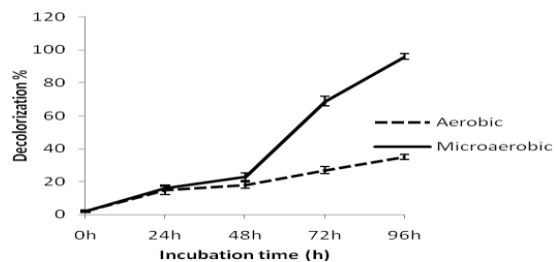
(A)

(B)

(C)

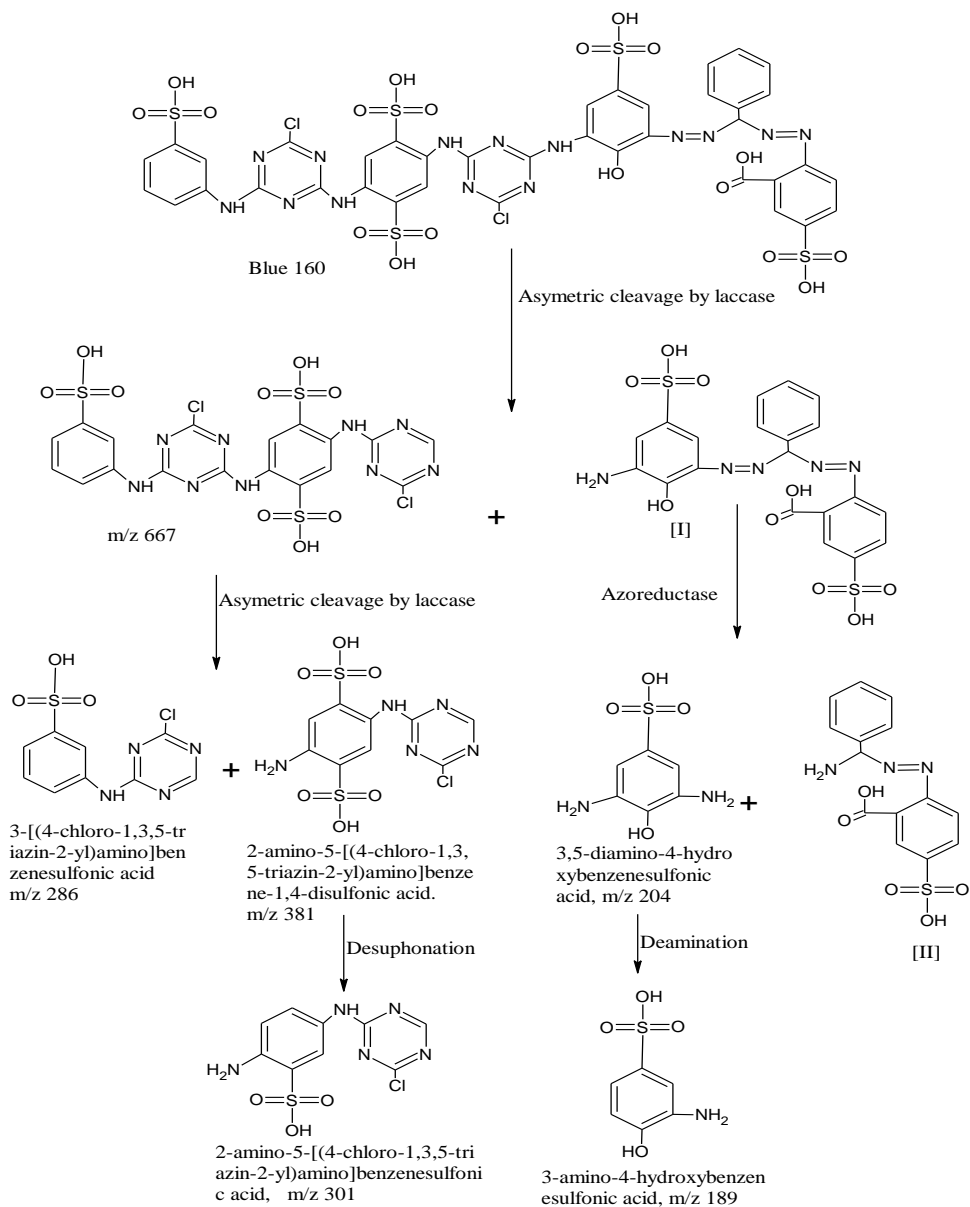**Fig.3** Proposed Pathway for RB160 Degradation by *Microbacteium* sp. B12

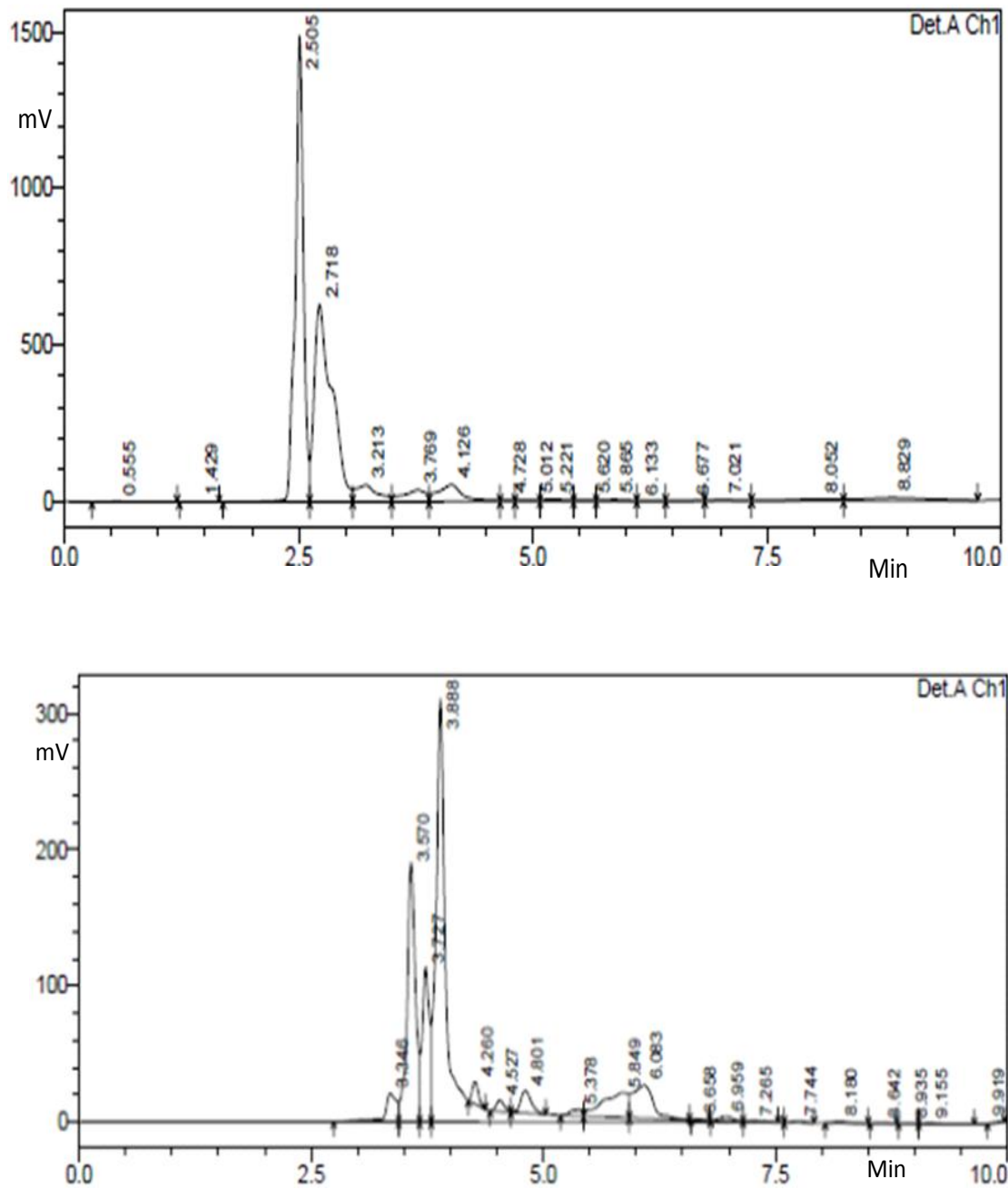**Fig.S1** HPLC Chromatogram of RB160 (a) Dye and its (b) Metabolites after 24 h

Table.S1 LC-MS Spectral Data of Metabolites Obtained after Degradation of RB160 by *Microbacterium* sp. B12

S.No	Retention time	m/z	Name of the metabolite	Mass spectra
1	3.48	188	3-amino-4-hydroxybenzen esulfonic acid	
2.	2.54	301	4-amino-N-(4,6-dimethylpyrimidin-2-yl) benzenesulfonamide	
3.	2.7	381	2-amino-5-[(4-chloro-1,3,5-triazin-2-yl)amino]benzene-1,4-disulfonic acid	
4.	3.3	204	3,5-diamino-4-hydroxybenzenesulfonic acid	
5	3.6	286	3-[(4-chloro-1,3,5-triazine-2-yl)amino]benzenesulfonic acid	
6	4.3	667	2-[(4-chloro-1,3,5-triazin-2yl) amino-5-4{(4-choro-6-{(3 sulphophenyl)amino}-1,3,5-triazine-2-yl)amino)] benzene-1,4-disulphonic acid	

Phytotoxicity Studies

Use of bioassays such as phytotoxicity for monitoring the toxic effect of dyes as well as its metabolites in plants was suggested by many researchers (Lade *et al.,* 2012). Seed germination and plant growth bioassays are the most common techniques used to evaluate the phytotoxicity. In this case, the phtotoxicity study revealed that there was an inhibition of 25% germination in a solution containing 1000 mg/l of RB160 shown in Table 3. This study suggests that the dye RB160 was toxic to plants, while the metabolites formed after degradation were less toxic, which signifies the detoxification of dyes by *Microbacterium. sp.* B12, these results underlines the importance of *Microbacterium* for bioremediation of diazo dyes RB160 in terms of both decolourization and detoxification.

The ability of *Microbacterium* sp. B12 to decolourize the diazo reactive dye RB160 under a broad range of temperature, pH, agitation, suggested that the isolated strain could be useful in biological treatment of industrial wastewater. UV-visible spectroscopy analysis confirmed decolourization, HPLC and LC-MS analysis confirmed degradation of RB160.

In conclusion, we found that the decolourization and degradation of RB160 have not been reported by single microbial isolate. The present work showed decolourization and degradation of RB160 by *Microbacterium* sp. B12 within 96 h, at pH 5.0 and 35 °C under microaerobic conditions. The process could be further effectively developed by using physical and chemical mutagens for improved decolourization in less time 72h. HPLC and LC-MS analysis showed formation of new metabolites. Phytotoxicity study proves that, strains have potential to degrade dye in to non- toxic compounds. To our knowledge, this is first time reported the decolourization and degradation of reactive dye RB160 by *Microbacterium* sp. B12.

Acknowledgement

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