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Role of azoreductase enzyme produced by potential *Bacillus spp* in decolourization of azo reactive red

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ABSRACT

Biological methods through bacteria are most feasible and ecofriendly alternatives for the degradation of xenobiotics compound especially azo reactive dyes. The potential Bacillus spp., isolated from the textile effluent contaminated sites. The enzyme azoreductase that catalyze the reduction of azo groups, the reactive azo dyes was purified and characterized by Bacillus pp., which is capable for the reduction or cleavage of azo bonds. It was found that the azoreductase from the bacterial strains was an inducible enzyme and play a key in dye degradation and the toxicity of degraded products was assessed by germination and growth of plant.

Keywords: Azoreductase, *Bacillus pp.*, Degradation, Phytotoxicity test.

INTRODUCTION

Textile industry is used for large quantities of water and different chemicals [1]. The high quality water is a crucial factor in textile industry processes such as cleaning, rinsing, dyeing and washing [2]. Azo dyes are the largest and important group of dyes mainly due to the simple synthesis [3]. Azo dyes-contaminated effluents have emerged as a serious issue because of their negative impact on ecosystems, animals, plants and humans. Due to these polluted dye water is a very serious health issues are become apparent [4]. Various factors which reduce degradation of dyes include high water solubility, high molecular weight and fused aromatic ring structures it inhibit permeation through biological cell membranes[5,6].The dyes includes acidic, reactive, basic, disperse, azo, diazo, anthraquinone dyes which causes a considerable environmental pollution problems. Effluent from the industries containing reactive dyes causes serious environment pollution because the contaminated dyes in water is highly visible and affects transparency and aesthetic even though the concentration of the dyes is low [7].

Compared to both physical and chemical treatments technologies bioremediation is an alternative effective technique which is ecofriendly, cost effective, less sludge producing properties and is used for environmental cleanup applications in recent years [8]. A number of microorganisms have been found to be able to mineralize textile dyes including bacteria, yeasts and fungi [9,10,11].Developed enzyme systems for the decolourization and mineralization of azo dyes under certain environmental conditions [12]. Azoreductase is the key enzyme responsible for the cleavage of azo dye degradation in bacterial species.

The presence of oxygen normally inhibits the azo bond reduction activity so aerobic respiration may dominate use of the NADH, thus impeding electron transfer from NADH to the azo bonds [13]. Toxic compounds from dye effluent get into aquatic organisms, pass through the food chain and in humans it leads to various physiological disorders like hypertension, sporadic fever, renal damage, etc. Plant growth parameters are germination, seedling survival and seedling height have been taken as criteria to assess plant response to specific pollutants. Dyes used are considered as carcinogenic and mutagenic and the effluents reduce the rate of germination and growth of crop plants [14].The

bioremediation potential of treated remazol red RB dye for germination of significant seeds was determined under standard in vitro conditions.

MATERIALS AND METHODS

Microorganism and culture conditions

Bacillus spp., was isolated from textile effluent contaminates site of Satravada, Chittoor District of Andhra Pradesh, India. Pure culture was maintained on nutrient agar slants. These are enriched by growing into conical flasks containing 100ml medium in 250 ml Erlenmeyer flasks of Mineral salt medium [15]. Amended with 50mg/l dye as the sole source of carbon and nitrogen. Composition of Mineral salt medium used for decolourization process was (g/L): KH_2PO 4.8g; $K_2HPO_4$1.2; $NH_4NO_3$1.0; $MgSo_4.7H_2O$ 0.2; $Ca(No3)_2.4H2O$ 0.04; $Fe(So_4)_3$ 0.001; Distilled Water 1000ml; pH 7.0.100 ml mineral salt medium in flasks was inoculated with bacterial suspension and incubated at 37°C until complete decolourization was achieved under static condition. The Screening procedure in liquid medium was continued until complete decolourization of broth.

Dyestuff and characterization

The textile dyes, Remazol red RB dye widely used commercially by the small scale textile dyeing units, it is a activated mono azo vinyl compound and water soluble dye and provided by a textile dyeing unit.

Decolourization assay

Bacillus spp., was grown at 37°C in 250 ml Erlenmeyer flasks containing 100 ml mineral salt medium to study the effect of initial dye concentration on the decolourization in static condition, The aliquot (5ml) of the culture media was withdrawn at different time intervals, centrifuged at 10,000 rpm for 10 min. Decolourization was monitored by measuring the absorbance of supernatant at 518nm.The percentage decolourization was calculated as follows.

$$\text{Decolourization (\%)} = \frac{\text{Initial absorbance - Final absorbance}}{\text{Initial absorbance}} \times 100$$

Assay of azoreductase activity

The reaction mixture consists of 400 µl of 50 mM potassium phosphate buffer (pH 7.4) with 200 µl of sample and 200 µl of Remazol red RB dye. Reaction mixture without NADH was pre incubated for 4 minutes and the reaction was started by the addition of 200 µl of NADH and the total reaction mixture was 1ml. Dye decolourization was followed by monitoring the decrease in colour intensity at 518 nm at room temperature. The linear decrease of absorption was used to calculate the azoreductase activity [16]. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the oxidation of one micromoles of dye reduced per minute [17].

NADH-DCIP reductase activity

NADH-DCIP reductase activity was measured [18]. The assay mixture contained 50 µM DCIP, 50 µM NADH in 50 mM potassium phosphate buffer (pH 7.4) and 0.1 ml enzyme solution to make 5.0 ml total volume. From this, 2ml reaction mixture was assayed at 590 nm using extinction coefficient of 19 mM/cm [19].

Partial purification of Azoreductase enzyme

The enzyme responsible for dye decolourization has been partially purified by a combination of different purification processes. Crude extract was subjected to ammonium sulphate $(NH_4)_2SO_4$) precipitation at 40% saturation to remove impurities, followed by 60% saturation in a second step to precipitate the azoreductase. The precipitated proteins from the second precipitation step have been collected by centrifugation, and the pellet has been dissolved in 30 ml of phosphate buffer (50 mM, pH7.0).The precipitated proteins were desalted by dialysis against phosphate buffer (50 mM, pH7.0) overnight. The desalted solution after dialysis has been filtered through a 0.45 µm filter and was subjected to anion exchange chromatography using DEAE cellulose as column bed in 1.5 cm diameter with bed length 10 cm and applied 1.5 ml aliquots of the resulting solution to the column. Protein was eluted at 5.4 cm/h. with the sample buffer and with step wise increasing concentration of buffer containing NaCl with range 0-200 mM and highest concentration up to 1M. The 1.5mL fractions were collected for each concentration of NaCl. The protein concentration and enzyme activity has been determined at every steps of purification.

Sodium dodecyl sulphate- polyacrylamide gel electrophoresis of partially purified proteins (SDS-PAGE)

In order to ascertain the presence of the enzyme, Sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) was the method applied to determination of the enzymes. The separating gel consisted of 10% (W/V) acrylamide N, N- methylene- bis- acrylamide (Sigma, USA) at a concentration such that the ratio of monomers to this

was 30: 08; 0.375 M Tris- HCl (pH 8.8) and 0.1% sodium dodecyl sulphate. It was chemically polymerized with 0.05% (V/V) TEMED (Merck, FRG). The solution was cast into slabs and was over layered with butanol to exclude contact with air. The stacking gel containing 4 % (W/V) acrylamide, 0.12 M Tris- HCl (pH 6.8), 0.1 % SDS, 0.05 % (W/V) ammonium persulphate, 0.05% (V/V). TEMED was over layered on separating gel. Samples (50- 200 µg) were mixed with an equal volume of sample buffer having 0.0625 M Tris- HCl (pH 6.8), 10 % (V/V) glycerol, 5% 2-mercaptoethanol, 2% SDS and 0.002 % bromophenol blue, heated in a boiling water bath for 5 minutes. After cooling samples were loaded into the slots. The sample, crude fraction was stacked at 50 V and run at 100 V for 6 hours using 0.025 M Tris, 0.192 M glycine buffer (pH 8.3) containing 0.1 % SDS as the electrode buffer. The molecular weight of the enzyme was determined by comparing the electrophoretic mobility of the enzyme with reference (low molecular weight calibration proteins).

Zymogram analysis

Polyacrylamide gel electrophoresis (PAGE) was carried out as described previously [20]. with some modifications for native PAGE, *i.e.*, both the detergent component sodium dodecyl sulfate (SDS) and 2-mercaptoethanol (mercaptoethanol) were omitted. 12% polyacrylamide gels were utilized and broad-range molecular weight markers from Pharmacia were used as standards. For activity staining, the method [16].was used. The gel was immediately immersed in approximately 50 ml of 50mM coomassie blue for 25 minutes. After removal of the staining solution, the gel was transferred to a tray filled with solution containing (2mM NADH in 50 mM phosphate buffer, pH 7.0). Colourless bands of the active enzyme appeared after approximately 10 minutes within background of gel.

Phytotoxicity test

The effect of remazol red RB dye and its degradative metabolites on germination and growth of the fenugreek plant was evaluated. The degradation metabolites of dye were extracted in ethyl acetate were dried and dissolved in water to form the final concentration of 600ppm. The dye solutions were also prepared with dye concentration of 600 ppm for phytotoxicity studies. The seeds were germinated in sterile petri dishes, layered with sterile filter paper. Seeds were sterilized before transferring to the surface of the paper in petri dish [21]. The phytotoxicity study was carried out at room temperature (32 ± 2^{o}C) in relation to fenugreek seeds (*Trigonella foenum-graecum*) (50 seeds per plate) by watering separately 5ml samples of dye and its degradation product. Seeds germinated in water as used as a control. Length of plumule (shoot), radicle (root) and germination (%) were recorded after every day. Germination of seeds with dye and degraded dye was calculated after comparing with control. At the end of the germination experiment, the shoot and root length of seedlings was measured separately for dye, degraded dye and control samples [22].

RESULTS AND DISCUSSION

Extraction and analysis of degraded product

After complete decolourization 100ml of dye degraded samples was taken and centrifuged at 10,000 rpm for 10 minutes. Then the culture supernatant was treated with equal amount of ethyl acetate and separated organic phase and aqueous phases by using separating funnel. Organic phase filtrate was evaporated in the rotary evaporator or evaporated to dryness and dissolved in HPLC grade methanol [23]. The degraded products were analyzed by Protein analysis.

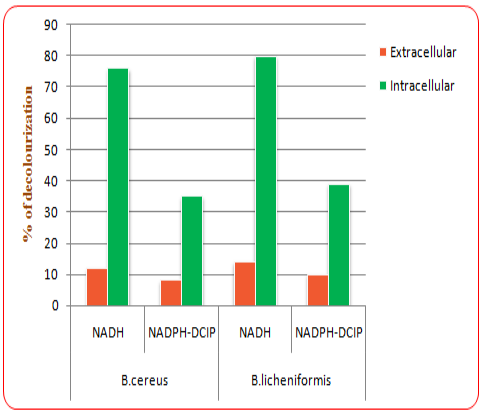


Fig 1: Dye decolourization by cell fractions in the presence of NADH and NADPH-DCIP

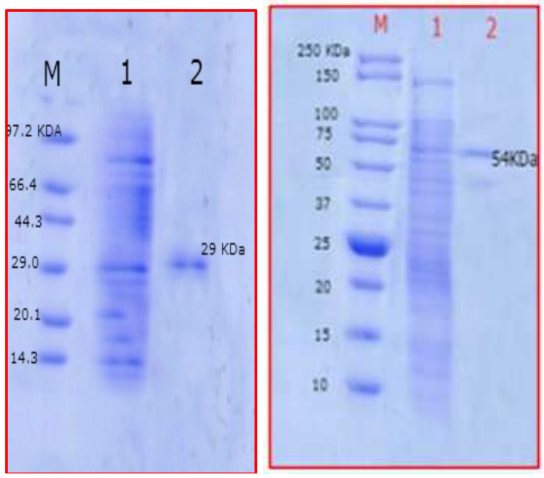
Enzyme activities in cell fractions

The enzyme activity was determined in cell fractions, extra and intracellularly. The effect of cofactors NADH and NADPH- DCIP were studied for both isolates. In NADH, extracellular enzyme activity for *Bacillus cereus* (11%)

and *Bacillus licheniformis* (13%), enhanced decolourization inracellulary in both *Bacillus cereus* (76%) and *Bacillus licheniformis* (79%).While in NADPH- DCIP, extracellular enzyme activity for *Bacillus cereus* (8%) and *Bacillus licheniformis* (10%), in intracellular for *Bacillus cereus* (35%) and *Bacillus licheniformis* (39%) were increased. Data revealed that NADH and NADPH- DCIP cofactors showed maximum decolourization as intracellular (Fig 1).

Purification of azoreductase enzyme

Crude protein extract obtained from *Bacillus cereus and Bacillus licheniformis* bacterial cells was found to decolourize Remazol red RB dye using NADH as electron donor. The absorbance of the enzyme assay mixture was taken at 502 nm by using spectrophotometer at the 5minutes of regular interval. The absorbance was found to be decrease, which indicates that the NADH is working as coenzyme for azoreductase. Intracellular azoreductase enzyme was identified. Each step of purification, the activity was assayed and it was found that the specific activity of the enzyme increased after each step of purification. Protein concentration was found to be (730 mg/ml;1320 mg/ml) in crude extract and the enzyme activity was found to be (1.58 U/mg;3.86 U/mg). The azoreductase enzyme involved in dye decolourization of was partially purified by a combination of purification methods by using ammonium sulphate (70% concentration) precipitation (0.063 U/mg; 340 µg/ml; 0.812U/mg;720mg/ml), dialysis (0.074U/mg;110mg/ml; 0.354U/mg; 220mg/ml), ion exchange column chromatography (0.089 U/mg; 90mg/ml; 0.186U/mg;160mg/ml). The enzyme was partially purified in higher quantity *ie.*, 8.16 and 10.7 folds by using DEAE-column with a yield of 15.2% and 23.2 % .The presence of band on SDS-PAGE as 29 KDa and 54 KDa approximately (Fig 2). Azoreductase is a key enzyme in azo dye degrading bacteria and catalyses the reductive cleavage of the azo bond. Maximum degradative enzyme activity was observed intracellularly, so the enzyme responsible for degradation of the azo dye may be considered membrane bound enzyme. Cofactors like NADH and NADPH- DCIP as well as the enzymes reducing these cofactors are located in the cytoplasm. Lysis of cells would release cofactors in the extracellular environment. Hence in cell extracts of lysed cells show higher azo dye reduction rates than intact or resting cells. The membrane transport system may be prerequisite for reduction of azo dyes by these cofactors in intact cells. So these catalyze reaction in the presence of reducing equivalents NADH and NADPH- DCIP. The enzymatic activity was faster with NADH than NADPH-DCIP reductase indicating that enzyme prefers NADH as a source of reduction. NADH was found to play an important role in dye degradation as a source of electron donor. Kudlich [24] support the suggestion that the membrane bound azoreductase activity, mediated by redox compounds is different from the soluble cytoplasmic azoreductase that is responsible for the reduction of non-sulphonated dyes that permeate through the cell membrane. Although the final reduction of the azo dyes in the cell supernatants is a dominantly chemical redox reaction, the redox mediators depend on cytoplasmic reducing enzymes to supply electrons. In ORB7106 cell-free extract from the pellet biomass showed more than (65%) decolourization of MR. However, the cell-free supernatant showed only (9-14%) decolourization of MR [25]. Intracellular azoreductase is responsible for the decolourization activity for azo dye degrading microorganisms [26].



A **B**

Fig 2: SDS- PAGE of Azoredutase enzyme (A,B)
Lane M : Low molecular weight Biorad Standard markers
Lane A : Molecular weight of partially purified enzyme A: *Bacillus cereus*(29 KDa) *and Bacillus licheniformis* (54 KDa)

Phytotoxicity test

In the present study, the phytotoxicity of the dye and its metabolites after degradation by *Bacillus cereus* and *Bacillus licheniformis* was observed. Phytotoxicity revealed the toxic nature of remazol red RB dye to the fenugreek seeds (*Trigonella foenum graecum*) and plants (Fig 3).Germination percentage of seeds with dye and degraded metabolites were compared with control and found to be (38.0%) for dye, degraded metabolites *Bacillus cereus* (80%) and *Bacillus licheniformis* (83%) and distilled water (92%).There is no significant difference in the root and shoot length in case of

fenugreek treated with the dye because growth of the plant is arrested when the seeds are soaked in the dye but in the case of metabolites the root and shoot length of fenugreek plant was significantly increased compared to control fenugreek plant. The phytotoxicity study showed good germination rate and as well as growth in the plumule and radical for both the plants in the metabolites extracted after decolourization compared to dye sample (Table 1). This indicates the detoxification of remazol red RB dye by the isolated bacterial cultures remazol red RB dye (Fig 4). Our results are similar with Kalyani [27] Red BLI with seeds of *Sorghum vulgare* and *Phaseolusmungo* showed more sensitivity towards dye while the products obtained after dye decolourization have less inhibitory effects. In the *Pseudomonas aeruginosa* strain BCH was able to detoxify the dye, Direct Orange 39 (1000ppm each day) effectively which was tested with *Triticum aestivum* and *Phaseolus mungo* [28]. Both germination, shooting and rooting from metabolites of dye for *Vignaradiata* (whole moong), *Triticum*spp (Wheat) and *Brassica juncea* (Mustardseeds) [29].

Fig 3: Bioassay of dye and degraded dye toxicity A) D. water B) Treated with *B. cereus* C) Treated with *B. licheniformis* D) Treated with dye

Table 1: Phytotoxic study of remazol red RB dye degradation metabolites on *Trigonellafoenumgraecum* germination

Seed type	Samples	Percentage of germination						Average shoot length (cm)	Average root length (cm)
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6		
Fenugreek seeds	Control (water)	0	23	48	59	84	92	6.3±0.01	3.2±0.01
	Dye	0	0	7	13	30	38	2.3±0.1	1.2±0.05
	Degraded dye by *B. cereus*	0	13	39	51	69	80	5.6±0.1	2.6±0.05
	Degraded dye by *B.licheniformis*	0	16	41	53	70	83	5.7±0.1	2.8±0.1

Values are the means of triplicates ±SD

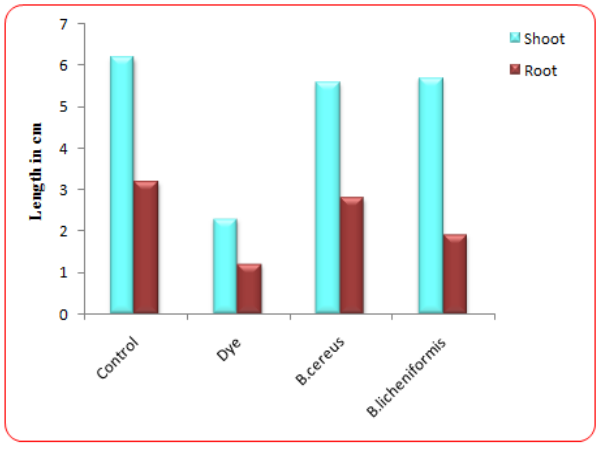


Fig 4: Phytotoxicity studies on root and shoot length of *Trigonella foenum graecum*

CONCLUSION

Both potential *Bacillus spp.*, isolated from the contaminated textile industrial effluent sites for the decolourization of remazol red RB dye. The azoreductase enzyme was play a key role in dye degradation and also phytotoxicity studies revealed that the detoxification of remazol red RB dye by *Bacillus spp.*, results into the non-toxic metabolites.

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