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Decolorization of sulfonated azo dye Metanil Yellow by newly isolated bacterial strains: *Bacillus* sp. strain AK1 and *Lysinibacillus* sp. strain AK2

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ABSTRACT

Two different bacterial strains capable of decolorizing a highly water soluble azo dye Metanil Yellow were isolated from dye contaminated soil sample collected from Atul Dyeing Industry, Bellary, India. The individual bacterial strains *Bacillus* sp. AK1 and *Lysinibacillus* sp. AK2 decolorized Metanil Yellow (200 mg L^{-1}) completely within 27 and 12 h respectively. Various parameters like pH, temperature, NaCl and initial dye concentrations were optimized to develop an economically feasible decolorization process. The maximum concentration of Metanil Yellow (1000 mg L^{-1}) was decolorized by strains AK2 and AK1 within 78 and 84 h respectively. These strains could decolorize Metanil Yellow over a broad pH range 5.5–9.0; the optimum pH was 7.2. The decolorization of Metanil Yellow was most efficient at 40 °C and confirmed by UV–visible spectroscopy, TLC, HPLC and GC/MS analysis. Further, both the strains showed the involvement of azoreductase in the decolorization process. Phytotoxicity studies of catabolic products of Metanil Yellow on the seeds of chick pea and pigeon pea revealed much reduction in the toxicity of metabolites as compared to the parent dye. These results indicating the effectiveness of strains AK1 and AK2 for the treatment of textile effluents containing azo dyes.

1. Introduction

Azo dyes constitute the largest class of dyes with the greatest variety of colors, having wide applications in textile, food, printing and cosmetic industries [1]. Azo dyes are characterized by the presence of one or more azo bonds –N=N–, which are responsible for their coloration. When such a bond is broken the compound loses its color [2]. The annual world wide production of azo dyes was estimated to be around one million tons and more than 10,000 structurally different azo dyes are currently in use [3,4]. It is reported that about 300,000 tons of different dyestuffs are used per year for textile dyeing operations. Thus the dyestuff, textile, paper, and leather industries are the major consumers of synthetic azo dyes and produce effluents that are usually resistant to the physico-chemical treatments and consequently served as the major source of water pollution. It is estimated that 10–15% of the total dye consumed in dyeing processes may be found in wastewater. This led to severe contamination of both surface and ground water [5,6]. Unless the wastewater is properly treated, these dyes may significantly affect the photosynthetic activity of aquatic life due to reduced light penetration as well as toxic to some aquatic life [7].

Metanil Yellow (Acid Yellow 36) is a highly water-soluble dye is extensively used for the coloring of soap, spirit lacquer, shoe polish, bloom sheep dip, for the preparation of wood stains, dyeing of leather, manufacture of pigment lakes and for staining paper [8]. Though Metanil Yellow is a non-permitted colorant, but still it is widely used as a colorant in sweet meat, ice creams, soft drinks and beverages. Due to its orange-yellow color, the dye is also extensively used for coating turmeric. The dye is highly suitable for the preparation of colored water-fast inks [9].

Toxicity data reveals that oral feeding or intraperitoneal and intratesticular administration of Metanil Yellow in animals produces testicular lesions due to which seminiferous tubules suffer damage and results in the decreased rate of spermatogenesis [10]. Studies have shown that 13.6% of the orally administered dose of Metanil Yellow (15 mg 200 g^{-1} rat) is retained even after 96 h in the gastrointestinal tract, which may be responsible for decreased mucin secretion from the intestinal mucous cells [11]. On oral consumption, it causes toxic methaemoglobinaemia [12] and cyanosis [13] in humans, while skin contact results into allergic dermatitis [14]. Metanil Yellow also has tumour-producing effects and can create intestinal and enzymic disorders in human body [11]. Though it is not mutagenic but can alter the expression of genes [15]. Thus, keeping the hazardous effects of this dye in view, attempts have been made by various researchers to remove Metanil Yellow from wastewater [10,16,17].

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Removal of Metanil Yellow from wastewater has become a major scientific interest. Several physico-chemical methods have been used to eliminate the Metanil Yellow from colored effluents in wastewater. These methods are rather costly and sometimes produce hazardous byproducts [18]. Therefore, there is still a demand to develop alternative methods for dye decolorization. The innovative biological methods are able to provide a more natural and complete clean-up of the pollutants in a more economical way. Microbial decolorization is an eco-friendly, cost-competitive, alternative to chemical decomposition process [19].

In the present investigation, we have reported the isolation and identification of micro-organisms from textile effluent contaminated soil capable of decolorizing Metanil Yellow. The various parameters such as initial dye concentration, temperature, pH and NaCl concentration have been optimized to achieve the maximum dye decolorization. The intermediate metabolites formed during the degradation of Metanil Yellow have been characterized by using various analytical techniques. The information obtained from this study is expected to provide the basis for the development of practical decolorization process for Metanil Yellow using these bacterial strains as biocatalysts. To our knowledge, this could be the first report on the decolorization of sulfonated azo dyes by *Lysinibacillus* sp.

2. Materials and methods

2.1. Chemicals and decolorization medium

The dyes Metanil Yellow, Amaranth, Congo Red, Reactive Black 5, Ponceau S and Fast Red were gifted from Mayura Dyes, Mumbai, India and all other chemicals were procured from S.D Fine Chemicals, Mumbai, India. The culture media ingredients were purchased from Hi-Media, Mumbai, India. The composition of decolorization medium was ($g\,L^{-1}$): K_2HPO_4 6.3; KH_2PO_4 1.8; NaCl 5; NH_4NO_3 1; $MgSO_4{\cdot}7H_2O$ 0.1; $MnSO_4$ 0.1; $CaCl_2{\cdot}2H_2O$ 0.1; $FeSO_4{\cdot}7H_2O$ 0.1; $NaMoO_7{\cdot}7H_2O$ 0.006; pH 7.2 ± 0.1. This medium was supplemented with yeast extract ($2.5\,g\,L^{-1}$) and Metanil Yellow ($200\,mg\,L^{-1}$). All other chemicals used in this study were of analytical grade.

2.2. Isolation and identification of bacterial strains

Dye contaminated soil sample was collected in and around the Atul Dyeing Industry, Bellary, India. A soil sample of 10 g was agitated in 50 mL of saline (0.8% NaCl) solution and kept on an orbital shaker at 180 rpm for 30 min. The supernatant was collected after allowing the soil to settle down for 30 min. The microorganisms having the ability to degrade dye were screened by inoculating 1 mL of supernatant on nutrient agar plates ($g\,L^{-1}$; peptone 5, yeast extract 2.5, NaCl 5, agar 25) containing $100\,mg\,L^{-1}$ of Metanil Yellow and incubated at 37 °C for 1 week. The colonies were selected on the basis of their ability to form clear zones on these plates. Such colonies were subsequently transferred to nutrient broth containing Metanil Yellow.

2.3. 16S rDNA sequencing

Single colony was suspended in 20 μl of Tris–HCl–EDTA–saline (pH 8.0). The bacterial suspension was incubated for 10 min at 95 °C and centrifuged at 18,600 × *g* for 2 min. The supernatant was transferred to fresh tubes and used as total genomic samples. The bacterial 16S rDNA gene was amplified from the total genomic DNA using universal eubacteria specific primers viz: 16F27 (5′-CCA GAG TTT GAT CMT GGC TCA G-3′) and 26R1525XP (5′-TTC TGC AGT CTA GAA GGA GGT GWT CCA GCC-3′) (Life Technologies, Pvt. Ltd., India), which yielded a product of approximately 1500 bp. The PCR conditions were 35 cycles at 95 °C denaturation for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, in addition one cycle of extension at 72 °C for 10 min. The PCR product was purified by PEG–NaCl precipitation and the purified sample was directly sequenced using Big Dye-terminator kit as described earlier [20]. The sequencing reactions were run on ABI-PRISM automated sequencer (ABI-3730 DNA analyzer). The nucleotide sequence analysis of the sequences was done at Blast-n site at NCBI server (www.ncbi.nih.gov/BLAST). The alignment of the sequences were done using CLUSTALW program V1.82 at European Bioinformatics site (www.ebi.ac.uk/clustalw). The analysis of 16S rDNA gene sequence was done at Ribosomal Database Project (RDP) II (http://rdp.cme.msu.edu). The phylogenetic tree was constructed using the aligned sequences by the neighbor joining method using Kimura-2-parameter distances in MEGA 2.1 software [21].

2.4. Decolorization studies of Metanil Yellow in liquid medium

The decolorization experiments were performed in 250 mL Erlenmeyer flasks containing decolorization medium. Metanil Yellow ($200\,mg\,L^{-1}$) was added to decolorization medium and inoculated with 2 mL of cultures broth (6×10^9 CFU mL^{-1}). The flasks were incubated at 40 °C under static condition till the decolorization was completed. The samples were withdrawn at different time intervals and analyzed for decolorization efficiency. The decolorization was monitored spectrophotometrically (Specord 50 BU., Germany) by reading the decrease in absorbance (430 nm) of the dye in culture supernatant. Uninoculated controls were used to compare abiotic color loss during the decolorization studies. Decolorizing activity is expressed in terms of percentage decolorization [22].

2.5. Assay of azoreductase

Azoreductase activity was measured according to the method described previously [4]. The bacterial cultures grown in the medium containing Metanil Yellow ($200\,mg\,L^{-1}$) was centrifuged at 10,000 rpm for 10 min at 4 °C. The cell pellet was washed twice with 50 mM potassium phosphate buffer (pH 7.0) and resuspended in the same buffer. The cells were disrupted in cold condition by sonication (30 s, 70% output, 5×) using Vibra Cell Ultrasonicator (Model VC 375, USA). The sonicated cells were centrifuged at 15,000 rpm for 30 min at 4 °C and the supernatant was used as the crude enzyme source. To 50 mM phosphate buffer (pH 7.0) containing 150 μM Metanil Yellow, 100 μL of crude enzyme was added and this reaction mixture (1 mL) was pre-incubated for 3 min followed by the addition of 2 mM NADH. The azoreductase activity was determined spectrophotometrically at room temperature by monitoring the decrease in absorbance at 340 nm. One unit of enzyme activity was defined as the amount of enzyme that catalyses the 1 μmol of substrate per minute. All experiments including enzyme assay were carried out in triplicate.

2.6. Study of physico-chemical parameters

Decolorization ability of strains AK1 and AK2 on Metanil Yellow was studied at different pH (4–9) and temperatures values (15–50 °C). It was observed that pH 7.2 and temperature 37 °C were found to be optimal for the decolorization activity. Based on the optimal decolorization, pH 7.2 and temperature of 37 °C were selected to study the decolorization process under various physico chemical factors such as dye concentration ($200–1000\,mg\,L^{-1}$), NaCl concentration ($5–20\,g\,L^{-1}$) and continuous addition of Metanil Yellow in the decolorization medium. Further, the decolorization of various azo dyes was studied by incubating the medium containing respective dye with individual strains AK1 and AK2. Decoloriza-

tion of different dyes was monitored by measuring the change in absorbance of culture supernatants at the maximum absorption wavelength (λ_{max}) of the respective dye. These experiments were performed in 250 mL Erlenmeyer flask under static condition in triplicate.

2.7. Analysis of degradation products of Metanil Yellow

The complete decolorized culture broths were centrifuged and the resulting supernatant was clarified by passing through 0.45 μm filter. The filtrate was extracted with equal volume of ethyl acetate and the organic phase was dried in an evaporator (Heidolph, Laborota 4000, Germany). The resulting residue was dissolved in methanol. The metabolic intermediates were analyzed and separated by TLC (Silica gel G, solvent-ethyl acetate:butanol:water:glacial acetic acid 3:3:1:0.2, v/v) and the chromatograms were observed under UV light (254 nm) and then exposed to iodine vapors in an iodine chamber. The extracted metabolites were also analyzed by HPLC (Waters, reverse phase C-18 column), 25 °C, with an isocratic condition, eluent – methanol:water (50:50, v/v). The injection volume was 10 μL and the flow rate was 1 mL min^{-1}. Gas chromatography–mass spectroscopy (GC/MS) analysis was carried out using a Shimadzu System.

2.8. Phytotoxicity studies

Phytoxicity studies of control dye, Metanil Yellow and its degradation products of ethyl acetate extracts (acidic and neutral fractions) were investigated. Tests were carried out on two kinds of seeds commonly used in the Indian agriculture; Pigeon pea (*Cajanus cajan*) and Chickpea (*Cicer arietinum*). The concentration of Metanil Yellow (0.4 g L^{-1}) showing inhibitory effect on the growth of seeds was selected for further studies. Ten seeds were taken to germinate on a bedded filter paper with daily watering of 10.0 mL solutions for respective samples (control dye/acidic/neutral fractions). Simultaneously, a control set with the plain water was carried out. The toxicity was measured in terms of percent germination and lengths of plumule and radical after 10 days. Relative seed germination, relative root elongation and germination index (GI) were calculated by the following formulae [23];

$$\text{Relative Seed Germination (\%)} = \frac{\text{No. of Seeds germinated in the extract}}{\text{No. of Seeds germinated in the control dye}} \times 100$$

$$\text{Relative root elongation (\%)} = \frac{\text{Mean root elongation in the extract}}{\text{Mean root elongation in the control dye}} \times 100$$

$$\text{Germination Index (\%)} = \frac{(\%\,\text{Seed germination}) \times (\%\,\text{Root elongation})}{100}$$

3. Results and discussion

3.1. Identification and phylogenetic position of bacterial isolates

Two different bacterial strains having remarkable Metanil Yellow decolorization capacity were isolated from dye contaminated soil sample collected in and around the Atul Dyeing Industry, Bellary, India. The identification of the strains was done on the basis of morphological and biochemical characteristics (Table 1) and 16S rDNA gene sequences. Bacterial strains AK1 and AK2 were identified as, *Bacillus* sp. strain AK1 and *Lysinibacillus* sp. strain AK2 respectively. To analyze the phylogenetic position, the 16S rDNA sequences of the strains AK1 and AK2 were determined. Fig. 1a and b shows the phylogenetic relationship between the isolated bacterial strains and other related bacteria found in the GenBank

Table 1
Characterization of bacterial isolates.

Sl. no.	Characteristic	*Bacillus* sp. AK1	*Lysinibacillus* sp. AK2
Morphological characteristics			
1.	Shape	Rod short	Rod short
2.	Gram staining	+	+
3.	Spore staining	+	+
4.	Motility	Motile	Motile
Biochemical tests			
1.	H_2S production	–	+
2.	Gelatin hydrolysis	–	–
3.	Citrate utilization	+	–
4.	Catalase activity	+	+
5.	Lactose utilization		
5a.	Gas production	–	–
5b.	Acid production	+	–

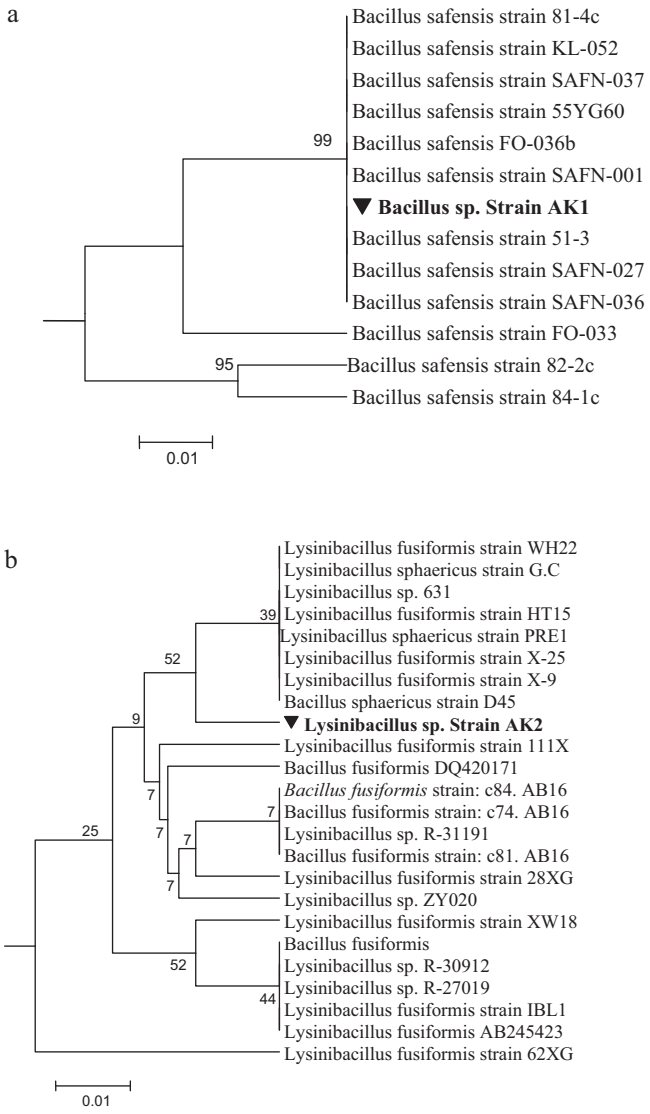

Fig. 1. Phylogenetic tree: (a) *Bacillus* sp. Strain AK1 and (b) *Lysinibacillus* sp. Strain AK2. Numbers at the nodes show the % bootstrap value.

Table 2
Decolorization of Metanil Yellow by various methods.

Method	Decolorization	Reference
1. Adsorption	86.9 mg/g	[17]
	99%	[10]
2. Photocatalytic degradation	90%	[16]
3. Electrochemical oxidation	90%	[18]
4. Microbial system		
Oenococcus oeni ML34	30%	[41]
Bacillus sp. AK1	100%	Present study
Lysinibacillus sp. AK2	100%	Present study

database. The homology assay results indicated that the strains AK1 and AK2 were in the phylogenetic branch of the genus *Bacillus* and *Lysinibacillus* respectively. *Bacillus* sp. is usually dominant in the activated sludge system, decolorizing and eventually degrading azo dyes [24,25]. Chen et al. [26] reported the phenol tolerance of indigenous phenol degraders such as *Klebsiella* sp. and *Lysinibacillus* sp. isolated from Northeast Taiwan. To our knowledge this is the first report on decolorization of azo dyes by *Lysinibacillus* sp. Both of these bacterial strains AK1 and AK2 are deposited in National Collection of Industrial Microorganisms (NCIM), Pune, India with accession No NCIM 5332 and 5333 respectively for public access. The 16S rDNA gene sequences available in GeneBank database with the accession number FJ888394 and FJ888395 respectively.

3.2. Time course for decolorization of Metanil Yellow in liquid medium

These two bacterial cultures were subjected to further screening for their ability to decolorize Metanil Yellow in liquid medium. The strain AK2 exhibited dye decolorization at a faster rate as compared to strain AK1 when incubated under static condition. At an early 6 h of incubation 37% and 9% of decolorization was exhibited by both the strains AK2 and AK1 respectively. It could be seen that AK2 exhibited high decolorization activity and could achieve 100% decolorization within 10–12 h. However, the decolorization activity of Metanil Yellow by strain AK1 was very slow and it could show only 22% decolorization within 12 h and 99% decolorization could be achieved within 27 h. Vijaykumar et al. reported that 99% decolorization of sulfonated azo dyes by *Kerstersia* sp. under static condition [4]. The maximum decolorization (100%) of Metanil Yellow by *Bacillus* sp. AK1 and *Lysinibacillus* sp. AK2 observed in the present study was compared with those of other methods used to decolorize Metanil Yellow in Table 2.

3.3. Azoreductase activity

The major mechanism involved in the microbial biodegradation of synthetic azo dyes is based on their biotransformation by enzymes [27]. The initial step involved in the biodegradation of azo dyes is the reductive cleavage of azo bond (–N=N–) with the azoreductase [28]. The decolorization of Metanil Yellow by azoreductase activity was monitored over the time intervals. Fig. 2 shows the decolorization of Metanil Yellow along with the respective azoreductase activity with time intervals for strains AK1 and AK2. A significant increase in the azoreductase activity of 1615 nmol/mg protein/min and 1630 nmol/mg protein/min was observed in the strains AK1 (Fig. 2a) and AK2 (Fig. 2b) respectively. Further, a significant induction in the activity of azoreductase was observed in the cell free extracts of both the strains AK1 and AK2 during Metanil Yellow decolorization when compared to the control cells. Such inductive pattern of azoreductase was observed during decolorization of sulfonated azo dyes by *Kerstersia* sp. strain VKY1 [4] and *Galactomyces geotrichum* MTCC 1360 [29]. From these results it is evident that the strains AK1 and AK2 degrade Metanil Yellow by enzymatic reduction with azoreductase.

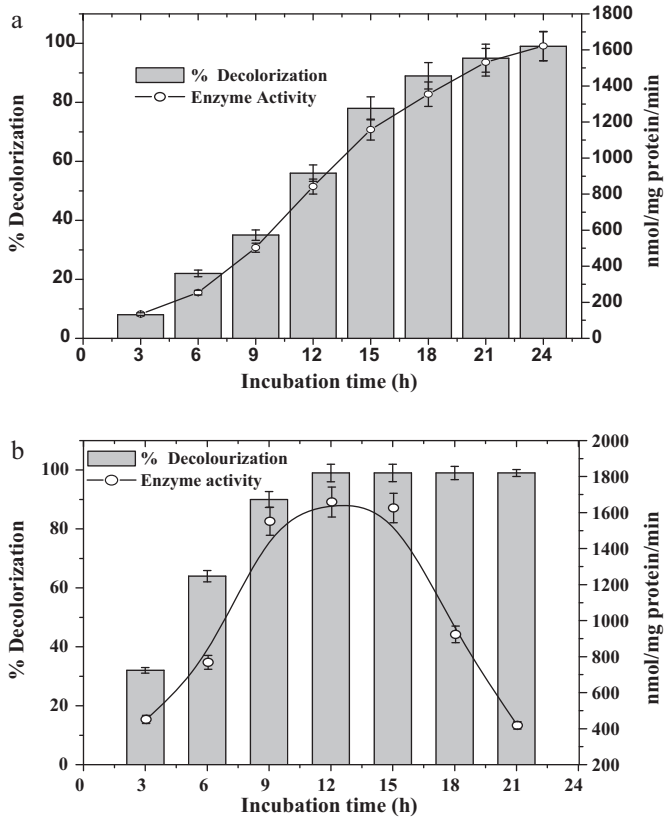

Fig. 2. Azoreductase activity of (a) *Bacillus* sp. AK1 and (b) *Lysinibacillus* sp. AK2.

3.4. Decolorization of structurally different azo dyes with strains AK1 and AK2

Dyes of different structures were often used in the textile processing industry, and therefore, the effluents from the industry are markedly variable in composition [30]. Strains AK1 and AK2 were tested for their ability to decolorize six structurally different azo dyes such as Metanil Yellow, Amaranth, Congo Red, Fast Red, Ponceau S and Reactive Black 5. The bacterial strains AK1 and AK2 had shown effective decolorization of all the six structurally different azo dyes (Table 3). The rapid decolorization of all azo dyes was observed by strain AK2 as compared to strain AK1. The slower decolorization with individual strains was due to structural differences, higher molecular weight and presence of inhibitory groups likes $–NO_2$ and $–SO_3Na$ in the dyes [30]. The present study confirms the ability of strains AK1 and AK2 to decolorize six structurally different azo dyes with decolorization efficiency of more than 50% in short time. Thus, the strains AK1 and AK2 could be successfully

Table 3
Decolorization of various azo dyes by *Bacillus* sp. AK1 and *Lysinibacillus* sp. AK2.

Azo dye	λ_{max} (nm)	*Bacillus* sp. AK1 (% decolorization)	*Lysinibacillus* sp. AK2 (% decolorization)
Metanil Yellow	430	99	100
Amaranth	520	97	99
Congo Red	502	80	98
Fast Red	512	62	71
Ponceaus S	470	46	58
Reactive Black	596	31	43

The cultures were incubated with azo dye at 37 °C under static conditions; 12 h for strain AK1 and 24 h for strain AK2.

employed for the treatment of industrial wastewater containing Metanil Yellow, Amaranth, Congo Red, Fast Red, Ponceaus S and Reactive Black 5 efficiently.

3.5. Effect of initial dye concentration and repeated use of cells

The percentage decolorization of Metanil Yellow by strains AK1 and AK2 was carried out at different initial dye concentrations (100–1000 mg L^{-1}). It was observed that decrease in percentage of decolorization of Metanil Yellow with increase in concentration under static condition by both the strains. Strain AK2 could effectively decolorize Metanil Yellow with decolorization percentage of 100% for 200 mg L^{-1} and 62% for 1000 mg L^{-1} dye concentration during 12 h and 78 h of incubation respectively. The decolorization percentage of 100% for 200 mg L^{-1} and 75% for 1000 mg L^{-1} during 24 h and 84 h of incubation respectively with strain AK1. Lower decolorization percentage at high dye concentration was reported and was expected to be due to the inhibitory effects of high dye concentration [2]. Similar observations have been recorded earlier for decolorization of synthetic dyes using a batch decolorization assay [31]. The repeated use of cells of strains AK1 and AK2 degrade Metanil Yellow (200 mg L^{-1}) up to nine cycles, but with an increase in time and reduction in percentage decolorization. The initial four cycles of dye decolorization with AK1 and AK2 degraded 100% dye at 12 h and 24 h respectively, but for the next remaining five cycles increase in time with reduced percentage of dye decolorization was observed for both the strains (data not shown).

3.6. Effect of temperature on decolorization of Metanil Yellow

The temperature effect on the decolorization of Metanil Yellow was significant for both the strains. When the decolorization of the dye was tested for a wide range of temperatures from 15 to 50 °C, it was observed that the increase in decolorization of Metanil Yellow with increase in temperature and was optimum at 37 °C for both the strains AK1 and AK2. Further increase in the temperature increases the decolorization of dye up to 40 °C and above this temperature a decreased dye decolorization was noticed (Fig. 3a). To understand the effect of low temperature, room temperature and high temperature on the decolorization of dye, the assay was carried out at different temperature range from 15 to 50 °C. The decrease in dye decolorization at high temperature can be attributed to the decline in microbial activity that led to the inactivation of the enzyme and eventually the loss of cells viability [32]. These results further showed that there is no thermal deactivation of decolorization activity under operational temperatures. Therefore, these strains AK1 and AK2 could acclimatize to broad range of temperature. Kapilkumar et al. [33] observed that the decrease in the decolorization efficiency of mixed cultures for color removal beyond 35 °C, which was predicted as thermal deactivation of the decolorization enzymes.

3.7. Effect of pH on decolorization of Metanil Yellow

The effect of pH on the decolorization of Metanil Yellow by both the strains was determined over a wide range of pH (4.0–9.0). Both the bacterial strains AK1 and AK2 showed maximum dye decolorization at pH 7.2 (Fig. 3b). At this optimum pH, the strain AK1 showed 98.5% and that of strain AK2 showed 99.9% of decolorization of Metanil Yellow. At pH 8.0, both the strains AK1 and AK2 showed 80% and 84% decolorization of Metanil Yellow respectively. Whereas at pH 4.0, both the strains showed only 8.0% and 15% dye decolorization respectively. Similar results were also reported by Sukumar et al. [34]. Chan and Kuo reported that the neutral pH would be more favorable for decolorization of the azo dyes and is suitable for industrial applications [35].

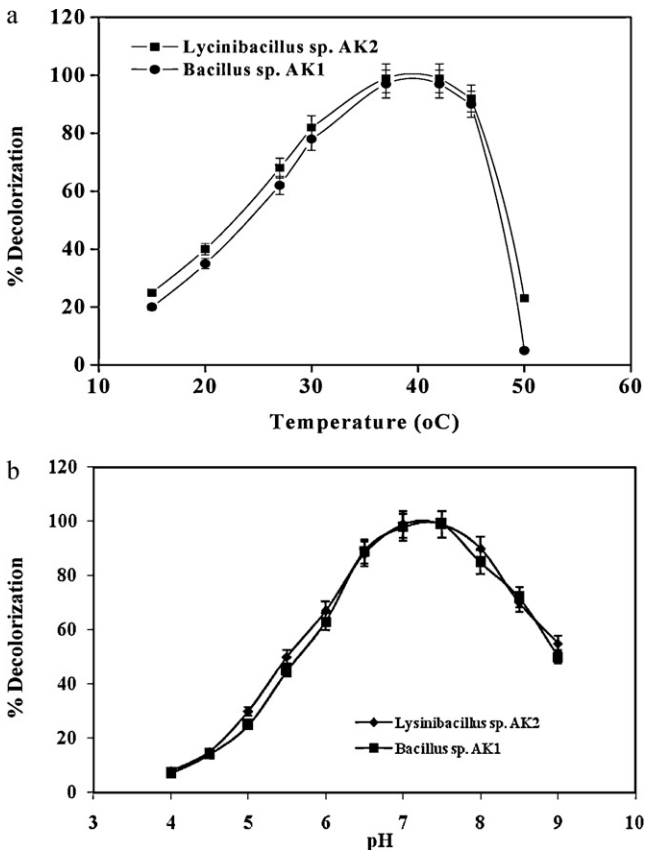

Fig. 3. Effect of (a) temperature and (b) pH on the decolorization of Metanil Yellow by both the strains AK1 and AK2.

3.8. Effect of NaCl concentration on decolorization of Metanil Yellow

The high salt concentration is a consequent product of batch processes in both the dye manufacturing and dye-consuming industries where the salt concentration is up to 15–20% [36]. Generally, sodium concentration above 3 g L^{-1} can cause moderate inhibition of most bacterial activities [37]. In the present study, the effect of NaCl concentration on the decolorization of Metanil Yellow by both the strains was examined. Both the strains AK1 and AK2 Exhibit 99% decolorization at 5 g L^{-1} NaCl concentration. Further, the NaCl concentration up to 10 g L^{-1} did not show any effect on dye decolorization by both cultures. At this concentration of NaCl, 90% and 95% of decolorization of Metanil Yellow was achieved by strains AK1 and AK2 respectively. Further, increase in concentration of NaCl results in decreased percentage of decolorization. The concentration of 20 g L^{-1} of NaCl drops the percentage decolorization up to 41% and 44% by strains AK1 and AK2 respectively.

3.9. Analysis and characterization of Metanil Yellow degradation products

The decolorization of Metanil Yellow by strains AK1 and AK2 was investigated spectrophotometrically at 430 nm at different hour of incubation period (Fig. 4). After complete decolorization of Metanil Yellow by strain AK1 within 12 h and by strain AK2 within 24 h, the spent medium was extracted with ethyl acetate and the residue was subjected for TLC, HPLC and GC/MS analysis. The TLC analysis of ethyl acetate extract of the spent medium showed two spots with different R_f values (0.28 and 0.86) for both strains AK1 and AK2. The R_f value of 0.28 corresponded well with the authentic metanilic acid and that of the R_f value 0.86 coincided with the R_f value of

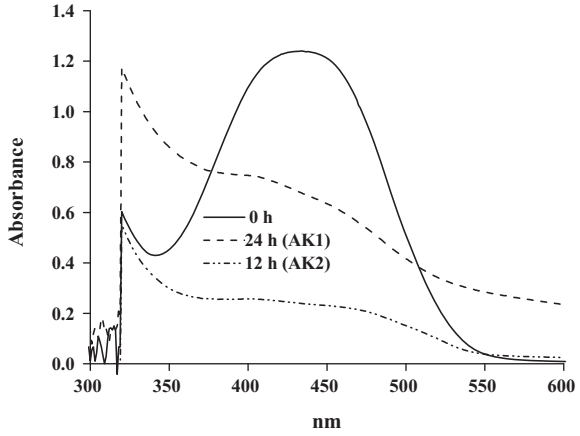

Fig. 4. UV–visible spectrophotometric analysis of Metanil Yellow decolorization by *Bacillus* sp. AK1 and *Lysinibacillus* sp. AK2.

authentic p-aminodiphenylamine. The HPLC analysis for the ethyl acetate extract of the spent medium taken at the beginning of static incubation showed a major peak with R_T value of 28.21 min (Fig. 5a), which represents the R_T value of authentic Metanil Yellow. This corresponding peak was absent in the dye decolorization medium treated with strains AK1 and AK2 (Fig. 5b). The metabolites extracted from the spent medium showed different R_T values. Metanil Yellow showed a major peak with R_T value 28.21, whereas Metanil Yellow degradation products showed major peaks with R_T values 24.1 and 2.8 for both strains AK1 and AK2. The peak with R_T value 2.8 corresponds to Metanillic acid and the peak with R_T value 24.1 corresponds to p-aminodiphenylamine [38]. GC/MS analysis was also carried out to investigate the metabolites formed during the biodegradation of Metanil Yellow by both strains. The mass spectrum analysis of both the strains (Fig. 6a and b) displayed the *m*/*z* value at 183, which corresponding to Metanillic acid and *m*/*z* value at 195, which corresponding to p-aminodiphenylamine. Based upon the TLC, HPLC and GC/MS results as well as the presence

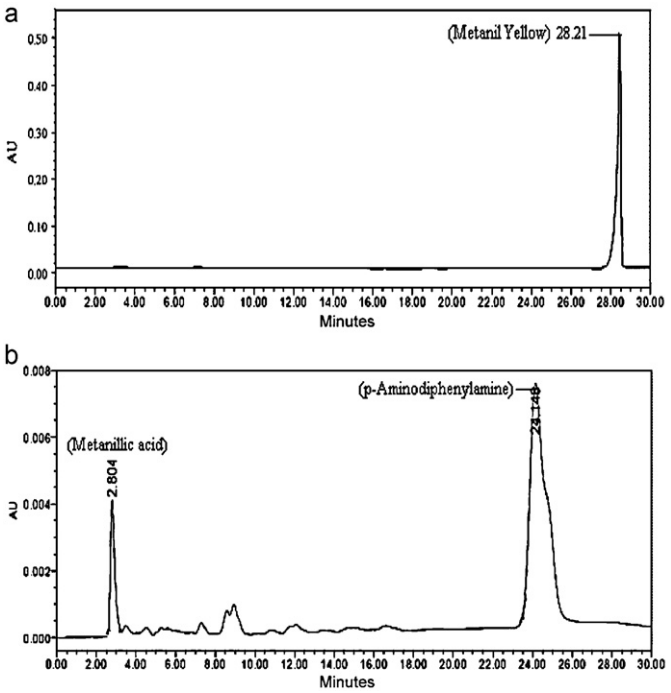

Fig. 5. HPLC analysis of (a) Metanil Yellow and (b) its degradation products by both the strains AK1 and AK2.

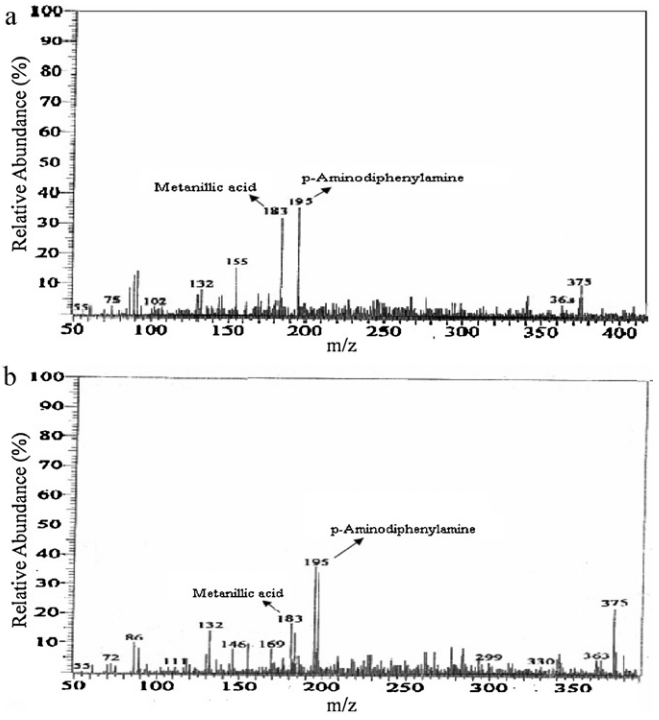

Fig. 6. GC/MS analysis of metabolites of Metanil Yellow by (a) *Bacillus* sp. AK1 and (b) *Lysinibacillus* sp. AK2.

of azoreductase activity, a partial degradation pathway of Metanil Yellow by both the strains AK1 and AK2 is shown in Fig. 7.

3.10. Phytotoxicity studies of Metanil Yellow and its degradation products

Seed germination and plant growth bioassays are the most common techniques used to evaluate the phytotoxicity of toxicants [30,39,40]. We investigated the phytotoxicity of Metanil Yellow and acidic and neutral fractions of ethyl acetate extracts of the cultures grown spent medium. Table 4 illustrates the significant different lengths of plumule and radical of the seeds of pigeon pea (*C. cajan*) and chickpea (*C. arietinum*) in the water, control dye (Metanil Yellow) and degradation products (acidic and neutral fractions) of strains AK1 and AK2 treated samples. The relative seed germination and relative root elongation of pigeon pea was 85.71 and 55.8% for AK1 respectively and that of AK2 was 86.25 and 65.3% respectively; whereas that of chickpea was 83.33 and 28.2% for AK1 respectively and that of AK2 was 85.12 and 30.2% respectively. However, germination index of pigeon pea and chick-

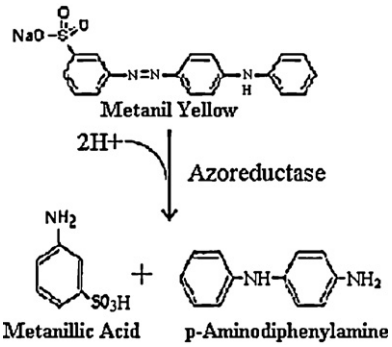

Fig. 7. A proposed pathway of Metanil Yellow degradation by both the strains AK1 and AK2.

Table 4
Phytotoxicity of Metanil Yellow and its degradation products on Chickpea and Pigeon pea.

Parameter	Plain water	Chickpea					Pigeon pea					
		MY	Products AK1		AK2		Plain water	MY	Products AK1		AK2	
			Acidic	Neutral	Acidic	Neutral			Acidic	Neutral	Acidic	Neutral
Germination (%)	100	70	90	85	80	80	100	60	80	83	79	75
Plumule (cm)	11.9 ± 0.46	2.7 ± 0.11	6.98 ± 0.14	8.19 ± 0.10	7.3 ± 0.21	10.07 ± 0.04	11.90 ± 0.53	3.1 ± 0.28	8.74 ± 0.21	9.14 ± 0.09	8.54 ± 0.16	10.41 ± 0.07
Radical (cm)	12.73 ± 0.31	1.31 ± 0.21	8.24 ± 0.13	9.40 ± 0.11	7.5 ± 0.08	9.73 ± 0.12	11.47 ± 0.28	3.27 ± 0.06	8.91 ± 0.24	9.19 ± 0.21	9.01 ± 0.28	9.03 ± 0.26

MY: Metanil Yellow. Values are the mean of ten germinated seeds of three sets SEM (±). Data was analyzed by one-way ANOVA test.

pea was 478 and 234 respectively. Phytotoxicity results suggested that degradation products of Metanil Yellow were much less toxic to the seeds of pigeon pea and chick pea. Jadhav et al. [39] showed the mean of plumule length and radical length of common wheat (*Triticum aestivum*) was 10.4 ± 0.13 and 10.4 ± 0.06 cm, respectively in 5000 ppm concentration of the tested samples. Saratale et al. [40] showed the phytotoxic effect of Navy Blue HER on the germination of *Phaseolus mungo* and *Sorghum vulgare.* Both the plant seeds inhibited 90% germination when seeds were treated with 1500 ppm concentration of Navy blue HER. On the contrary, no phytotoxic effect (100% germination) was observed at the same concentration of degradation products.

4. Conclusion

Bacterial decolorization proves to be a very efficient method for complete decolorization of sulfonated azo dyes (Metanil Yellow, Amaranth, Congo Red, Fast Red, Ponceau S and Reactive Black 5). The two bacterial strains *Bacillus* sp. strain AK1 and *Lysinibacillus* sp. strain AK2 are potential for decolorization and partial degradation of azo dye Metanil Yellow under static condition. The induction of azoreductase by Metanil Yellow was observed in both the strains. An enzyme azoreductase would be a good material for further research on the enzymological mechanism of dye decolorization in bacteria. Further, the strains AK1 and AK2 are proved to be efficient for conventional wastewater treatment and bioremediation of recalcitrant azo dyes under normal parameters. Furthermore, phytotoxicity studies of both the strains suggested that the degradation products of Metanil Yellow were much less toxic to the seeds of pigeon pea and chick pea. Therefore, *Bacillus* sp. AK1 and *Lysinibacillus* sp. AK2 are the highly promising microorganisms both for the application in the treatment of dyeing wastewater and in bioremediation of recalcitrant Metanil Yellow and other azo dyes. However the complete degradation of Metanil Yellow is under progress from these two strains.

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References

[1] B. Amith, D. Sivanesan, K. Kannan, C. Tapan, Kinetics of decolorization and biotransformation of Direct Black 38 by *C. hominis* and *P. stutzeri*, Appl. Microbiol. Biotechnol. 74 (2007) 1145–1152.
[2] O. Guven, P. Baris, K. Ali, E.O. Esra, Y. Ihsan, K. Ismail, Decolorization of Acid Black 210 by *Vibrio harveyi* TEMS1 a newly isolated bioluminescent bacterium from Izmir Bay, Turkey, World J. Microbiol. Biotechnol. 24 (2008) 1375–1381.
[3] O. Anjaneya, M. Santoshkumar, S.N. Anand, T.B. Karegoudar, Biosorption of Acid Violet dye from aqueous solutions using native biomass of a new isolate of *Penicillium* sp., Int. Biodeter. Biodegrad. 63 (2009) 782–785.
[4] M.H. Vijaykumar, A. Parag, Vaishampayan, S.S. Yogesh, T.B. Karegoudar, Decolorization of naphthalene-containing sulfonated azo dyes by *Kerstersia* sp. Strain VKY1, Enzyme Microb. Technol. 40 (2007) 204–211.
[5] J. Anna, K.R. Wilkplazka, M. Janina, W. Elzbieta, L. Wladyslaw, Andrzej, Fungi and their ability to decolorize azo and anthraquinonic dyes, Enzyme Microb. Technol. 30 (2002) 566–572.
[6] G.B. Cinthia, O. Larissa, G.M.S. Cristina, M.P. Rosane, Decolorization of synthetic dyes by solid state cultures *Lentinula (Lentinus) edodes* producing manganese peroxidase as the main ligninolytic enzyme, Bioresour. Technol. 94 (2004) 107–112.
[7] P. Waranusantigul, P. Pokethitiyook, M. Kruatrachue, E.S. Upatham, Kinetics of basic dye (Methylene Blue) biosorption by giant duckweed (*Spirodela polyrrhiza*), Environ. Pollut. 25 (2003) 385–392.
[8] M. Das, S. Ramchandani, R.K. Upreti, S.K. Khanna, MetanilYellow: a biofunctional inducer of hepatic phase I and phase II xenoblastic-metabolising enzymes, Food Chem. Toxicol. 35 (1997) 835–838.

[9] E.W. Zimmerman, Colored waterproof drawing inks, Ind. Eng. Chem. 25 (1933) 1033–1034.
[10] M. Alok, V.K. Gupta, M. Arti, M. Jyoti, Process development for the batch and bulk removal and recovery of a hazardous, water-soluble azo dye (Metanil Yellow) by adsorption over waste materials (Bottom Ash and De-Oiled Soya), J. Hazard. Mater. 151 (2008) 821–832.
[11] S. Ramachandani, M. Das, A. Joshi, S.K. Khanna, Effect of oral and parental administration of Metanil Yellow on some hepatic and intestinal biochemical parameters, J. Appl. Toxicol. 17 (1997) 85–91.
[12] S.M. Sachdeva, K.V. Mani, S.K. Adval, V.P. Jalpota, K.C. Rasela, D.S. Chadha, Acquired toxic methaemoglobinaemia, J. Assoc. Phys. India 40 (1992) 239–240.
[13] S.S. Chandro, T. Nagaraja, A food-poisoning out break with chemical dye. An investigation report, Med. J. Arm. Forc. India 43 (1987) 291–300.
[14] B.M. Hausen, A case of allergic contact dermatitis due to Metanil Yellow, Contact Dermat. 31 (1994) 117–118.
[15] S. Gupta, M. Sundarrajan, K.V.K. Rao, Tumour promotion by Metanil Yellow and Malachite Green during rat hepatocarcinogenesis is associated with dysregulated expression of cell cycle regulatory proteins, Teratog. Carcinog. Mutagen. 1 (2003) 301–312.
[16] S. Mohamad, V. Daniel, F. Corinne, M.C. Jean, Photocatalytic degradation of azo dye Metanil Yellow: optimization and kinetic modeling using a chemometric approach, Appl. Catal. B: Environ. 77 (2007) 1–11.
[17] P.K. Malik, Use of activated carbons prepared from sawdust and rice-husk for adsorption of acid dyes: a case study of Acid Yellow 36, Dyes Pigments 56 (2003) 239–249.
[18] M.R. Villanueva, A.R. Hernandez, J.M.H. Peralta, R.B. Erick, A. Marco, A. Quiroz, Enhancing the electrochemical oxidation of Acid-Yellow 36 azo dye using boron-doped diamond electrodes by addition of ferrous ion, J. Hazard. Mater. 167 (2009) 1226–1230.
[19] P. Verma, D. Madamwar, Decolorization of synthetic dyes by a newly isolated strain of *Serratia maerascens*, World J. Microbiol. Biotechnol. 19 (2003) 615–618.
[20] V.J. Pidiyar, K. Jangid, M.S. Patole, Y.S. Shouche, Studies on cultured and uncultured microbiota of wild *Culex quinquefasciatus* mosquito midgu based on 16S ribosomal RNA gene analysis, Am. J. Trop. Med. Hyg. 70 (2004) 597–603.
[21] S. Kumar, K. Tamura, I.B. Jakobsen, M. Nei, MEGA2: molecular evolutionary genetics analysis software, Bioinformatics 17 (2001) 1244–1250.
[22] C. Yatome, K.T. Maiti, B.C. Bhattacharyya, Degradation of crystal violet by *Nocardia crallina*, Appl. Microbiol. Biotechnol. 38 (1993) 565–569.
[23] S. Ademir, A. Ferrerira, T. Regina, M. Rosim, Plant bioassay to assess toxicity of textile sludge compost, Sci. Agric. 62 (2005) 286–290.
[24] V.V. Dawkar, U.U. Jadhav, D.P. Tamboli, S.P. Govindwar, Efficient industrial dye decolorization by *Bacillus* sp. VUS with its enzyme system, Ecotoxicol. Environ. Saf. 73 (2010) 1696–1703.
[25] N.K. Toshiaki, S. Mifumi, N. Nobuhiko, C. Thalerng, N. Tadaatsu, Decolorization of molasses wastewater by *Bacillus* sp. under thermophilic and anaerobic conditions, J. Biosci. Bioeng. 87 (1999) 119–121.
[26] B.Y. Chen, W.M. Chen, Y.P. Wu, C.C. Hsueh, K.L. Lin, C.T. Chang, Revealing phenol tolerance of indigenous phenol degraders isolated from Northeast Taiwan, J. Taiwan. Inst. Chem. Eng. (2010), in press, doi:10.1016/j.jtice.2010.01.010.
[27] C. Raghukumar, D. Chandramohan, J. Michel, C.A. Reddy, Degradation of lignin and decolorization of paper mill bleach plant effluent (BPE) by marine fungi, Biotechnol. Lett. 18 (1996) 105–106.
[28] J.S. Chang, T.S. Kuo, Y.P. Chao, J.Y. Ho, P.J. Lin, Azo dye decolorization with a mutant *Escherichia coli* strain, Biotechnol. Lett. 22 (2000) 807–812.
[29] S.U. Jadhav, M.U. Jadhav, A.N. Kagalkar, S.P. Govindwar, Decolorization of Brilliant Blue G dye mediated by degradation of the microbial consortium of Galactomyces geotrichum and *Bacillus* sp., J. Chin. Inst. Chem. Eng. 39 (2008) 563–570.
[30] P.S. Patil, U.U. Shedbalkar, D.C. Kalyani, J.P. Jadhav, Biodegradation of Reactive Blue 59 by isolated bacterial consortium PMB11, J. Ind. Microbiol. Biotechnol. 35 (2008) 1181–1190.
[31] N.B. Haq, A. Nadia, A. Muhammad, Optimization of culture conditions for enhanced decolorization of Cibacron Red FN-2BL by *Schizophyllum commune* IBL-6, Appl. Biochem. Biotechnol. 149 (2008) 255–264.
[32] C.I. Pearce, J.R. Lioyd, J.T. Gutherie, The removal of color from textile wastewater using whole bacterial cells: a review, Dyes Pigments 58 (2003) 179–186.
[33] M.G. KapilKumar, T.R. Dastidar, Sreekrishnanb, Effect of process parameters on aerobic decolorization of reactive azo dye using mixed culture, World Acad. Sci. Eng. Technol. 58 (2009) 963–965.
[34] M. Sukumar, A. Sivasamy, G. Swaminathan, Decolorization of textile dye effluent by genetically improved bacterial strains, Appl. Biochem. Biotechnol. 136 (2007) 53–61.
[35] J. Chan, T. Kuo, Kinetics of bacterial decolorization of azo dye with *Escherichia coli* NO3, Bioresour. Technol. 75 (2000) 107–111.
[36] B. Manu, S. Chaudhari, Decolorization of indigo and azo dyes in semicontinuous reactors with long hydraulic retention time, Process Biochem. 38 (2003) 1213–1221.
[37] L.A. De Baere, M. Devocht, P.V. Assche, W. Verstraete, Influence of high NaCl and NH_4Cl salt levels on methanogenic associations, Water Res. 18 (1984) 543–548.
[38] C.V. Nachiyar, K. Vijayalakshmi, D. Muralidharan, G. Suseela Rajakumar, Mineralization of metanilic acid by *Pseudomonas aeruginosa* CLRI BL22, World J. Microbiol. Biotechnol. 23 (2007) 1733–1738.
[39] J.P. Jadhav, G.K. Parshetti, S.D. Kalme, S.P. Govindwar, Decolorization of azo dye Methylred by *Saccharomyces cerevisiae* MTCC463, Chemosphere 68 (2007) 394–400.
[40] R.G. Saratale, G.D. Saratale, J.S. Chang, S.P. Govindwar, Decolorization and biodegradation of textile dye Navy Blue HER by *Trichosporon beigelii* NCIM-3326, J. Hazard. Mater. 166 (2009) 1421–1428.
[41] M. Amani, D.E. Ashwany, Decolorization of Fast Red by metabolizing cells of *Oenococcus oeni* ML34, World J. Microbiol. Biotechnol. 24 (2008) 1521–1527.