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Bioremediation of hazardous azo dye methyl red by a newly isolated *Bacillus megaterium* ITBHU01: Process improvement through ANN-GA based synergistic approach

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Methyl red (MR), a commonly used azo dye, poses serious environmental consequences due to its toxic, recalcitrant and refractory nature. In the current study, a bacterial isolate, ITBHU01, with remarkable characteristics to decolourize MR dye, was characterized by its phenotypic features and phylogeny using fatty acid methyl ester (FAME) profiling and 16S rDNA sequence homology. At artificial neural network linked genetic algorithm (ANN-GA) based optimal conditions of parameters *viz*., initial MR concentration of 258.54 mg/L, pH of 7.21, the temperature of 38.2°C, and salinity of 26.3 g/L, the maximum decolourization of 98.1% was obtainedby static incubation of 24 h. FTIR spectra revealed the bacterial degradation of MR dye by cleavage of azo bonds present in the dye. ESI-MS analyses have shown formation of two prime metabolic products as N,N-dimethyl-p-phenylenediamine and 2-aminobenzoic acid indicating the symmetric cleavage of azo (–N=N–) bond; however, the presence of additional peaks illustrated about the formation of different other metabolites also. Purified azoreductase with a molecular weight of 28.0±0.2 kDa was found primarily responsible for reductive cleavage of azo bond leading to biodegradation of MR. Phytotoxicity assay for bacterial treated dye on seeds of *Solanum lycopersicum*, *Sorghum bicolor* and *Triticum aestivum* had significantly shown that there was no inhibition in their seed germination confirming the non-toxic nature of degraded metabolites.

Keywords: Artificial neural network-Genetic algorithm, Azoreductase, Biodegradation, Phytotoxicity assay, 16S rRNA gene, *Solanum lycopersicum*, *Sorghum bicolor*, *Triticum aestivum*

Azo dyes are xenobiotic compounds with one or more azo linkages (–N=N–) and aromatic rings, widely used in the leather, textile, food, cosmetics, pharmaceutical and paper industries[1]. Over 10000 commercially existing dyes are identified with a marking rate of nearly 8×10^5 tons per year, out of which at least 10% dyestuffs in different forms are released into the ecosystem[2] as observed as residuals in industrial wastewater. It has become a grave environmental concern because of their recalcitrant, toxic, potentially mutagenic and carcinogenic nature causing long-term health issues. The industrial effluent containing azo dyes affect photosynthesis of aquatic plants by reducing light infiltration[3] and also be lethal for some aquatic animals due to the occurrence of a breakdown product of dyes[4]. Dye effluents need proper treatment before disposal. Though a large spectrum of treatment methods is available for curing wastewater containing dyes including different conventional physical and chemical processes, it still requires attention. The aerobic wastewater treatment processes are usually inefficient to decolourize and degrade the dyes present in the effluents to the recommended levels[5]. Though electrochemical demolition, physicochemical flocculation, coagulation, and adsorption are tertiary treatment methods, these are complex and cost-intensive[6]. Additionally, a lot of sludge is produced during these treatment methods, which poses another issue. The anaerobic treatment methods also could not be regarded as safe, as they produce mutagenic and highly toxic aromatic amines, which further are not metabolizable under these conditions[7].

Biological decolourization is seen as an avenue to transform, degrade or mineralize azo dyes[8]. Moreover, such bio-decolourization and bioremediation are eco-friendly and cost competitive

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compared to the conventional decomposition processes. Different classes of algae, fungi, yeast and bacteria have been reported to decolourize broad range of dyes from wastewater with high efficiency[9]. However, none of the microorganisms, except for bacteria, were found to biodegrade the dye effluent without any shortcomings *viz*., a low pH necessity for optimal decolourization, extended hydraulic retention time, extended time duration for absolute decolourization, specific nutritional requirement, etc.[10]. Biological colour removal using bacteria is interesting because of their potential to decolourize and mineralize most of the azo dyes efficiently and at much faster rate. Further, bacterial degradation requires smaller time as compared to other microorganisms due to their rapid growth rate. Many bacteria illustrate capability to degrade azo dyes aerobically and anaerobically[11].

The present investigation deals with application of our laboratory isolate *Bacillus* strain, for complete degradation of MR dye under static conditions. This strain proved successful in biodegradation of another dye Orange G already[12]. Decolourization studies were performed on different parameters affecting the process of decolourization and performance of the microorganism such as pH, the concentration of dye, salinity and temperature. Modeling and optimization of these parameters were precisely done by response surface methodology (RSM) and artificial neural network hybridized with genetic algorithm (ANN-GA) based approach. Additionally, the enzyme responsible for dye decolourization was partially purified to evaluate the mechanism behind decolourization of MR. Further, phytotoxicity assay was also performed to explore possible usability of treated effluent for irrigation purpose.

Materials and Methods

Azo dye and chemicals

The textile grade dye Methyl red (MR; C.I. No.13020, $C_{15}H_{14}N_3NaO_2$, Molecular weight: 291.28) was procured from S.D. Fine Chemical Ltd. (Mumbai, India) and its chemical structure named as 2-(N, N-dimethyl-4-aminophenyl) azobenzene carboxylic acid (Fig. 1). Rest of the chemicals used in the study was of analytical grade and purchased from Himedia, Merck and Sisco Laboratories, India.

Strain isolation and cultivation

The microbial sources were obtained formerly from the soil samples collected from waste contaminated

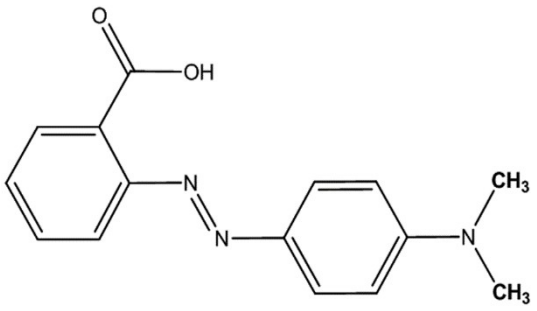

Fig. 1—Chemical structure of methyl red

site at Sir Sundar Lal Hospital, Banaras Hindu University, Varanasi and their preliminary characterization based on colonial morphology, cellular characteristics such as Gram staining, motility, presence or absence of spores, and biochemical characteristics as in Bergey's Manual of Determinative Bacteriology was performed[13]. To screen their dye degrading potential, the isolates were cultivated in enrichment culture plates containing (in g/L): Peptone 10.0, Glucose 10.0, NaCl 5.0 and 100 ppm (mg/L) of MR with 2% agar (pH 7.0) and incubated at 35°C. MR was used as the indicator of microbial activity. Microbial colonies, around which clean zones of decolorization increased quickly, were chosen. The bacterium (designated as *Bacillus* spp. strain ITBHU01) showing largest zone was selected for subsequent MR degradation experiments. The pure culture of the promising bacterium was maintained in nutrient agar (NA) slant. The slant was incubated at 35°C for 24 h and stored at 4±1°C. The stock culture was sub-cultured into fresh medium every 3-4 weeks.

Identification of microorganism by 16S rDNA sequence

The phylogenetic as well as generic characterization of *Bacillus* spp. ITBHU01 was done based on its fatty acid methyl ester (FAME) analysis and 16S rDNA sequence homology. FAME analysis was carried out by Royal Life Sciences Pvt. Ltd., Secunderabad, India; a MIDI Sherlock®, USA based laboratory. Further, the amplification of 16S rRNA gene of ITBHU01 strain was done by PCR using two universal eubacterial oligonucleotide primers, 16SF 5'-AGAGTTTGATCCTGGCTCAG-3' and 16SR 5'-AAGGAGGTGATCCAGCC-3'[14]. The primers were constructed by Integrated DNA Technology (IDT®, USA). PCR was performed in a thermocycler (Biometra, Germany) containing 25 µL volume of reaction buffer using 10 pmol each of the primers, 20-30 ng genomic DNA of the isolate, 0.25 mM

dNTPs (MBI, Fermentas), 2.5 mM $MgCl_2$ and 1 unit of Taq DNA Polymerase (Bangalore Genei, India). The PCR conditions details were as: an initial denaturation for 4 min, denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s with total 30 cycles of amplification and a final extension step at 72°C for 5 min. The amplified PCR product was purified by Gel/PCR DNA fragment extraction kit (Geneaid, Taiwan) and the sequencing was performed in both directions with same primer (used for PCR) using an automated sequencer (3730XL DNA Analyzer, Applied Biosystem, HITACHI, USA).

The homology search analysis of the resulted 16S rDNA gene sequence was done by BLASTN method using EzTaxon online server version 2.1[15] and finally deposited to NCBI GeneBank database (Accession no. HM436653). Preliminary multiple sequence alignment and a phylogenetic tree construction were performed, respectively, using Clustal W and Mega software version 4.1[16].

Acclimatization

The acclimatization was performed by gradually exposing *Bacillus* spp. ITBHU01 to the higher concentrations of MR solution. The strain ITBHU01 was grown for 24 h at 35°C in 250 mL Erlenmeyer flasks with 50 mL of medium containing (in g/L): peptone 10.0, Glucose 10.0, NaCl 5.0 (pH 7.0). During this study, the nutrient broth was successively added with increasing MR concentration. The acclimatized bacterium was used throughout the study.

Inoculum development and decolorization studies in shake flasks

The inoculum was developed by transferring one loop full of the acclimatized bacterial cells from the slant culture into an Erlenmeyer flask (250 mL) containing 100 mL of a basal semi-synthetic medium (BSM) composing of (in g/L): KH_2PO_4 0.68, K_2HPO_4 1.73, $MgSO_4.7H_2O$ 0.1, NaCl 0.1, $FeSO_4.7H_2O$ 0.03, $CaCl_2.2H_2O$ 0.02, glucose 5.0, peptone 5.0 (pH 7.0)[12]. The flask was incubated in an orbital shaker at 35°C and 160 rpm for 24 h for inoculum development. *Bacillus* spp. strain ITBHU01 with 2% inoculums ($OD_{600}\approx2.0$) was inoculated in 250 mL Erlenmeyer flask with 100 mL BSM containing MR (250 g/L) and incubated at 35°C for 48 h at static and shaking conditions (160 rpm). The effect of inoculum size of *Bacillus megaterium* ITBHU01 was evaluated at different inoculum volumes (1-8%, v/v). The samples of 2.0 mL from inoculated and control medium were withdrawn at regular interval of 4 h was observed and centrifuged at 10000×g for 15 min at 4°C. The obtained clear cell-free extract was used for determination of percentage decolorization of MR dye.

Decolourization assay

MR dye decolorization in cell free extracts was monitored at 430 nm (λ_{max}) by a Double-beam UV-Vis 1700 PharmaSpec spectrophotometer (Shimadzu, Japan) against media blank and growth was examined turbidometrically by evaluating the absorbance at 600 nm. Un-inoculated media with 100 mg/L of MR dye served as abiotic control. The percentage of decolorization was calculated from the difference between initial and final values, using the given formula:

$$\%Decolorization = \frac{A_0 - A_1}{A_0} \times 100$$

where, A_0 and A_1 represented the initial and final absorbance of the dye, respectively[17]. All the decolorization experiments were performed in triplicate, and the average values were used in calculations.

Determination of azoreductase activity

The bacterial culture was grown in BSM containing 250 mg/L of MR until the dye was completely decolorized. The culture sample was withdrawn and centrifuged at 10000×g for 10 min at 4°C. The cell pellet was collected, washed with 50 mM phosphate buffer (pH 7.0), and re-suspended in the same buffer. However, the cell free broth was utilized to detect the extracellular azoreductase activity. The suspended cells were ultrasonicated (Vibra Cell, Sonics, USA) at 20 MHz, 80% amplitude with four cycles of 15 s and the content was centrifuged again at 10000×g to remove the cell debris for 10 min. The supernatant was analyzed for intracellular azoreductase activity.

Azoreductase activity was assayed based on a standard method as described by Zimmermann *et al*.[18]. The reaction mixture containing 50 mM phosphate buffer (pH 7.0), 2 mM NADH, 20 µM Methyl red, and 0.1 mL of cell-free extract in a total volume of 1 mL was incubated for exactly 30 min at 37°C. Dye decolorization was monitored by measuring absorbance at 430 nm. Similarly, other reducing agents such as NADPH and FAD could also be used

to measure azoreductase activity by adding at the concentration of 2 and 0.2 mM of NADPH and FAD, respectively in place of NADH. One international unit (U) of azoreductase activity is defined as the amount of enzyme that reduces 1.0 micromole of dye per minute at specified conditions. The Protein content of the crude protein extract was determined by Lowry's method[19] using bovine serum albumin as the standard for determination of specific activity, *i.e.*, enzyme units per milligram protein.

Experimental design and process parameters optimization

Response surface methodology

Response surface methodology (RSM) is a commonly used statistical technique, which expresses the process parameters of a system in the form of a polynomial model providing a statistical relationship among them. The effects of four process parameters, namely, pH (4-10), the initial concentration of dye (200-800 mg/L), temperature (20-50°C), and salinity (5-35 g/L) on dye decolorization have been studied using central composite design (CCD) in RSM. The experimental plan and levels of all the four variables are represented in Table 1. A full factorial CCD was applied to create a set of 31 experiments using the statistical software MINITAB v15.1.0.0, USA. A second order polynomial regression model comprising a summative response (Y) of all the linear, square, and linear interactive terms for tested parameters, could be expressed as follows:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n} \sum_{j=1}^{n} \beta_{ij} X_i X_j \quad \ldots (1)$$

Table 1—CCD design matrix and comparative results predicted through RSM and ANN model for the dye decolourization (%)

pH (X_1)	Initial Methyl red concentration (mg/L)(X_2)	Temperature (°C) (X_3)	NaCl concentration (g/L)(X_4)	Dye decolourization (%)		
				Experimental	Predicted decolourization (%)	
					RSM	ANN
Dataset used for training of ANN model						
7	500	35	35	35.5	34.9	35.8
4	500	35	20	33.2	34.9	89.3
5	700	45	10	40.2	44.1	90.1
10	500	35	20	44.5	44.4	89.2
7	500	35	20	59.4	62.8	89.3
7	500	20	20	45.5	43.3	87.1
9	300	25	10	52.4	55.3	52.7
7	500	35	20	63.2	62.8	58.9
5	300	25	30	25.4	29.3	56.8
7	200	35	20	77.5	75.9	52.0
9	700	25	30	22.5	25.8	75.4
5	300	45	10	63.5	59.7	77.1
5	700	25	10	38.9	35.4	88.5
5	700	25	30	22.5	21.9	87.7
9	300	45	30	44.7	47.8	89.2
7	800	35	20	48.7	51.9	76.1
7	500	35	5	58.4	60.6	92.7
7	500	35	20	63.5	62.8	58.2
7	500	50	20	50.3	54.1	93.3
7	500	35	20	63.8	62.8	91.7
5	300	25	10	45.2	45.8	56.8
5	700	45	30	26.4	23.1	87.4
5	300	45	30	36.4	35.6	57.9
7	500	35	20	60.3	62.8	92.2
Dataset used for testing of ANN model						
9	300	25	30	46.7	42.2	90.1
9	700	25	10	35.8	35.9	93.1
7	500	35	20	67.4	62.8	92.7
9	700	45	10	48.3	43.9	58.4
7	500	35	20	65.2	62.8	53.2
9	700	45	30	27.5	26.2	55.4
9	300	45	10	68.6	68.5	85.7

where β_0 is constant, n represents the number of variables, β_i is the slope or linear effect of the input variable X_i, β_{ii} denotes the quadratic effect of input factor X_i and βij stands for linear by linear interaction effect between the input variable X_i and X_j.

Artificial neural network linked genetic algorithm (ANN-GA)

The artificial neural networks (ANNs) had been widely employed for approximation and simulation of different linear and nonlinear functions; however, the genetic algorithm (GA) was applied to attain the optimized input space generated by the ANN model[20]. Therefore, a feed forward neural network, also called as multilayer perceptron (MLP), with back propagation algorithm was applied to build a predictive mathematical model with four inputs (*i.e.*, pH, initial concentration of dye, temperature and salinity), one hidden layer, and one output node (percent dye decolourization). The input and output data were normalized by scaling within the uniform range (0.1-0.9) as given by the following equations:

$$X' = 0.8\frac{X - X_{\min}}{X_{\max} - X_{\min}} + 0.1 \quad \ldots (2)$$

The learning rate, during the training of the neural network, was varied in such a way that led to achieving the most favourable coefficient of determination (R^2) for the neural network. The R^2value also explains the goodness of fit of the constructed ANN model. The performance of the network was figured out regarding mean squared error (MSE), which represents the statistical divergence between experimental and model predicted values.

GA was applied to the resulted ANN parameters with the objective function of maximization and condition of GA parameters (set by default) as following:population type, double vector; original population size, 100; crossover probability, 0.8; elite count, 20; crossover function, @crossoversinglepoint; migration direction, forward; selection function, @selectionroulette; mutation function, @mutationgaussian; total generations, 100. The ANN-GA based modeling and optimization analysis were conducted using MATLAB program (v7.0, Mathworks Inc., MA, USA).

Partial purification of azoreductase

Preparation of crude enzyme

For purification of azoreductase enzyme, the bacterial culture was grown in 2 L of the optimized medium. The cell-free fermentation broth was collected by centrifuging the 24 h old culture at 10000×g for 10 min at 4°C. The cell pellet was suspended in 50 mM phosphate buffer (pH 7.0) containing 1 mM lysozyme and 20% glycerol. The cells were ultrasonicated in cold condition at 20 MHz, 60% output for 5 cycles of 30 s each with 2 min intervals. Cell debris was removed by centrifugation at 15000×g for 20 min and the supernatant, thus obtained, was mixed properly with cell-free broth to be used as crude enzyme preparation.

Fractionation with ammonium sulphate

The crude cell-free extract was initially fractionated by graded precipitation using ammonium sulfate. First, the solid $(NH_4)_2SO_4$ was added to the supernatant to attain a saturation concentration of 20% and the precipitated protein was collected by centrifugation at 15000×g for 30 min, re-suspended in a minimal volume of 50 mM phosphate buffer (pH 7.0). The supernatant resulted after centrifugation was again subjected to addition of $(NH_4)_2SO_4$ to achieve a final concentration of 40% of the salt saturation and resulting protein was collected and re-suspended again. The enzyme activity of azoreductase was evaluated at each step of ammonium sulphate fractionation.

The fraction with azoreductase activity was dialyzed using dialysis bag (molecular weight cutoff 12-14 kDa; Himedia, Mumbai) for 24 h against three changes of the same buffer. The dialyzed solution was further concentrated 5-fold using an Amiconmembrane concentrator (molecular weight cutoff 10 kDa).

DEAE-Cellulose column chromatography

The enzyme concentrate obtained from the previous step was put onto a DEAE-Cellulose ion exchange column (2.0×10.0 cm) pre-equilibrated with 50 mM phosphate buffer (pH 7.0). The adsorbed proteins were eluted with a linear gradient of 0-0.6 M NaCl in the same buffer. The active fractions eluted were assayed for azoreductase activity, pooled, desalted and further utilized for electrophoretic analysis.

Determination of molecular mass and activity staining of the enzyme

The proteins with azoreductase activity were evaluated with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as described by Laemmli[21] using a 12% separating gel (pH 8.8) and a 5% stacking gel (pH 6.8) using

standard protein markers (range, 14.3–97.4 kDa; PMWM, Genei). The gels were stained subsequently with Coomassie brilliant blue R250.

Activity staining of the electrophoretic gel was carried out for tracing the presence of azoreductase enzyme by washing the gel with 50 mM phosphate buffer (pH 7.0). A renaturation method was performed to recover the enzyme activity within the gel. The gel was incubated in unbuffered 1% Triton X 100 for 1 h, washed again thrice with the same buffer, stained with 100 µM MR solution, and subsequently transferred into the phosphate buffer containing 2 mM NADH maintained at 37°C. The azoreductase activity was confirmed by the presence of clear colorless band against dye background within the gel.

Biodegradation analysis

The quantitative evaluation of the dye was performed by a UV-Vis spectrophotometer (1700 PharmaSpec Shimadzu, Japan). However, the biodegradation analysis was done by extracting metabolites from culture supernatant with equal volumes of dichloromethane, desiccating it over anhydrous Na_2SO_4 and finally evaporating to dryness. The residue obtained was subjected to FTIR spectroscopy at mid-IR region (400-4000 cm^{-1}) by FTIR using Thermo NICOLET 5700 spectrophotometer. The output spectrum from the spectrophotometer was analyzed using OMNIC software. Further, to find out the degradation products, the Electron spray ionization mass spectrometry (ESI-MS) studies were carried out using SL 1200 system (Agilent) with ion trap detection in the positive ion mode. From culture medium amended with MR, 50 mL of decolorized samples were collected after 48 h of decolorization, centrifuged at 10000 rpm for 15 min and filtered through 0.45 mm membrane filter. The filtrate was further extracted twice with ethyl acetate and evaporated in a vacuum evaporator at 40-45°C. The resulted residue was then dissolved in a solvent of 50% acetonitrile-water with 0.1% formic acid and used for ESI- MS analysis.

Phytotoxicity study

Phytotoxicity studies of Methyl red and its degraded metabolic products after bacterial action were performed to explore the usability of treated water on irrigation as well as an impact on the ecosystem. The tests were carried out according to the procedure as described by Kalyani *et al*.[23] by germinating three kinds of plant seeds, which are important crops of Indian agriculture *viz*. *Sorghum bicolor*, *Triticum aestivum* and *Solanum lycopersicum* in the presence of untreated and treated MR solutions. All the tests were carried out in triplicate, and the values were averaged to minimize the experimental error.

Results and Discussion

Characterization of Methyl red decolorizing strain

Among all the bacterial isolates, the strain ITBHU01 has shown maximum dye decolorizing potential. Based on Bergey's Identification flow chart of Determinative Bacteriology, cellular and colonial morphological features, the isolate was tentatively named as *Bacillus* spp. strain ITBHU01 as described in the previous studies[12]. One unit in terms of O.D. at 600 nm of bacterial suspension corresponded to approximately 3.2×10^8 CFU/mL or 0.929 mg dry cell weight/mL.

The total fatty acid methyl ester (FAME) profile of *Bacillus* sp. ITBHU01 was analyzed by gas chromatography using MIDI system (Sherlock® Microbial Identification System version 4.0, MIDI Inc., Newark, DE). The gas chromatogram showing the methylated cellular fatty acid content is given in Fig. 2. The main cellular fatty acid contents of the isolate were detected as: anteiso-C15:0 (52.62%), iso-C15:0 (16.09%), iso-C14:0 (8.26%), iso-C16:0 (4.53%), C16:1ω11C (4.16%), C16:0 (3.16%), C14:0 (2.72%), anteiso-C17:0 (2.03%) (Table 2). The RTSBA6.0 database matches from Sherlock® software showed the Similarity Index (SI) value of 0.600 with *Bacillus megaterium*-GC group B. Similarity Index (SI) value depicts the degree of closeness of the cellular fatty acid composition of an unknown sample in comparison to the mean cellular fatty acid composition of the strains used to create the library entry listed as its match. SI value, 1.000 shows an exact match of the cellular fatty acid make-up of the unknown sample to the mean of library entry results[23].

Based on BLASTN analysis of 16S rRNA gene using EzTaxon online server version 2.1, the isolate had found to have 96.8 % similarity with *Bacillus megaterium* SR28C (accession no. AM900770). Therefore, the strain was considered as a close relative to *Bacillus megaterium*. The phylogenetic dendrogram constructed by the neighbor-joining method also indicated that the isolate ITBHU01 was a discrete strain in the *Bacillus megaterium* cluster (Fig. 3). In the phylogenetic dendrogram, the drop-

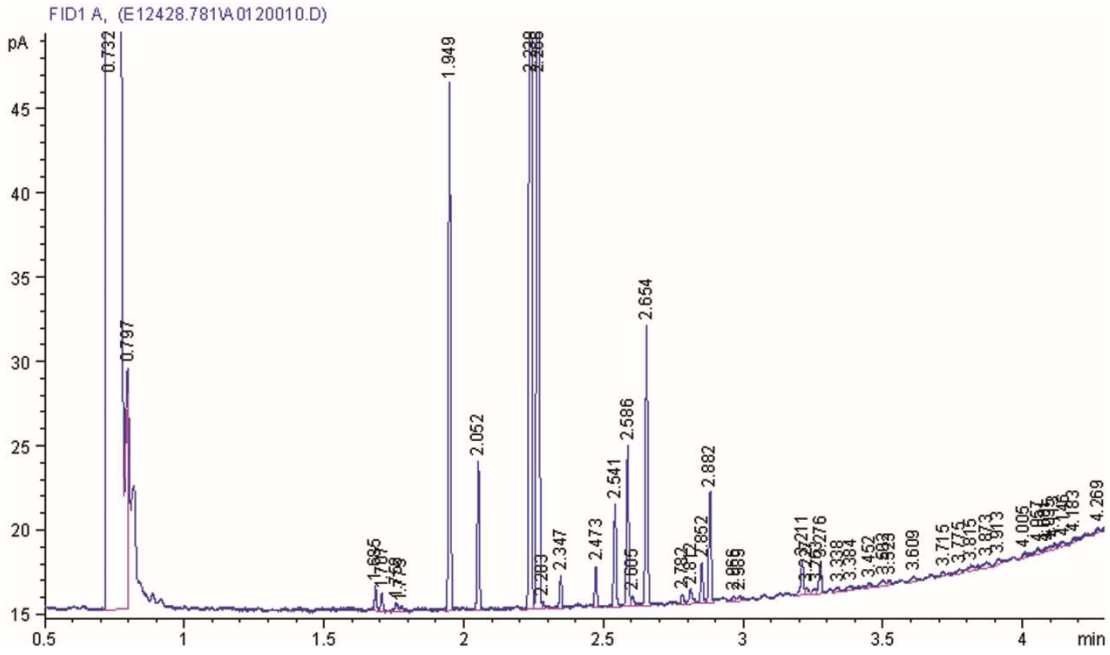

Fig. 2—Gas chromatogram of methylated cellular fatty acids of *Bacillus* sp. ITBHU01

Table 2—Fatty acid compositions of *Bacillus* sp. strain ITBHU01

Retention Time (min)	Peak Name	Percent (%)
0.7322	solvent peak	----
1.6848	13:0 iso	0.49
1.7070	13:0 anteiso	0.35
1.7750	13:0	0.18
1.9494	14:0 iso	8.26
2.0521	14:0	2.72
2.2381	15:0 iso	16.09
2.2659	15:0 anteiso	53.62
2.3472	15:0	0.00
2.4731	16:1 w7c alcohol	0.77
2.5412	16:0 iso	4.53
2.5864	16:1 w11c	4.16
2.6543	16:0	3.16
2.7818	17:1 iso w10c	0.22
2.8516	17:0 iso	0.80
2.8816	17:0 anteiso	2.03
2.9660	17:0	0.15
3.2113	18:1 w9c	0.76
3.2765	18:0	0.62
3.3383	17:0 iso 3OH	0.18
3.4518	17:0 3OH	0.17
3.5016	19:0 anteiso	0.25
3.7745	20:0 iso	0.19

down scale represents Jukes-cantor distance, which shows genetic distances regarding substitutions per nucleotide position. However, upper scale represents evolutionary rate in millions of years.

Effect of static and shaking conditions on dye decolorization

The decolourization of MR was studied under a static and shaking condition (160 rpm) at pH 7.0 and 35°C using the acclimatized culture of

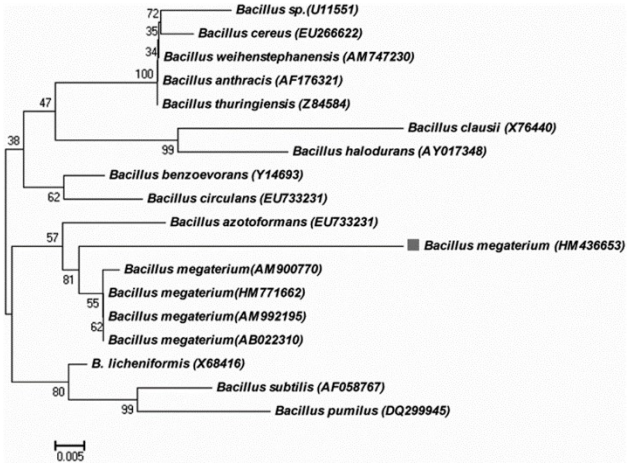

Fig. 3—Phylogenetic dendrogram constructed from sequence alignment of 16S rRNA genes using the neighbor-joining method, for *Bacillus megaterium* strain ITBHU01 [■] and related strains (Numbers in parentheses following the name of the strains are GenBank sequence accession numbers)

Bacillus megaterium ITBHU01 with an initial dye concentration of 250 mg/L. The shaking conditions were observed to support more biomass production of the bacterium (1.448±0.11 g/L) as compared to that of static conditions (0.512±0.05 g/L). However, it was found that under static anoxic condition, the maximum dye decolourization of MR was 74.4% within 24 h as compared to 26.6% under agitation, respectively (Fig. 4A). To ensure whether the dye decolourization was the attribute of microbial metabolic activities or change in pH, the final pH of

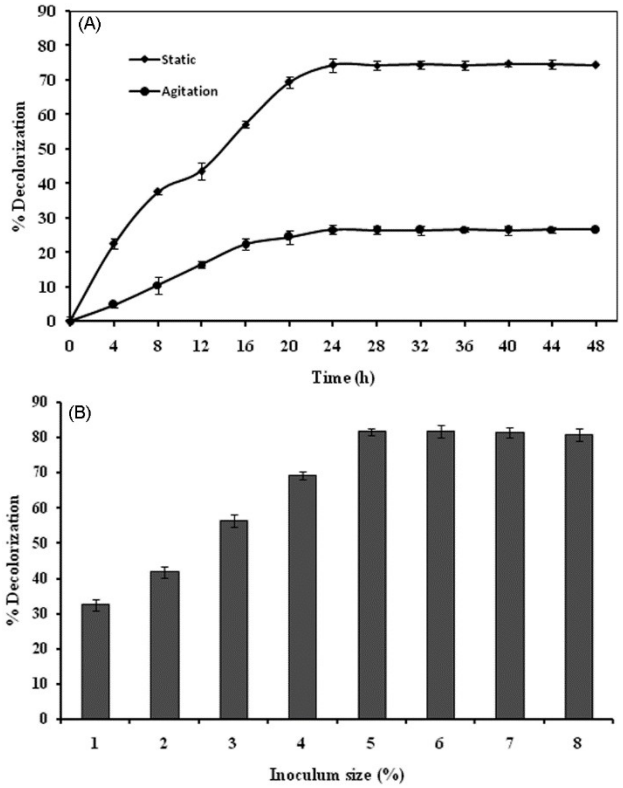

Fig. 4 — (A)Effect of agitation on the dye decolorization efficiency of *Bacillus megaterium* ITBHU01 studied for 48 h in synthetic medium containing Methyl red (250 mg/L) at 35°C, pH 7.0 under static and shaking conditions; (B) Effect of Inoculum size on the dye decolorization efficiency of *Bacillus megaterium* ITBHU01

the fermented fluid was measured, which showed negligible change in pH in the range of 6.4-7.6 under static condition. The results confirmed that MR decolorization occurred due to microbial action. The result bears similarity with those of studies on *Pseudomonas desmolyticum*[24], *Pseudomonas luteola*[25], *Escherichia coli* No3[26], *Escherichia coli*[27] and a microbial consortium JW-2[28]. It was found out that under agitation conditions, the presence of oxygen deprives the azoreductase of attaining electrons needed for cleavage of azo dyes. Whereas, under static anoxic conditions these electrons are available to azoreductase from NADH to decolourize azo dyes[29]. Further, the static conditions were preferred to investigate bacterial dye decolourization.

Effect of inoculum size

The inoculum size of *Bacillus megaterium* ITBHU01 was varied from 1 to 8% (v/v). Maximum decolorization of 81.6% was observed at 5% inoculum volume at pH of 7.0, the temperature of 35°C and static condition (Fig. 4B). Increasing the inoculum size beyond 5% had no significant increase in percentage decolorization. Whereas, for inoculum size lower than 5%, lower percentage decolorization committed to the fact that less number of bacterial cells were not able to decolorize the azo dye with better efficiency.

Predictive modeling and optimization

Response surface methodological approach

Response surface method includes a full factorial search through pattern evaluation of the different level of important parameters, identification of feasible interactions and determination of optimum working conditions. All 31 experimental runs in CCD were performed individually, and statistical analysis of the obtained results was carried out, which was expressed in the form of a quadratic polynomial equation correlating maximum dye decolorization (Y) with independent factors *viz*., pH (X_1), initial MR concentration (X_2), temperature (X_3), and salinity (X_4) as shown below:

$$Y = 62.84 + 3.17X_1 - 8.01X_2 + 3.58X_3 - 8.54X_4 - 10.29X_1^2 + 0.49X_2^2 - 6.27X_3^2 - 6.69X_4^2 - 2.24X_1X_2 - 0.17X_1X_3 + 0.84X_1X_4 - 1.3X_2X_3 + 0.76X_2X_4 - 1.9X_3X_4$$

The optimized conditions of pH, dye concentration, temperature and salinity were found to be 7.6, 221.41 mg/L, 40.6°C and 12.27 g/L, respectively, for a predicted maximum dye decolourization of 84.2%. Statistical analysis of the data showed a value of 94.72 and 91.84% for R^2 and adjusted R^2, respectively, whereas the average percentage error was estimated to be 9.87%. The experimental validation of RSM predicted the optimum condition of parameters had represented the percentage of dye decolourization of 86.8±0.3%.

Although the RSM parameters showed an adequate statistical fitness, a considerable difference in predicted and experimental results of maximum dye decolourization, as well as substantial value of average percentage error, had revealed the necessity of further optimization of process parameters using a more accurate modeling technique for efficient deduction of probable non-linearities within results. Therefore, the further optimization was carried out by implementing a synergistic ANN-GA based modeling technique.

The hybrid ANN-GA based approach

Artificial neural network is a superior and more powerful modeling technique as compared to RSM as

it represents the nonlinearities in a much better way[20]. Training of the network was carried out by utilizing the 80% (≈24) of CCD data as a training set; however, the rest 20% (≈7) data were served as test data for substantiating the performance of the trained network. The appropriate number of neurons in the hidden layer was accustomed by executing the program at varying numbers of neurons and evaluating the generalization capability of network regarding obtained MSE value at its minimum. Finally, network architecture with a topology of 4-6-1 (*i.e*. 6 neurons in hidden layer) and learning rate of 0.3 was found suitable for dye decolorization by the bacterial system. After completion of training, the network performance was validated using the test dataset. The data processing was conducted between input and hidden layer by implementing tangent sigmoid transfer function (*tansig*), whereas linear transfer function (*purelin*) was used to communicate the data between hidden and an output layer. The training of network was done by utilizing the training function (*trainlm*), which process the data according to the Lavenberg–Marquardt algorithm[30]. Some key codes were: %creating neural network: net=newff (minmax [*input matrix*]) [6 1],{'tansig','purelin'}, 'trainlm', 'mse'); %divide dataset into training and test dataset: net.divideParam.trainRatio=80/100; net.divideParam. testRatio=20/100. %training of ANN: net=train (*net*, *input vector*, *target vector*). % setting the maximum number of training epochs: net. trainParam. epochs=1000. %setting the performance of network in terms of 'mse': net.performFcn='mse' and %setting the 'mse': net.trainParam.goal=0.001. %simulating the output function: Y=sim (*net, input vector of testing dataset*). The goal of least MSE during network training was achieved after 311 epochs with the value for the training data set, E_{trn}=0.187, while MSE value for test data set, under the similar conditions of the network, was E_{tst}=0.387 (Fig. 5A). The percentage average error between the experimental and ANN predicted dye decolourization results for the training and test set data were 3.14 and 10.23, respectively. An R^2 value between the model-predicted dye decolourization and overall experimental data, including training and test data, was 0.9997 (Fig. 5B). Comparably smaller values of the MSE and average prediction error, and better values of R^2, for both training and test set outputs, had clearly illustrated that the constructed network architecture encompasses good approximation characteristics.

The best possible set of pH, dye concentration, temperature and salinity for maximum dye decolorization was obtained from the input space of ANN by the implementation of GA. As in Fig. 5C, the

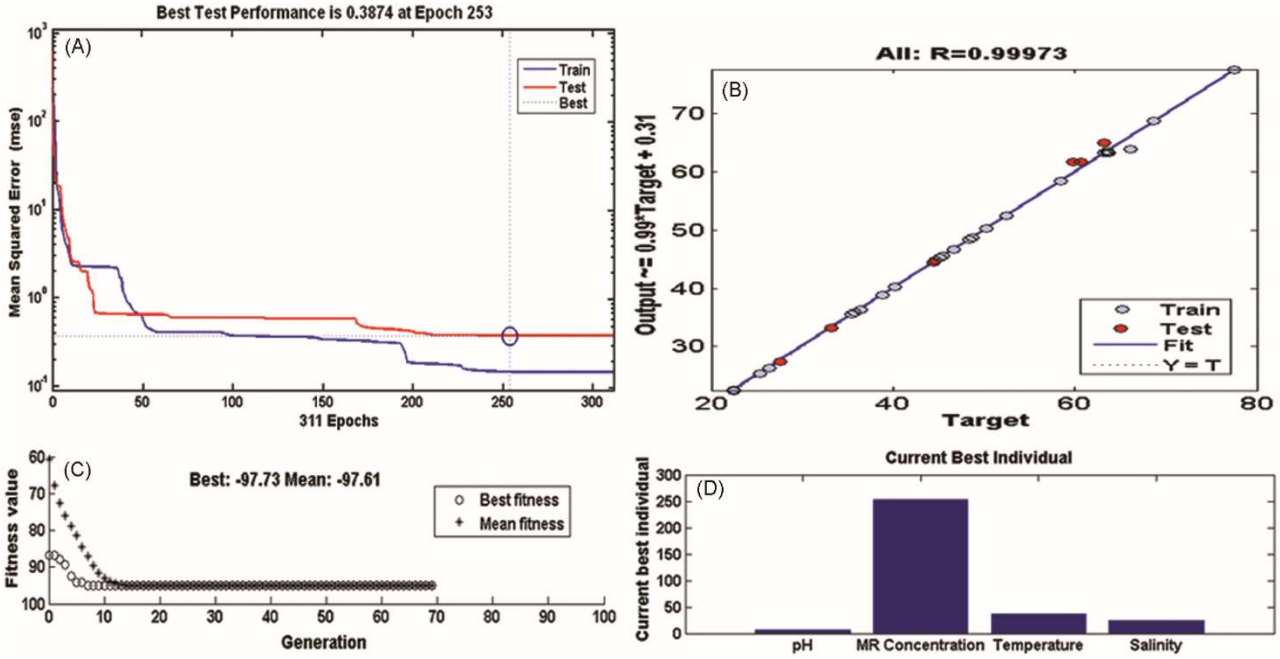

Fig. 5—(A) Mean square error of the network during training and test data till final adaptation; (B) correlations of prediction distribution coefficient (R^2) for training and test set of data; (C) best fitness plot showing the progressive performance [% dye decolorization] of GA till the achievement of optimum solution; and (D) best experimental individual showing optimum values of all four parameters

best fitness plot clearly explained the gradual convergence of results during the iterations of GA to attain optimized condition. The ANN predicted dye decolourization at GA optimized condition was 97.6% under the conditions, pH of 7.21, initial MR concentration 258.54 mg/L, temperature 38.2°C, and salinity 26.3 g/L (Fig. 5D). Unless the ANN-GA model showed a satisfactory fitness, the performance of batch experiments at model-specified conditions was doneto validate the conditions, which had resulted into maximum dye decolorization of 98.1±0.2% illustrating the close accordance with ANN-GA predicted results. In our previous reports, optimum pH and temperature for Orange G degradation wereachieved to be 6.9 and 38°C as a comparison to present pH *i.e.* 7.21 and 38.2°C for MR degradation showing accordance to each other[12]. Reports by other coworkers had also described the biodegradation abilities of different other bacteria near neutral pH and normal range of temperature[17,25]. However, the biodegradation of MR dye by *B. megaterium* ITBHU01 (*i.e.* 98.1%) was comparatively higher than it for Orange G (*i.e.* 94.48%).

Purification of azoreductase

The ability to decolorize textile dyes makes *Bacillus megaterium* ITBHU01 a significant strain regarding industrial effluent treatment[12]. A protein having azoreductase activity was partially purified from the strain using following purification steps: ammonium sulfate fractionation at 20-40% saturation and then, concentrating subsequently the dialyzed fraction up to five-fold using a membrane concentrator followed by ion exchange chromatography using DEAE-Cellulose (Table 3). About 15.3-fold purification and 41.4% recovery of finally purified azoreductase with a specific activity of 6.58 U/mg was achieved. The purity of the enzyme after each step was judged by SDS-PAGE (Fig. 6A), while activity staining was done for locating the presence of the azoreductase (Fig. 6B). Based on the presence of electrophoretic band and respective clear zone of azoreductase activity in zymogram, the molecular weight of the enzyme was estimated to be 28.0±0.2 kDa. Maier *et al.*[31] have reported an azoreductase from *Bacillus* sp. Strain SF with mass 61.6 kDa. However, other azoreductases purified from *Pseudomonas* sp. and *Enterobacter agglomerans* have shown the molecular masses of 30 kDa[18] and 28 kDa[32].

Table 3—Summary of purification steps of azoreductase produced by *B. megaterium* ITBHU01

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Fold purification
Crude extract	963.90	418.00	0.43	100	1.0
$(NH_4)_2SO_4$ precipitation	327.59	301.38	0.92	72.1	2.14
Amicon membrane concentrator	306.12	288.42	0.94	69.0	2.19
DEAE-Cellulose	26.31	173.10	6.58	41.4	15.3

Biodegradation product analysis

UV-Vis absorbance of the dye samples withdrawn at a regular time interval of 4 h after incubation within the wavelength range of 300-800 nm was examined using a UV-Vis spectrophotometer to observe the biodegradation rate of MR dye by *B. megaterium*. The peak observed at 430 nm (λ_{max}) for the control sample (0 h) was disappeared without any shift in wavelength attaining a plateau and indicated that the decolorization was due to dye degradation into intermediate products (Fig. 7).

FTIR spectra of control and treated sample were recorded. In the spectrum of the control dye (Fig. 8A), the absorption peak at 1601 and 1312 cm^{-1}were attributed to the presence of –N=N– stretching of the azo group on the aromatic structure and –N=N– stretching in α substituted compounds, respectively[33]. The peaks at 2929 and 1723 cm^{-1} were assigned to C–H asymmetric stretching and aromatic –CH stretching vibration, respectively. The peaks at 825,

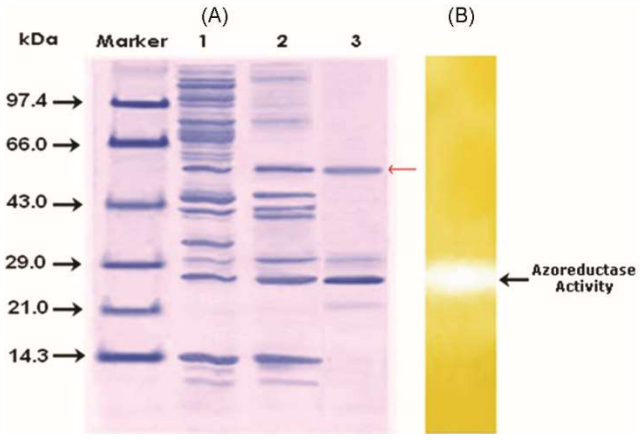

Fig. 6—SDS–PAGE pattern of bacterial homogenate showing the presence of azoreductase. (A) SDS-PAGE (12 % gel) showing the lane of: Marker, 1-crude extract, 2-ammonium sulphate, and 3-DEAE-Cellulose purified enzyme (red arrow showing the probable presence of laccase enzyme); and (B) activity staining of purified azoreductase (decolorized region showing the presence of azoreductase at 28 kDa)

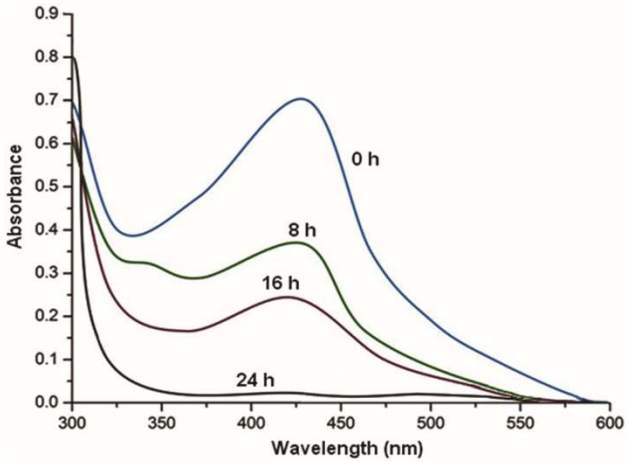

Fig. 7—UV-Vis spectrum of Methyl red and its decolorization products at every 8 h (lowering of peak at 430 nm indicates removal of methyl red). Blue line shows the spectrum for the control dye (0 h) and black line is for the degraded products (24 h)

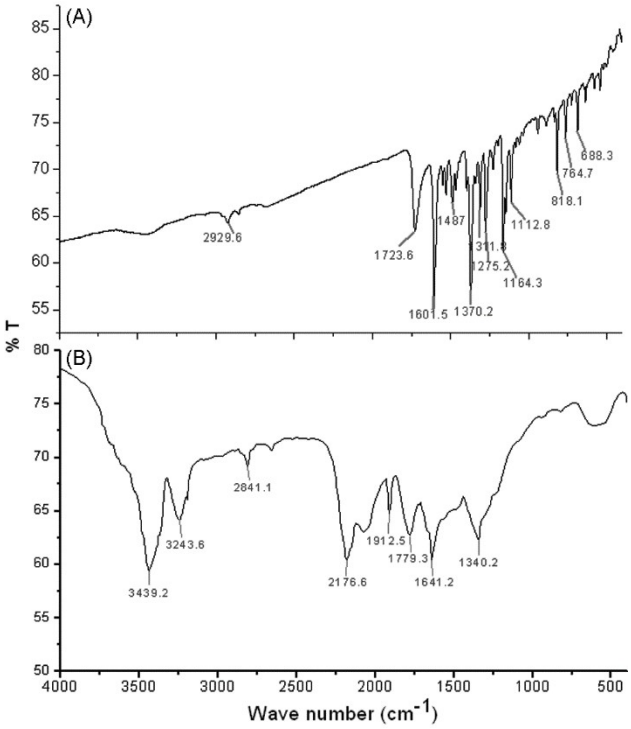

Fig. 8—FTIR spectrum of (A) control methyl red; and (B) degraded sample of methyl red dye extracted after 24 h

765 and 688 cm^{-1} represented aromatic nature of parent dye compound[34]. However, the IR spectrum recorded after complete dye decolourization showed a characteristic change in the positions of peaks when compared to the spectrum of control dye. The absence of the peaks at 1601 and 1312 cm^{-1} had pointed out that the breakdown products of MRlacked azo bond (–N=N– group) confirming the previously obtained UV-Vis results about azo bond dissociation, which might have occurred due to the activity of azoreductase enzyme (Fig.8B). As reported in the case of Orange G[12], biodegradation of this dye was also resulted due to cleavage of an azo bond (-N=N-) with resulting into formation of intermediates like phenyl hydrazine and naphthalene derivatives, perhaps due to the activity of azoreductase enzyme. The spectrum exhibited a peak at 3439 cm^{-1} for N-H stretch and a peak at 2841 cm^{-1} for CH_3 stretch. The appearance of a peak at 3243 cm^{-1} was the indicative of the presence of –OH stretching of an aromatic group, which represented the formation of new metabolites containing hydroxyl group during the degradation of the dye. This result might be due to the activity of laccase enzyme, which can catalyze the oxidation of phenolic and other aromatic compounds. The presence of laccase, in the present case, was verified from electrophoretic gel band showing a presence of a protein band at 58±1.2 kDa. The laccases purified from other bacterial sources such as *Bacillus subtilis* and *Bacillus licheniformis* were reported in the range of molecular mass of 56-65 kDa by other coworkers[35,36]. The activity of laccase enzyme was further confirmed by performing individual assays using 2, 2'-azinobis (3ethylbenzothiazoline-6-sulphonic acid) (ABTS) according to the protocol as described by Koschorreck *et al*.[37]. The peak observed at 1641 cm^{-1} was due to the conjugation of C=C and C=O groups, intimating that this peak could belong to a carbonyl group in a carboxylic acid, ester or conjugated aldehyde groups attached to an aromatic ring[33].

Followed by UV-Vis and FTIR spectra, ESI-MS spectra was recorded for identification of degradation products of methyl red caused by *B. megaterium* ITBHU01. ESI-MS analysis of the supernatant obtained after decolourization confirms degradation of MR dye to intermediate compounds (Fig. 9). The metabolites identified by the ESI-MS spectrum were (M_1) N,N-dimethyl-p-phenylenediamine with molecular weight 136 (m/z) and (M_2) 2-aminobenzoic acid with molecular weight 137 (m/z), but the spectrum showed an m/z peak at 138 indicating the presence of protonated molecule (M_2+H^+). During the degradation, there is symmetric cleavage of azo bonds in MR due to the activity of azoreductase enzyme resulting in the formation of a N,N-dimethyl-p-phenylenediamine and 2-aminobenzoic acid. Some additional peaks in the spectrum were observed at

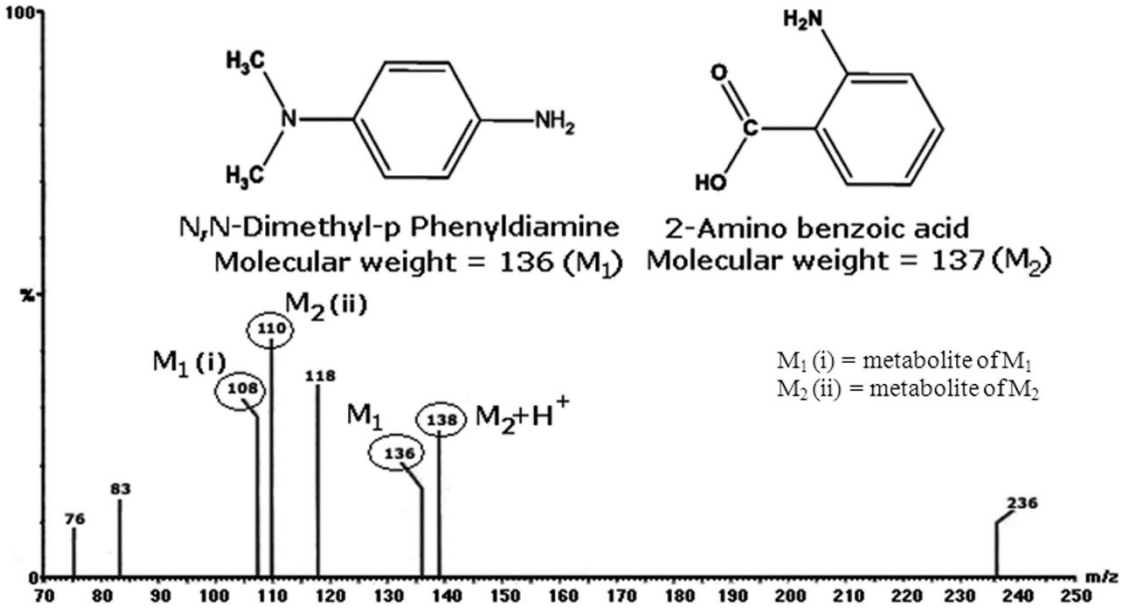

Fig. 9—ESI-MSspectraofdegradationofmethylred by*Bacillus megaterium* ITBHU01 revealing the degradation mechanism

Table 4 — Phytotoxicitystudies of methyl red and its degradation products by *B. megaterium* ITBHU01

Parameter studied	*Sorghum bicolor*			*Triticum aestivum*			*Solanum lycopersicum*		
	BSM	Untreated MR dye	Treated MR dye	BSM	Untreated MR dye	Treated MR dye	BSM	Untreated MR dye	Treated MR dye
Germination (%)	100	65	100	100	55	100	100	70	95
Plumule length (cm) (± S.D.)	5.76±0.32	2.11±0.24	4.81±0.14	6.32±0.26	2.48±0.08	5.28±0.42	3.52±0.22	1.08±0.20	2.78±0.16
Radical length (cm) (± S.D.)	6.14±0.21	3.08±0.42	5.21±0.44	8.66±0.11	3.05±0.40	6.86±0.36	4.47±0.12	1.54±0.31	4.11±0.08

The seed germination parametersshown in the table as means ± S.D. (n = 3)

m/z of 110 and 108 with a considerable amount of relative intensity, which could be due to the presence of other degradation products. Ziegler *et al*.[38] had reported the presence of catechol (m/z: 110) as an intermediate compound obtained after the degradation of 2-aminobenzoic acid under partial aerobic conditions. The peak of catechol, in the present case, would be due to the activity of laccase enzyme secreted in fermentation broth of *Bacillus megaterium* ITBHU01. Yemashova *et al*.[39] had accounted that 1,4-phenylene-diamine (m/z: 108) was a metabolic product, attained after the transformation of N,N-dimethyl-p-phenylenediamine, which strengthen the probability of a further breakdown of N,N-dimethyl-p-phenylenediamine to 1,4-phenylenediamine (m/z: 108) in the present case also.

Several authors have already reported N, N-dimethyl-p-phenylenediamine and 2-aminobenzoic acid as the metabolic products obtained after degradation of MR due to microbial activity as in the case of *Enterobacter agglomerans*[40], *Galactomyces geotrichum* MTCC 1360[41] and *Klebsiella pneumoniae* RS-13[42].

Phytotoxicity study

The phytotoxicity assay was performed to evaluate the nature of degraded products of MR dye by *Bacillus megaterium* ITBHU01 as well as to access the probable impact of treated effluent on ecosystem and fertility of soil on disposal. As clear from the results shown in Table 4, untreated (control) MR dye (at the concentration of 258.54 mg/L) was quite toxic for crop seeds used in our study *viz*. *S. bicolor*, *T. Aestivum* and *S. lycopersicum* showing 35%, 45% and 30% inhibition, respectively, on seed germination. However, in contrast with that of the untreated MR dye, the results of phytotoxicity assays for degradation products of MR did not exhibit any inhibitory or hazardous outcomes, moreover, these degradation products supported good seed

germination with significant shoot and root growth rate in *S. bicolor*, *T. Aestivum* and *S. Lycopersicum* (Table 4). These outcomes for effective treatment of MR can establish *Bacillus megaterium* ITBHU01 as a promising bacterial strain in effluent treatment industries.

Conclusion

The results, thus, obtained have successfully established Methyl red degrading efficiency of *Bacillus megaterium* ITBHU01. The bacterial culture has got tremendous potential in wastewater treatment. The ability of the strain to tolerate, decolourize and degrade azo dyes at high concentration and transforming them to non-toxic metabolites endows it an edge over traditional methods for treatment of textile industry wastewater. The presence of enzymes, such as azoreductase and laccase imparted a novel bioremediation property to the strain *B. megaterium* ITBHU01. The strain could be used as an eco-friendly substitute to conventional physicochemical methods of dye effluent treatment (such as electrochemical, photochemical or chemical methods) as well as to overcome the threat posed by azo dyes and its intermediates.

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