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Accelerated Production of Oxygen-Insensitive Azoreductase from Mutant *Pseudomonas* Species for Degradation Azo Dyes under Aerobic Condition

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Bacteria producing an oxygen insensitive intracellular azoreductase were isolated from a dye contaminated area and identified as a *Pseudomonas* species B1 by 16s rRNA sequencing. Maximum azoreductase production (0.39 U/mL) was observed in the mutant strain within 30 h of incubation under optimized conditions at pH 6.5, temperature 35 °C, glucose 2 %, sodium nitrate 1 % and 2 % of inoculum concentration. Azoreductase was purified by ammonium precipitation method and followed by anion exchange chromatography. Mutant *Pseudomonas* species B1 showed 2-fold increased level of azoreductase production and purified up to 94-fold with a recovery of 18 %. Native PAGE analysis revealed that the purified enzyme was a monomer with a molecular weight of 29 kDa. The K_m and V_{max} values were 0.09 mM and 6.7 U mg^{-1} of protein for NADH and 0.04 mM and 4.7 U mg^{-1} of protein for naphthol blue black, respectively. Furthermore, the purified enzyme could effectively degrade 78 % of naphthol blue black under aerobic conditions, as monitored by UV-visible, FTIR-spectroscopy, HPLC and GCMS. Phytotoxicity and microbial toxicity assays showed that the degradation products of naphthol blue black were less toxic than the dye itself.

Keywords: Azoreductase, *Pseudomonas sp.*, Random mutagenesis, Dye decolorization, Phytotoxicity.

INTRODUCTION

Azo dyes are synthetic aromatic compounds that are characterized by the presence of one or more azo bonds (R_1-N=N-R_2). These dyes are the largest chemical class of artificial dyes has wide range of applications in various branches of textile industries. Unfortunately, every year 15 % of the azo dyes were released into the environment during manufacture of dyes and causes serious problem in an aquatic habitat [1-4]. Azo dyes are recalcitrant in nature because they are highly stable against the chemicals, light and aerobic conditions [5,6]. Currently, there is several treatment techniques have been employed for the removal of colour from the dye containing textile effluent but they are not effective because of the high cost and the secondary pollution which can be generated by the excessive use of chemicals [7]. In contrast, bacterial degradation of azo dyes is considered to be an eco-friendly and cost-competitive method for restoration. Generally, bacterial degradation of azo dyes under anaerobic condition produces toxic aromatic amines, which are carcinogenic and mutagenic. However, to eradicate these toxic aromatic amines, the aerobic degradation of bacteria is only possible because they further oxidize the reduced products *via* deamination or hydroxylation reactions. To understand the mechanism of biological treatment of azo dyes in wastewater, it is essential to discover azo dye-degrading microorganisms and to study the enzymes involved. Mostly, bacterial biodegradation was achieved with the help of intracellular and extracellular oxido-reductive enzyme such as azoreductases [8].

In recent years, several azoreductases producing bacteria have been identified and characterized in bacterial species, including *Escherichia coli, Bacillus subtilis, Enterococcus faecalis, Rhodobacter sphaeroides* and *Pseudomonas aeruginosa* [9]. The direct use of enzyme for the treatment of dye containing effluent has more advantageous when compared to the use of microorganisms because they only act on substrates, operate in mild conditions and are safe, easy to maintain and reuse. Moreover, the time required for decolourization processes by the bacterial cells could also be substantially longer [10]. The class of azoreductase family consists of enzymes with different structure, functions and evolution that catalyzes an NAD(P)H dependent reductive cleavage of the azo bond of azo dyes [11]. Oxygen-insensitive azoreductase produced from bacteria is generally classified in to two types such as monomeric flavin-free enzymes containing a putative NAD(P)H binding motif and polymeric

flavin-dependent enzymes. Currently, many researchers have been focused on accelerated production of azoreductase under optimized conditions but till now, there is no study conducted for enhancement of azoreductase production by strain improvement. Keeping this in view, the present study was undertaken for the accelerated production of oxygen-insensitive azoreductase using mutant strain and to evaluate the efficacy of purified enzyme for bioremediation of azo dyes.

EXPERIMENTAL

All chemicals were purchased from Hi-Media Laboratories (Mumbai, India). NADH and the azo dyes naphthol blue black, reactive black 5, ponceau S and trypan blue were purchased from Sigma Aldrich, (India). All chemicals used were of highest purity available and of an analytical grade.

Isolation and screening of azo dye degrading bacteria: Azo dye contaminated soil samples and textile effluent samples were collected in sterilized containers from the four textile industrial sites Avinasi, Sulthan peetai, Veerapandi and Naranapuram, located in and around Tirupur, (Tamilnadu, India). An aliquot of 100 µL of effluent and 100 mg soil sample was added to 100 mL of nutrient broth containing a mixture of the azo dyes (carbon source) at a concentration of 50 mg/L in a 250 mL Erlenmeyer flask. After 72 h incubation, 100 µL were aseptically transferred into basal minimal medium (BMM) (7.5 mg/L NaCl, 3.5 mg/L $CaCl_2 \cdot 2H_2O$, 3 mg/L $MgSO_4 \cdot 7H_2O$) to check the ability of bacteria to decolourize the selected dyes. After complete decolourization, the 1 mL of suspension was spread on basal minimal medium-agar plates containing 50 mg/L of all selected azo dyes and incubated at 35 °C for 72 h. The clear zone around bacterial colonies indicated the ability of the organism to reduce azo dyes was selected and preserved at 4 °C for further analysis.

16s rRNA sequencing and phylogenetic analysis: The isolated potent bacterial strain was identified using 16s rRNA sequence analysis. Genomic DNA from the isolated bacteria strain B1 was purified using (Instra-Gene TM Matrix Genomic DNA isolation kit, Chennai, India), as per the manufacturers protocol. Amplification of 16S rRNA gene sequence was performed with the help of universal primers 8f (5'-AGAGTTTGATCCATGGC-3') and 1492R (5'-GTTACCTTGTTACGACTT-3'). The amplification products were resolved on gel electrophoresis (Yaazh Xenomics, Chennai, India), stained with ethidium bromide and photographed under UV light. The amplified product was further purified and sequenced by dideoxy chain-termination method using a kit (ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems, Chennai, India) as described by the manufacturer. The DNA-based tree was constructed using the aligned sequences by the neighbor-joining method using Kimura-2-parameter (K2P) model in MEGA 5 software [12].

Strain development through random mutagenesis: A loop-full of isolated azo degraders was aseptically transferred to basal minimal medium broth containing 10 mg/L of azo dyes and incubated at 37 °C for 24 h. The young bacterial cells were taken from the culture media by centrifugation at 6000 x g for 20 min. The collected cell pellet containing 10^8 cells was resuspended in sterile deionized water. Two steps of mutagenesis approach were performed for the development of highly mutated and stable bacterial strains. In the first step, 10 mL of the wild strains B1 cell suspension were outspread onto the flats Petri-plate under UV-A mutation at 320 nm at 25 cm distance, receiving radiation for regular intervals of 10, 20, 30, 40, 50 and 60 min. In order to avoid photoreactivation the petri plates were placed in a dark room, immediately after UV radiation. The mutated bacterial strains possessing a higher rate of azoreductase activity was screened out. During the second step, all the best mutant cultures obtained from first mutation were again subjected to ethidium bromide mutagens (100 µg/mL) at specified time intervals ranging from 0 to 120 min and double mutated strains were obtained. Among all mutated strains, the best potential strains were screened out for the hyper production of azoreductase and further work was continued on that mutant.

Optimization of parameters: The effect of various carbon sources (2 % w/v) such as glucose, fructose, maltose and sucrose and nitrogen sources (1 % w/v) such as yeast extract, peptone, urea and sodium nitrate, temperature (5-50 °C), pH (3-9), substrate concentration (0-50 µM) and inoculum concentration (1-5 %) containing 10^8 cells/mL were investigated for the production of azoreductase using mutant *Pseudomonas* sp. under static conditions.

Production of azoreductase: The cultivation medium used for azoreductase production contained (g/L): KH_2PO_4, 3.5; $Na_2HPO_4 \cdot 7H_2O$, 7.5; yeast extract, 10.0; peptone, 20.0; NH_4SO_4, 2.5; $MgSO_4 \cdot 7H_2O$, 4.5; $MnSO_4 \cdot H_2O$, 0.2; iron citrate $5H_2O$, 0.7; and 2.5 % (v/v) SL-6 trace element solution as described previously by [13]. The medium was sterilized at 121 °C for 0.5 h. Two production experiments were conducted separately with two different conical flasks each containing 500mL of the above mentioned medium with 5 mg/L of each selected azo dye, inoculated separately with wild and mutant strain (2×10^8 cells) and incubated in the incubator shaker (150 rpm) at 37 °C for 24 h under aerobic condition.

Azoreductase assay: Azoreductase activity was determined using the azo dyes as substrates according to Nachiyar and Rajakumar [14]. The assay mixture contained 2.8 mL of 50 mM sodium phosphate buffer (pH 7.0) with 50 µL of 0.2 mM electron acceptor substrates (azo dye), 50 µL of 2 mM of NADH and 100 µL of enzyme solution in 3 mL of reaction mixture. The reaction mixture without NADH was pre incubated for 5 min at 30 °C and then the reaction was initiated by the addition of NADH in an oxygenic condition. Azoreductase activity was detected by following the disappearance of selected each azo dye at its maximum absorbance wavelength. One unit of (U) azoreductase activity was defined as the amount of enzyme required to reduce 1 µmol of substrate (azo dye) min^{-1} under the assay conditions. All reactions were performed in triplicate.

Determination of protein concentration: Protein concentration was measured according to Lowry *et al.* [15] using bovine serum albumin (BSA) as a standard protein.

Purification of azoreductase: The purification techniques were carried out at 4 °C. The young bacterial cell cultures

were harvested by centrifugation at 8000 x g for 20 min and the pellet was washed twice with 50 mmol/L phosphate buffer (pH 7.0). Cells were disrupted in the cold by sonication using Qsonicas sonicators (15 s, 70 % outputs, 500 watts) and then cell debris was removed by centrifugation at 5000 x g for 15 min. The resulting supernatant fraction was precipitated with 80 % ammonium sulfate and centrifuged at 11000 x g for 20 min and the pellet was dissolved in 30 mL phosphate buffer. The enzyme collected was applied to anion exchange chromatography (DEAE cellulose) (Bio-Rad) equilibrated with 100 mM sodium phosphate buffer, pH 7.0, at a flow rate of 0.4 mL/min. The column was washed with 200 mM NaCl in 100mM phosphate buffer, pH 7.0. The concentrated enzyme solution was then applied to a Sephadex G-100 (1.6 cm × 50 cm) column equilibrated with 15mM Tris-HCl buffer. The retained proteins were eluted with 600 mM NaCl in 100 mM phosphate buffer, pH 7.0. Fractions with azoreductase activity were pooled, concentrated and then stored at 4 °C.

Native gel electrophoresis: The purified enzyme and high standard markers were mixed with the bromophenol blue before the sample was loaded on the gel. The separating gel for non-denaturing polyacrylamide gels contained 10 % acrylamide and 0.26 % bis and was prepared in accordance with the instructions provided by Sigma Chemical Co., St. Louis, Mo. The stacking gel contained 4.5 % acrylamide and 0.5 µg of riboflavin per mL. The electrode buffer contained 5 mM Tris and 37.3 mM glycine (pH 8.3). The purified azoreductase of 30 µg were loaded on the gel along with the high standard marker. A constant current of 6 mA was applied to the gel until the bromophenol blue was close to the anode end of the gel. All steps for the preparation and running of the gel had to be performed under aerobic conditions at 4 °C to ensure the activity of the enzyme. Native gel was stained using Coomassie blue R-250 (Sigma).

Kinetic studies of purified azoreductase: The enzyme kinetic study was performed according to the method of Misal *et al.* [16]. Initial velocity of enzyme reaction was performed by varying the concentration of one substrate, naphthol blue black (from 0.05 to 0.5 mM) or NADH (from 0.05 to 0.5 mM), while the concentration of the other substrate was kept constant (NADH or naphthol blue black). Michalis constants (K_m) and maximal velocity (V_{max}) for the reduction of naphthol blue black and the oxidation of NADH by the purified azoreductase were determined from Lineweaver-Burk plots.

Decolourization assay: The decolourization assay was carried out separately for each selected azo dye in a sterile screw capped tube. The effects of dye concentration and enzyme activity on the decolourization of azo dye by the purified enzyme were assessed. The final reaction mixture (5 mL) contained 2 mL of azo dye (25-100 mg/L), 1.5 mL of 0.1 mM phosphate buffer (pH 7.0), 1.5 mL of 0.1 mM NADH (a mediator) mixed thoroughly and then added with 500 µL of purified azoreductase (0.1-0.5 U/mL) from mutant *Pseudomonas* B1. A reaction was initiated by adding azoreductase and the mixtures were incubated at 30 °C with mild shaking under aerobic condition. The control samples were run in parallel without the addition of azoreductase. The decolourization percentage was spectrophotometrically calculated as the relative decrease in absorbance at the maximal absorbance wavelength of each selected azo dyes. All reactions were performed in triplicate.

Characterization of degraded metabolites

FT-IR: FT-IR analysis was carried out to examine the changes occurs in the surface functional groups, before and after the enzymatical degradation of azo dyes. FTIR analysis was done on a Shimadzu 8400S spectrophotometer (Shimadzu Corporation, Japan) in the mid IR region of 4000-400 cm^{-1} with 16 scan speed). The samples were prepared using spectroscopic pure KBr (5:95); pellets were fixed in the sample holder and analyzed.

HPLC analysis: High performance liquid chromatography analysis was performed in an isocratic Waters 2690 system equipped with dual absorbance detector. Column specifications were C18 column with symmetry 4.6 mm 250 mm. HPLC grade methanol: water (80:20) was used as the mobile phase with flow rate 1 mL min^{-1} and HPLC run was carried out for 10 min.

GC-MS analysis: The GC-MS analysis of metabolites was carried out using Agilent GC system 7890 A, the MS 5975 C (Triple Axis Detector) with an integrated gas chromatograph with DB35MS column J&W 122-3832:340 °C:30 m × 250 µm × 0.25 µm. Helium was used as the carrier gas at a flow rate 1 mL/min. The injector temperature was maintained at 220 °C with oven conditions as 60 °C for 1 min and increased up to 200 °C with 15 °C min^{-1} to 310 °C for 5 min.

Phytotoxicity and microbial toxicity assay: Phytotoxicity assay was carried out to assess the toxicity effects of metabolite formed during the enzymatical degradation of naphthol blue black [17]. Two commonly available plants *Sesamum indicum* and *Vigna mungo* were selected for the phytotoxicity assay. Approximately, 15 seeds of each plant were sowed into a plastic sand pot containing 20 g of sterilized sand. Naphthol blue black and ethyl acetate extracted metabolites (dry) were dissolved separately in distilled water and the final concentration made was about 500 mg/L. The filtered textile effluent was directly used to assess its toxicity. Toxicity study was done by watering (5 mL) the seeds of each plant with naphthol blue black samples and extracted metabolites sample. The control was run by watering the seeds with distilled water. The watering of plants was done twice a day. Germination (%), length of plumule (shoot) and radical root were recorded after 10 days. The experiments were carried out at room temperature. The germination index (GI) was calculated according to Nouren and Bhatti [18]:

$$GI = GP \times La/Lc \quad (3)$$

where, GP is the number of germinated seeds expressed as a percentage of control values, La is the average value of root elongation in the dye solutions and Lc is the average value of root elongation in the control. The microbial toxicity assay was performed using nitrogen fixing aerobic bacteria, such as Azotobacter vinelandii MTCC 2460 and phosphate solubilizing bacteria, such as *Pseudomonas putida* MTCC 2476 on Mueller and Hinton agar plate having a composition of 1 % peptone, 0.5 % NaCl, 0.3 % yeast extract and 2.5 % agar. The data represents the mean and standard deviation of the three experiments.

Statistical analysis: Data were analyzed by one-way analysis of variance (ANNOVA) with Turkey-Kramer multiple comparison test.

RESULTS AND DISCUSSION

Isolating and developing a new strain for hyper-production of azo reductase will be more beneficial for effective degradation of textile dyes. It has long been reported that bacteria inhabits in industrial effluents utilizing its constituents as their source of energy. Likewise, the textile dye effluents examined in this study were appeared to harbor a diverse community of microorganisms. In the present study the soil and effluent collected from the textile dye contaminated area were subjected to enriched media containing mixture of selected azo dyes. The most potent and adapted bacterial culture was screened out using plate assay method and designated as B1. The formation of zone around the bacterial colony indicates the positive result for plate assay method. It thus provides evidence that textile dye effluent harbor a wide range of bacterial species that may even degrade the dyes to obtain their essential elements.

The isolated potent bacterial strain B1 were identified by 16s RNA sequencing. The 16S rRNA complete gene sequence of the isolated strain B1 was submitted to GenBank ID (GenBank accession no. KJ855516). The blast analysis revealed that the sequence strain B1 (796 bp) showed 99.17 % similarity with *Pseudomonas* species (Fig. 1). A phylogenetic tree was constructed using neighbor-joining model of the MEGA 5 program. The percentage of replicate trees in which

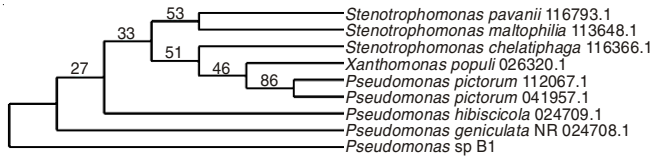

Fig. 1. Phylogenetic tree of isolated strain *Pseudomonas* species B1 & related species

the associated taxa clustered together in the bootstrap test (100 replicates) was presented next to the branches. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site [19,20]. In the present study, the attempt was also made to improve the isolated strain for accelerated production of azoreductase through random mutagenesis. After the induction of UV mutation, the mutation frequency increased with time of irradiation and reached a plateau after 35 min (Fig. 2a), where as in induction of EtBr mutation nearly 65 % kill rate were observed (Fig. 2b). The double mutated strain showed a 2-fold increase level of enzyme activity of azoreductase when compared to wild strain (Fig. 2c). The increase level of azoreductase activity is due to the DNA damage of the suppressor gene of azoreductase enzyme or probably may be due to the alteration in the nucleotide sequence by the intercalating agent. In most of the research related to enzyme technology, it has been reported that the hyper-production of enzyme depend on the strain, improvement strategies, types of mutagen, mutagenic concentration and the duration of exposition [21,22].

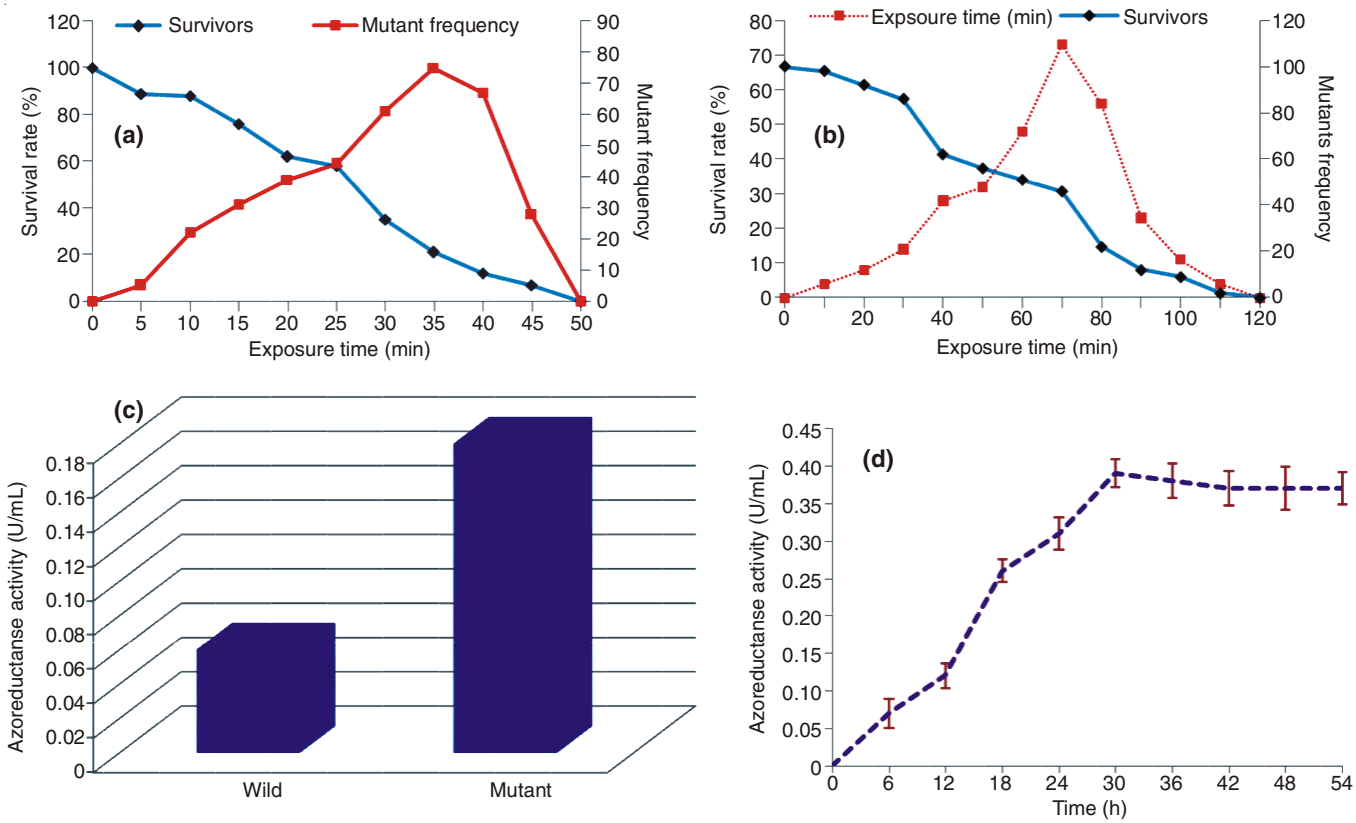

Fig. 2. Strain development using random mutagenesis for accelerated production of Azoreductase (a) Effect of UV radiation doses on survival rate and mutant frequency of *Pseudomonas* species (b) Effect of ethidium bromide doses on survival rate and mutant frequency of *Pseudomonas* species (c) Azoreductase activity from wild and mutant strain before optimization (d) Hyper-production of azoreductase under optimized condition by isolated mutant *Pseudomonas* sp. B1

The environmental condition of the industrial wastes keeps on changing and hence when using those wastes as substrate, an adaptation of microorganism to various temperature makes them more selective to bioprocess of enzymes. Keeping this in view, the present study was carried out to determine the optimum temperature ranging from 10 to 50 °C for the accelerated production of azoreductase from mutant *Pseudomonas* B1 were tested. It was observed that the mutant strain gradually raised azoreductase synthesis and attained maximum activity at 35 °C, whereas the production rate was very low at extreme temperature (Fig. 3a). Temperature optima for azoreductase reported in the literature ranges from 40 to 45 °C [23-25] and in alkali-thermophilic bacterium 80 °C [26]. But in most of the research, it was reported that the enzyme activity was quite stable for the mutant strain when compared to the wild strain even in various temperature [27]. Present result revealed that the mutant shows a more tolerant ability to a wide range of temperature for the accelerated production of azoreductase.

For determining the optimum pH, a range of pH 2.5 to 9 was tested, keeping the incubation temperature constant at 35 °C. Azoreductase production was significantly higher in the pH 6.5 with maximum production 0.31 U/mL, whereas the enzyme production declined by about 52 % at medium pH of 8.5 (Fig. 3b). Furthermore, for enhancing the production of azoreductase the medium is supplemented with the different carbon and nitrogen sources. The supplementation of glucose significantly increased the azoreductase production to 0.33 U/mL (Fig. 3c). However, the other carbohydrate shows the minimum synthesis of azoreductase with maximum decline activity in the presence of maltose. Hence, our result revealed that the mutant readily utilize the (2 % w/v) glucose as sole carbon source, when compared to other carbohydrate molecules. Among the entire nitrogen source selected sodium nitrate was found to be the most favourable nitrogen source with the maximum production of azoreductase 0.29 U/mL (Fig. 3d).

To determine the substrate specificity of azoreductase, the activity of the purified mutant enzyme was examined by using four different azo dyes as a substrate at ranges from 0-50 µM concentrations. Azoreductase showed the highest activity towards naphthol blue black and reactive black at 50 µM concentrations, while the activity was significantly reduced

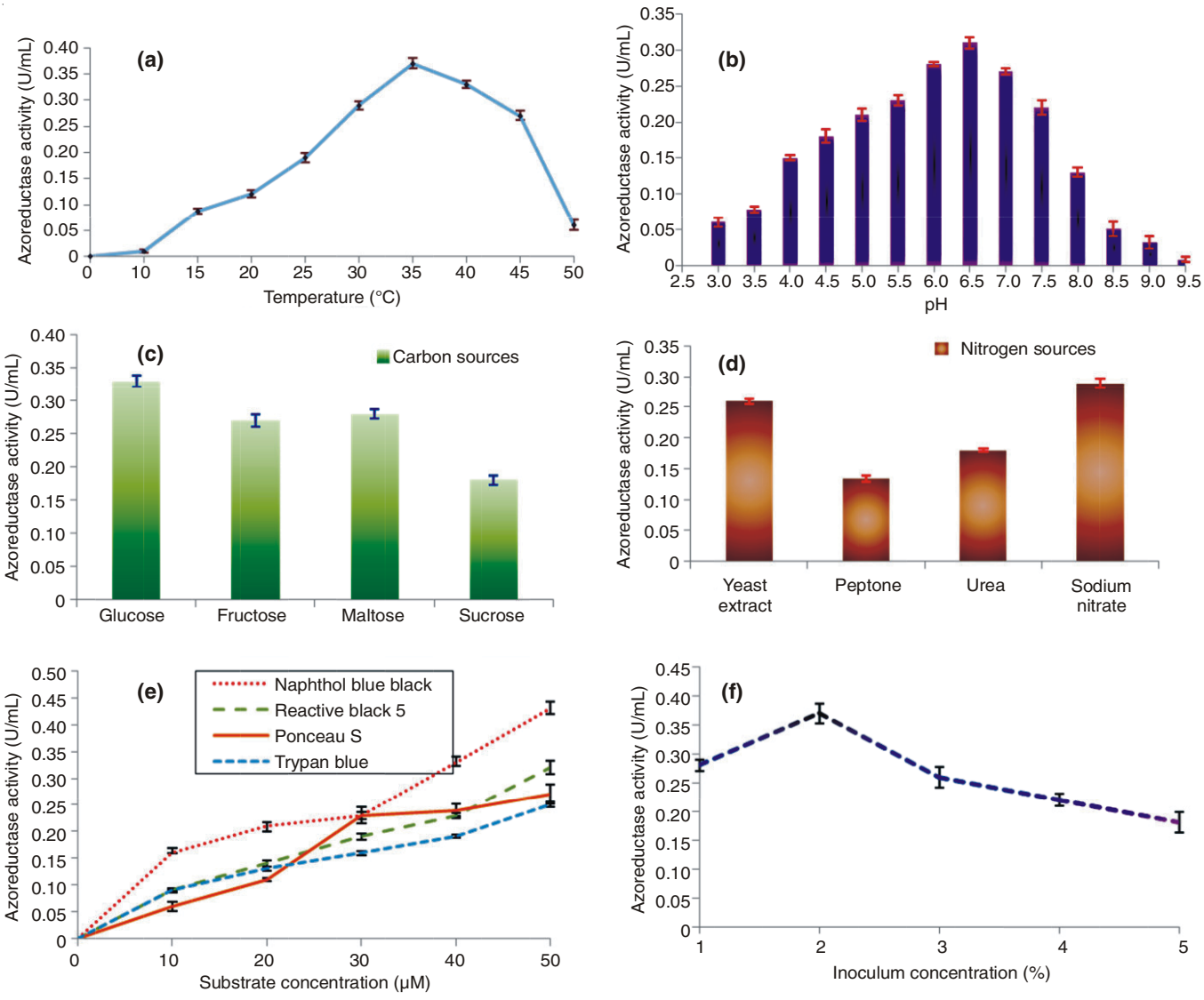

Fig. 3. Effect of (a) Temperature (b) pH (c) Carbon sources (d) Nitrogen sources (e) Substrate concentration and (f) Inoculum concentration for the accelerated production of azoreductase using mutant *Pseudomonas* sp. B1

in the other two selected dyes such as trypan blue and ponceau (Fig. 3e). The rate of activity of the enzyme depends on the chemical nature of the dyes, substrate specificity and concentration of the enzyme [28]. Generally, the inoculum concentration is one of the prime factors in the field of enzyme technology. Regarding to the inoculum concentration effect, the first known point is that excessive cell density leads to a decrease in the substrate/microorganism (S/M) ratio that causes a shortage of substrate to support microbial metabolism. In addition, the excess of cells can favour the formation of cell aggregates, which can hamper the penetration of light due to the self-shading effect or can even limit the diffusion of substrate into bacterial flux [29]. Our result revealed that the maximum enzyme production (0.37 U/mL) was achieved at 2 % inoculum concentration (Fig. 3f).

Hyper-production of azoreductase by mutant *Pseudomonas* B1 was investigated with different parameters under static condition. The maximum amount 0.39 U/mL of azoreductase was observed in the mutant *Pseudomonas* B1 at 30 h of incubation under static condition (Fig. 2d). Hyper-production of azoreductase was achieved under optimized conditions at pH 6.5, temperature 35 °C, glucose 2 %, sodium nitrate 1 % and 2 % of inoculum concentration.

Furthermore, the purification of intracellular azoreductase was carried out using sodium sulphate precipitation methods, followed by ion exchange column chromatography on DEAE-cellulose and the results of purification of azoreductase from the mutant *Pseudomonas* B1 strain are summarized in Table-1. About 94-fold purification of the enzyme from the initial culture broth was achieved with a recovery of approximately 18 %. The specific activity of the finally purified enzyme was 19.8 U/mg. Molecular weight of purified azoreductase of mutant *Pseudomonas* B1 was estimated to be 29 Kda by Native PAGE with protein molecular weight standards (Fig. 4). The mutant enzyme found to be monomer and the presence of single band in Native PAGE indicating that the enzyme was purified to homogeneity. Intracellular enzymes secreted by different microbial source had a similar molecular weight of azoreductase [30].

Enzyme kinetics for mutant azoreductase was carried out by varying the concentration of naphthol blue black or NADH and fixing the other substrate concentration at the same time. The values of K_m and V_{max} were found to be 0.09 mM and 6.68 U mg^{-1} of protein for NADH and 0.04 mM and 4.74 U mg^{-1} of protein for naphthol blue black, respectively. Since, the K_m value for naphthol blue black was found to be low, which indicate that the isolate mutant azoreductase has higher affinity towards a substrate.

In the present study, high recalcitrant nature of four azo dyes were selected based on structural complexity, pH and

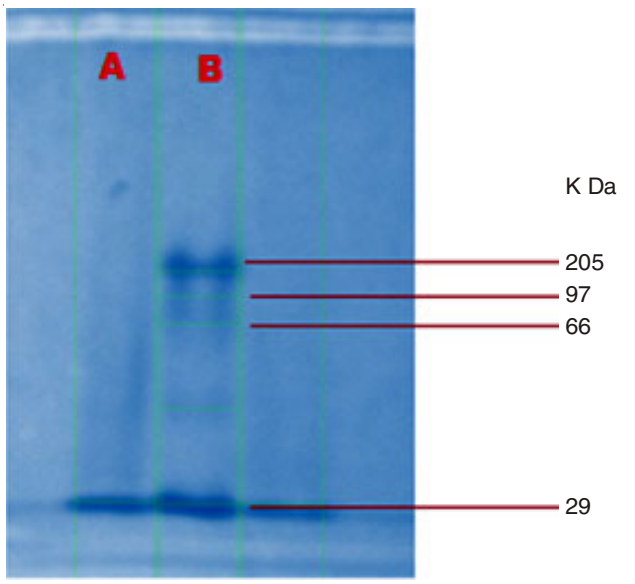

Fig. 4. Native PAGE of purified azoreductase from mutant *Pseudomonas* species B1 Lane-A: Purified azoreductase Lane-B: Standard markers

temperature stability and used as the model dye to evaluate the decolourization efficacy of purified enzyme from the mutant strain. Result of effective degradation of selected azo dyes was illustrated in Fig. 5a. Interesting the mutant enzymes was quite stable and very active, even in the presence of oxygen concentration and was able to degrade 79 % of naphthol blue black, within 30 min of incubation containing 0.4U/mL concentration of azoreductase (Fig. 5b). More than 48 % of the naphthol blue black was decolourized within 5 min when the dye concentration was below 50 mg L^{-1}, whereas 79 % of naphthol blue black was degraded at 30 min. For higher concentration (100 mg L^{-1} of dye), only 26 % of naphthol blue black was decolourized in the first 5 min. However, the final decolourization rate was 78 % after 30 min (Fig. 5c). The recombinant *E. coli* strain showed only 10 % decolourization of AR27 (1 mM) in 30 h incubation, which indicate that the overexpressed intracellular azoreductase AZR is not effective in bacterial decolourization [31-33]. However, the oxygen insensitive azoreductase produce from the mutant strain showed an effective degradation on naphthol blue black when compared to all the selected dye; this indicated the differences in specificity of the enzyme azoreductase from different dyes. It has been reported that the oxygen insensitive azoreductase enzyme synthesized from aerobic organisms able to oxidize the azo dyes by deamination or hydroxylation reactions [34,35]. To our best of knowledge, present results suggest that the azoreductase activity of mutant *Pseudomonas* B1 strain was much higher than some of the reported bacterial strain [36].

TABLE-1
SUMMARY OF AZOREDUCTASE PRODUCTION FROM MUTANT *Pseudomonas* SPECIES B1

Purification steps	Total protein (mg)	Total activity (U/mg total protein)	Specific activity (U/mg)	Purification fold	Yield (%)
Cell free extracts	1269	2791	0.22	1.0	100
$(NH_4)_2SO_4$ precipitation	325	1664	0.64	3.0	59
DEAE–cellulose	17	693	9.7	46	24
Sephadex G-100	7.7	504	19.8	94.2	18

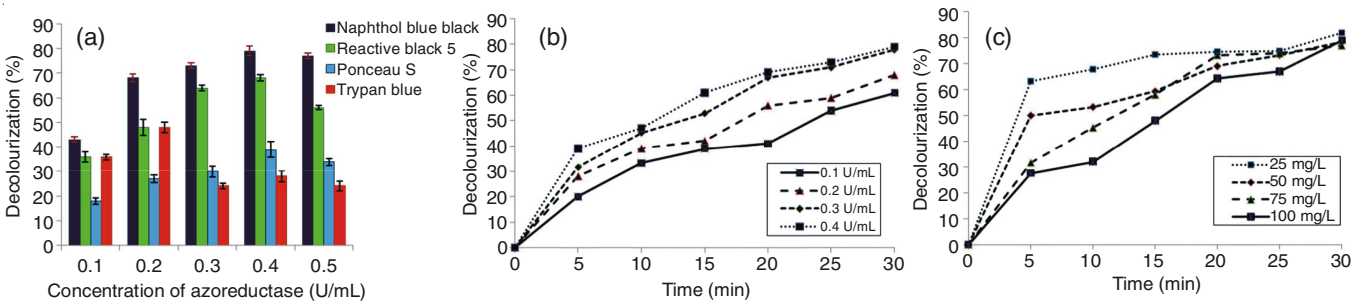

Fig. 5. (a) Decolourization of azo dyes by the purified azoreductase from mutant *Pseudomonas* Sp. B1 under pH 6.5, for 30 min incubation at 30 °C. Effects of (b) dye concentration and (c) azoreductase activity on decolourization of naphthol blue black by the purified enzyme

The azoreductase mediated degradation product of naphthol blue black was confirmed from FTIR analysis. The FTIR spectrum of extracted metabolites obtained after the enzymatic treatment showed significant change in the positions of the peaks (Fig. 6b), when compared to control dye spectrum (Fig. 6a). The spectrum of control dye displaced different peaks which represent the free -NH_2 group showing amide asymmetric stretching at 3413 cm^{-1}, C=H asymmetric stretching at 2952 cm^{-1}, C=C stretching of benzene ring at 1643 cm^{-1}, N=N stretching at 1452 cm^{-1} (indicate the azo group present in the dye), O-H deformation was observed at 1505 cm^{-1}, S=O stretching was notified at 1112 cm^{-1}, SO stretching of sulfonic acid was observed at 1052 cm^{-1} and the peaks at 694 cm^{-1} indicates the aromatic nature of the parental dye. Disappearance of the major peaks and appearance of new peaks in the FTIR spectrum of enzymatic degraded metabolites suggests the biotransformation of dye into distinct metabolites. Especially, the vanishing of peak at 1452 cm^{-1} in case of dye metabolites gives the evidence of azo bond cleavage. Two major peaks were observed in the HPLC elution profile of naphthol blue black control at retention time 3.4 and 8.5 (Fig. 6c) whereas, the detection of minor peaks with different retention time in the HPLC elution profile of enzymatic degraded metabolite clearly indicated the degradation of the parent dye (Fig. 6d).

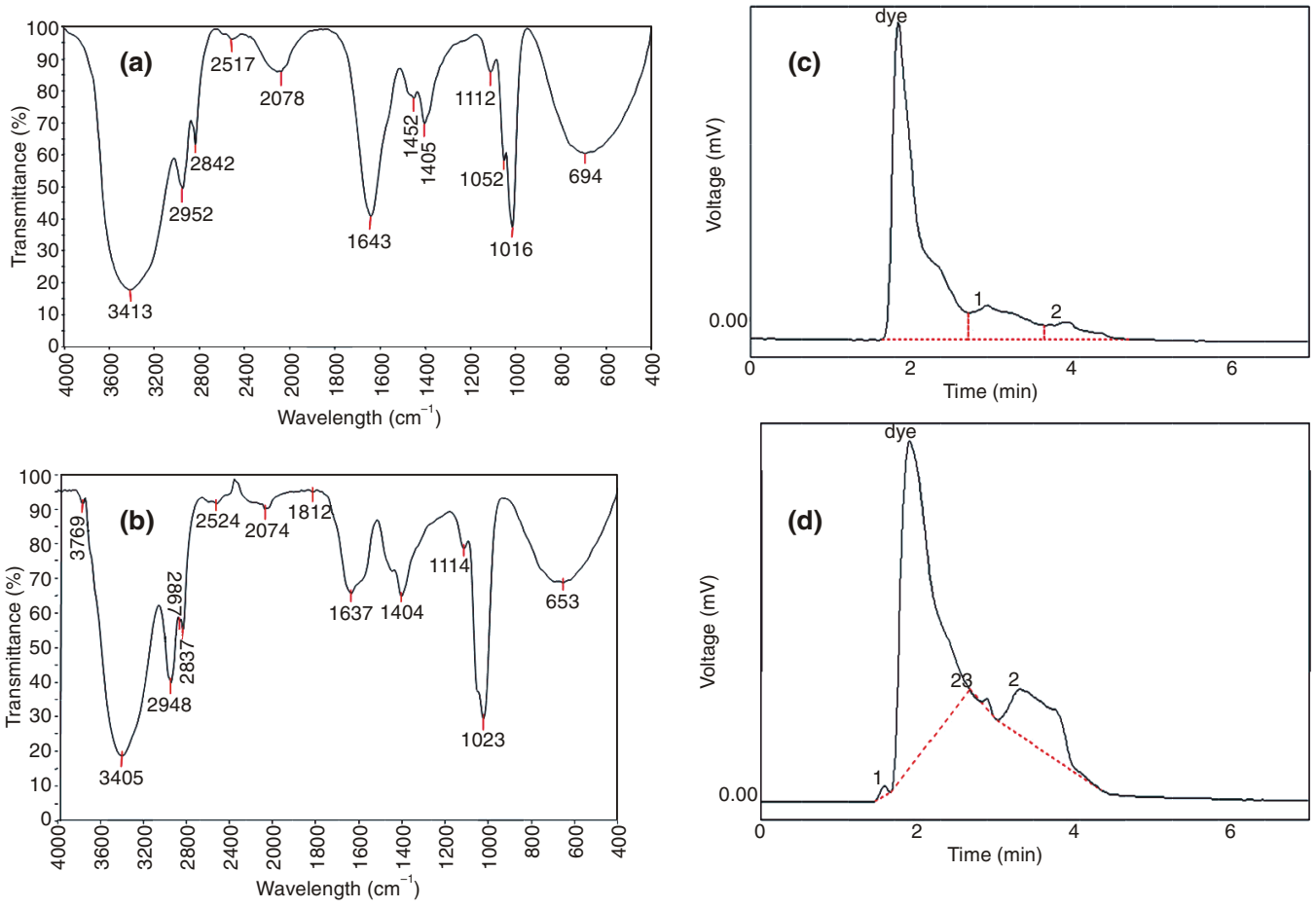

Fig. 6. (A) FTIR spectrum of control dye naphthol blue black (B) FTIR spectrum of enzymatical degraded metabolites of naphthol blue black by mutant *Pseudomonas* sp. (C) HPLC chromatogram of naphthol blue black before the enzymatical treatment with concentration of 100 mg/L (D) HPLC chromatogram of Naphthol blue black after the enzymatical treatment with concentration of 100 mg/L

TABLE-2
PHYTOTOXICITY STUDY OF NAPHTHOL BLUE BLACK AND ITS DEGRADED METABOLITES WITH *Sesamum indicum* AND *Vigna mungo*

Parameters	*Sesamum indicum*			*Vigna mungo*		
	Distilled water	Naphthol blue black	Metabolite	Distilled water	Naphthol blue black	Metabolite
Germination (%)	100	10	100	100	20	90
Plumule (cm)	9.23 ± 0.280	0.40 ± 0.03	8.30 ± 0.49	14.78 ± 0.55	0.55 ± 0.05	11.4 ± 0.16
Radical (cm)	1.28 ± 0.057	0.12 ± 0.06	1.05 ± 0.01	24.70 ± 0.87	1.70 ± 0.08	21.3 ± 0.73

Values are mean of three experiments standard error mean (SEM) (±), Seeds germinated in naphthol blue black and degraded metabolites are significantly different from the seeds germinated in distilled water at $P < 0.001$ by one-way analysis of variance (ANNOVA) with Tukey-Kramer comparison test.

TABLE-3
MICROBIAL TOXICITY STUDY OF NAPHTHOL BLUE BLACK AND ITS DEGRADED METABOLITES

Parameters	Test microorganism	Control	Naphthol blue black	Metabolite
Zone of inhibition	*Azotobacter vinelandii* MTCC 2460	0.62 ± 0.06	1.4 ± 0.03	0.69 ± 0.04
	Pseudomonas putida MTCC 2476	0.66 ± 0.10	1.4 ± 0.06	0.71 ± 0.09

Values are mean of three experiments standard error mean (SEM) (±), is significantly different from the control at, $P < 0.003$, by one-way analysis of variance (ANOVA) with Tukey-Kramer comparison test.

GC/MS analysis of degraded metabolites of naphthol blue black showed the presence of several peaks in the spectrum. However, at the end enzymatic degradation, two distinct metabolites naphthalene-1-carboxamide and phenyl nitrate were detected in GC/MS analysis along with several other metabolites due to the asymmetric cleavage of azo bond.

The result of the phytotoxicity tests demonstrated that the germination percentage and lengths of the plumule and radicle of *Sesamum indicum* and *Vigna mungo* seeds were significantly increased with degradation metabolites or with water treatment when compared to untreated naphthol blue black treatment (Table-2). However, germination index (GI) of untreated and enzymatic treated naphthol blue black was found to be 9.3 % and 82 % for *Sesamum indicum*, whereas 6.1 % and 77 % for *Vigna mungo* respectively. Similarly, in microbial toxicity assay there was significant reduction in zone of inhibition was observed in *Azotobacter vinelandii* MTCC 2460 and *Pseudomonas putida* MTCC 2476 in the presence of enzymatic degraded products of naphthol blue black, when compared with the untreated dye (Table-3). Our detoxification tests clearly confirmed that degraded metabolite was less toxic to untreated dye.

Conclusion

In the present study, we isolate a potential azo dye degrader and developed as a stable mutant strain for hyper-production of an oxygen-sensitive azoreductase through random mutagenesis. It was found that the oxygen-sensitive azoreductase obtained from mutant strain show remarkably high enzyme activities and found to be capable of degrading azo dyes in to non-toxic metabolite without any mediator. Furthermore, for better understating the molecular investigation is required to improve the expression of azoreductase gene for the hyper-production of an enzyme in upcoming era.

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