emergent
Life Sciences Research

Research Article

Production, partial purification and characterization of intracellular Azoreductase from bacterial isolates during biodecolorization of textile dye Acid Maroon V

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Abstract

Azoreductase is an important enzyme for the reduction of the azo linkage of dye during the biodecolorization process. In the present study, the production of intracellular azoreductase from bacterial cultures *Enterobacter dissolvens* AGYP1 (ED) and *Pseudomonas aeruginosa* AGYP2 (PA) was achieved during biodecolorization of synthetic textile dye Acid Maroon V. When compared with PA, the enzyme activity of ED was 2.31 times greater. The partial purification of azoreductase revealed specific activity of the dialyzed enzyme 51.72 U mg^{-1} protein (ED) and 33.06 U mg^{-1} protein (PA). Methyl Red served as the best substrate for the azoreductase enzyme with superior activity at optimal pH 7 and temperature 30 °C. 75-80% of azoreductase enzyme activity was maintained when Mg^{+2} and Ca^{+2} were present. The azoreductase activity was considerably increased with NADH as the most suitable electron donor. The kinetic study showed consequent Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) values of 50 µM and 2222 U ml^{-1} (ED) and 250 µM and 1000 U ml^{-1} (PA), respectively.

Keywords azoreductase, bacterial cultures ED and PA, characterization, decolorization

Received: 11 December 2022
Accepted: 13 March 2023
Online: 17 March 2023

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Emer Life Sci Res (2023) 9(1): 61-71

E-ISSN: 2395-6658
P-ISSN: 2395-664X

DOI: https://doi.org/10.31783/elsr.2023.916171

Introduction

Synthetic dyes offer a broad range of applications in various industrial sectors like textile, pulp and paper, leather, photography, pharmaceutical, food, cosmetics, etc. Such dyes are more widely accepted because of their simpler and more affordable synthesis method, a wide range of color tones, and longer durability when compared to natural dyes [1]. However, because the synthetic dye is xenobiotic and recalcitrant, it poses serious risks to the environment and biological systems [2]. Due to inefficient dyeing processes, the wastewater from the textile sectors contains 10 to 50% dyes [3-4]. Additionally, dye with a concentration of less than 1 ppm is visible in the receiving water body, which is aesthetically unpleasant. The textile dying business is classified as a "Red Industry" (the most polluting sector) in several nations as a result of the release of a massive volume of colored effluent [5]. As a result, people, researchers, and legislative agencies are becoming increasingly concerned with how to remediate colored industrial effluents [6]. Various types of physicochemical techniques are in use for the fate of dyes from industrial

waste water. However, such treatment processes have certain inherent limitations. Therefore, the biological method seems to be environmentally benign and the most feasible alternative to achieve the significant fate of synthetic dyes [7]. Different members of the biotic community like species of bacteria, actinomycetes, yeasts, fungi, algae, and plants have been utilized to decolorize, degrade, and mineralize a range of synthetic dyes. It is thought that utilizing bacterial agents to treat hazardous dyes is an efficient strategy. For the biodecolorization of the array of synthetic dyes, both pure and mixed bacterial cultures are widely used [1-2, 8-9]. The main processes of bacterial decolorization of synthetic dyes include bioaccumulation, biosorption, and enzymatic destruction. The oxidoreductase enzymes present in the bacteria catalyze several biochemical reactions and ultimately led to the cleavage of the complex molecular structure of dye [3]. One of the crucial reductive enzymes, azoreductase, breaks down the azo linkage (-N=N-) of the chromophore moiety of azo dye to produce colorless aromatic amines [10]. Azoreductases are divided into intracellular and extracellular, as well as flavin-dependent and flavin-independent categories, based on where they are located in the cell membrane, and whether they need a cofactor. The decolorization of several dyes by azoreductase has been documented in earlier reports [11-13]. A detailed explanation of the shift of substrate specificity of azoreductase with structural variability and relative presence of functional groups in dye molecules is given in the literature [14]. In the present study, the production of azoreductase from bacterial cultures during dye decolorization is investigated. Partial purification and characterization of azoreductase are also included in the present study.

Methodology

Dyes, bacterial cultures and culture media

The synthetic dyes used in the current study Acid Maroon V (C.I. Acid Red 119), Methyl Red (C.I. Acid Red 2), Remazol Brilliant Violet 5R (C.I. Reactive Violet 5), Acid Red F2R (C.I. Acid Red 151), Congo Red (C.I. C.I. Direct Red 28), Direct Red 12B (C.I. Direct Red 31) and Reactive Red M5B (C.I. Reactive Red 2) were procured from the textile industry at Ankleshwar, Gujarat, India. The bacterial cultures *Enterobacter dissolvens* AGYP1 (GenBank accession number HQ336043) and *Pseudomonas aeruginosa* AGYP2 (GenBank accession number HQ336042) studied for azoreductase enzyme production were isolated from dye-laden soil samples [15]. The experiments of decolorization of model dye Acid maroon V were executed in Mineral Salt Medium (MSM) containing g l^{-1}: sucrose, 4; $NH_4H_2PO_4$, 0.3; K_2HPO_4, 6; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.1; NaCl, 5; and pH 7±0.2. The Luria Bertani (LB) broth containing g l^{-1}: casein enzymatic hydrolysates, 10; yeast extract, 5; NaCl, 5; and pH, 7±0.2 was used for the preparation of bacterial inoculum.

Dye decolorization in liquid media

The dye removal experiment was carried out using 150 ml MSM supplemented with dye Acid Maroon V (100 mg l^{-1}) in 250 ml Erlenmeyer flasks. The individual bacterial cultures at 10% (v/v) inoculum of 0.5 OD_{600} were inoculated into the medium. The flasks were incubated at 30 °C under static conditions for color removal.

Intracellular azo reductase estimation

Preparation of crude extract

The bacterial cultures were inoculated separately in MSM containing 100 mg l^{-1}Acid Maroon V. After decolorization, the flask content was centrifuged at 4 °C for 15 min at 8000 rpm in the refrigerated centrifuge (Kubota 6200, Japan). The cell pellet obtained was rinsed thrice with physiological saline (0.85% NaCl) followed by 0.1M potassium phosphate buffer (pH 7). The washed cell pellet (1 g) was mixed with 8 ml of the same buffer and disrupted by ultrasonication in the cold condition for 45 s, 14 times with 30% outputs (Sonics Vibracell VCX 130 sonicator, USA). The removal of cell debris was performed by centrifugation at 10,000 rpm for 15 min at 4 °C. The clear supernatant obtained was

considered a crude enzyme source.

Azo reductase assay

The azoreductase activity was evaluated at 30 °C using a UV-visible spectrophotometer [16]. The reaction mixture (1 ml) for the azoreductase assay contained 0.1 M potassium phosphate buffer (pH 7), 24 µM Methyl Red, and an appropriately diluted enzyme solution. After 4 min of pre-incubation at 30 °C, the assay system was supplemented with 1 mM NADH. The decrease in Methyl Red absorbance was measured at 437 nm. One unit (U) of azoreductase activity was considered as the amount of enzyme needed to reduce 1 µM of Methyl Red per min.

Estimation of protein

The concentration of protein was determined as per Lowry's method using BSA as a standard.

Partial purification of azoreductase

Ammonium sulfate precipitation

The crude extract was mixed with ammonium sulfate and stirred constantly in cold conditions throughout 3 h to obtain 0-60% saturation. The mixture was kept in the refrigerator at 4 °C overnight. The resulting precipitates were separated by centrifugation at 10,000 rpm for 20 min at 4 °C. The precipitates were dissolved in a minimal volume of potassium phosphate buffer (0.1 M, pH 7). The supernatant and precipitates were analyzed for azo reductase activity and protein determination.

Dialysis

The precipitates obtained by 60% ammonium sulfate precipitation were dissolved in a small volume of potassium phosphate buffer (0.1 M, pH 7). The solution was dialyzed using dialysis membrane 150 (HiMedia) against the large volume of the same buffer for 12 h at 4 °C, with the intermittent change of buffer at 4 h intervals.

Characterization of partially purified azoreductase

Determination of substrate specificity

The azoreductase activity was determined using different dyes at an equimolar concentration as substrate. The dyes were Methyl Red (molar extinction coefficient 23,360 M^{-1} cm^{-1}), Acid Maroon V (molar extinction coefficient 8,800 M^{-1} cm^{-1}), Remazol Brilliant Violet 5R (molar extinction coefficient 10,700 M^{-1} cm^{-1}), Direct Red 12B (molar extinction coefficient 23,660 M^{-1} cm^{-1}), Acid Red F2R (molar extinction coefficient 19,730 M^{-1} cm^{-1}), Congo Red (Molar extinction coefficient 17,400 M^{-1} cm^{-1}) and Reactive Red M5B (molar extinction coefficient 15,340 M^{-1} cm^{-1}).

Determination of optimal pH and temperature

The azoreductase activity was studied over a wide range of pH (3 to 10) using different buffers (0.1 M citrate buffer for pH 3-5; 0.1 M potassium phosphate buffer for pH 6-8 and 0.1 M glycin-NaOH buffer for pH 9-10). To assess the effect of temperature, the reaction mixture was incubated at 25 to 55 °C temperatures. The enzyme activity was measured as indicated earlier using Methyl Red as substrate.

Effect of metal ions and inhibitors

The enzyme was pre-incubated with 2 mM of different divalent metals - Mg^{+2}, Ag^{+2}, Mn^{+2}, Cu^{+2}, Fe^{+2}, Ca^{+2}, Zn^{+2}, and Hg^{+2}, as well as different concentrations of EDTA and SDS for 30 min at room temperature. The residual activity was calculated under standard assay conditions. The enzyme activity without any metal and inhibitor (control) was considered 100%.

Kinetic studies of azoreductase

The initial velocity of azoreductase was evaluated in the presence of electron donors (NADH, NADPH, and FAD). To check the effect of NADH on azoreductase activity, different concentrations of NADH were taken (0.1 to 2 mM) with constant Methyl Red concentration (24 µM). Maximal velocity (V_{max}) and Michaelis constants (K_m) of azoreductase were determined from a double reciprocal Lineweaver-Burk plot using variable concentrations of Methyl Red.

Results and Discussion

Production and partial purification of azoreductase from bacterial cultures

In our study intracellular azoreductase could be produced by both bacterial cultures. The azoreductase activity of ED and PA was found to be 119.34±7.4 and 51.58±2.8 U ml^{-1} respectively after Acid Maroon V decolorization under static conditions (Table 1).

Table 1: Azoreductase activity of bacterial cultures

Bacterial culture	Azoreductase activity (U ml^{-1})	Specific activity (U mg^{-1} protein)
ED	119.34±7.4	18.05±1.12
PA	51.58±2.8	14.36±0.78

Nevertheless, compared to PA, ED showed 2.31 times more enzyme activity. Crude extracts of disrupted cells of ED and PA yielded 528.8 and 359 mg of protein, corresponding to 9547.2 and 5158 units of azoreductase enzyme respectively. After dialysis, azoreductase from ED and PA showed 2.86 and 2.3 fold purification with the recovery of 43% and 39%, respectively. The specific enzyme activities of the dialyzed samples were 51.72 U mg^{-1} protein (ED) and 33.06 U mg^{-1} protein (PA). The results thus displayed a quite considerable purification and recovery of azoreductase by applying salt precipitation and dialysis. Our findings are consistent with other studies that explained how intracellular azoreductase activity was induced together with other enzymes during bacterial decolorization of textile dyes [1, 5, 11]. The process of biodegradation of azo dyes is often commenced with the breakdown of the azo bond by azoreductase resulting in aromatic amines formation. Subsequently, the degradation of aromatic amines is mediated by various oxygenases [3]. The azoreductase catalyzed cleavage of azo linkage requires four electron transfer reactions in two stages, where two electrons are transferred to the azo bond of the dye in each stage [17]. In the process of decolorizing dyes, mainly two types of azoreductases - flavin-dependent and flavin-independent are involved. The flavin-dependent azoreductases are further categorized as NADH only, NADPH only, or both depending on coenzymes as electron donors [18]. Most often, anaerobic bacteria are known to produce azoreductase, some aerobic bacteria have also been known to produce intracellular azoreductase [19-20]. For instance, oxygen-insensitive azoreductase has been found in *Caulobacter subvibroides* strain C7-D [13] and *Pigmentiphaga kullae* K24 [2].

Characterization of partially purified azoreductase

Substrate specificity of azoreductase

Seven different dyes, each at an equimolar concentration were used to assess the azoreductase enzyme's selectivity for its substrate. The activity of azoreductase was found with all dyes used as the substrate. However, the highest enzyme activity was observed with Methyl Red dye, followed by Acid Maroon V (Table 2). This indicated Methyl Red as the most suitable substrate for the azoreductase from both bacterial cultures. The azoreductase of ED and PA showed the least preference for dyes Reactive Red M5B and Remazol Brilliant Violet 5R, respectively as substrates. The structural complexity of the dyes as well as the characteristics and configuration of the replacing groups in the dye molecule may contribute to the diversity in the substrate specificities [21]. Methyl Red is more

easily degradable than di-azo dyes because it is a mono-azo dye and doesn't contain sulfonated

Table 2. Substrate specificity of azoreductase from bacterial cultures

Substrate (Dye)	λ_{max} (nm)	Azoreductase activity (U ml^{-1})	
		ED	PA
Acid Maroon V	523	11.34±2	6.23±0.6
Methyl Red	437	287.07±12	205.33±8
Remazol Brilliant Violet 5R	559	1.3±0.05	0.8±0.02
Acid Red F2R	510	8.41±1.8	3.12±0.7
Congo Red	498	4.65±0.2	4.45±1.7
Direct Red 12B	524	3.27±0.01	2.56±0.03
Reactive Red M5B	538	0.6±0.02	1.7±0.05

groups. Many researchers have demonstrated substrate preference of the azoreductase to the structural diversity of the azo dyes [8, 22-24]. In one study, when the hydroxyl group was present at the para position and ortho position of the naphthol rings of Orange I and Orange II respectively, the decolorization of such dyes was faster by bacterial azoreductase [14]. Another study claimed that the activity of the azoreductase enzyme was inhibited when charged groups and electron-donating groups were close to the azo linkage in the dye. Moreover, the enzyme activity was accelerated in the presence of the phenyl group containing electron-withdrawing sulfo (SO_3^-) groups [13].

Effect of pH on azoreductase activity

The influence of pH on the activities of azoreductase from both bacterial cultures was determined in a wide range of pH (3-10). The data shown in Figure 1 demonstrated that the enzyme activity was discovered to be pH sensitive, and increased linearly with an increment of pH from 3 to 7.

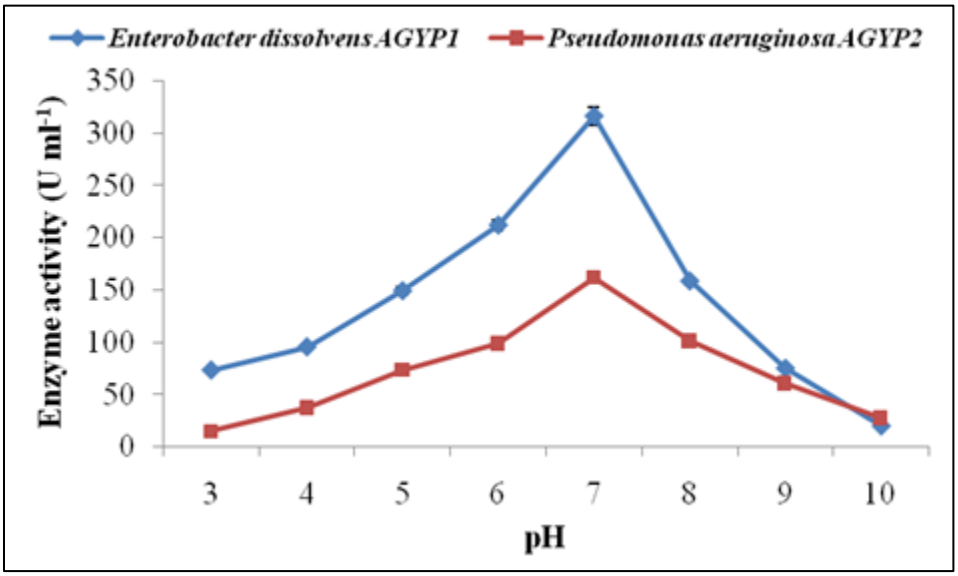

Figure 1. pH optima of bacteria azoreductase activity

However, maximum enzyme activity was observed at pH 7 for ED (315.58±8.4 U ml^{-1}) and PA (161.71±2.7 U ml^{-1}). At extremely acidic and alkaline pHs, the azoreductase activity was considerably decreased. In comparison to optimum pH 7, the enzyme activity was only 23.20 and 9.10% at pH 3,

and 6.29 and 16.83% at pH 10 for ED and PA respectively. Nevertheless, the azoreductase from both bacterial cultures was quite stable throughout a wide pH range (pH 4-9), indicating considerable Ph tolerance. Our results are consistent with earlier studies demonstrating that pH 7 was ideal for azoreductase activity [25-26]. In another study, the azoreductase from the bacterial consortium had maximum activity at pH 7 than other pH values. An individual member of the consortium, *Bacillus odyssey*, *B. thuringiensis*, *B. subtilis*, *B. cereus*, *Alcaligenes* sp., and *Nocardiopsis alba* also displayed the peak of azoreductase activity at pH 7 [9]. On the other hand, the azoreductase enzyme from *Rhodobacter sphaeroides* AS1.1737 exhibited its highest level of activity at pH 8 [27]. In the previous publication, a variable degree of pH's impact on the azoreductase produced by human gut bacteria was described [11]. It is well known that highly acidic and alkaline pH leads to denaturation of the enzyme, and altered enzyme configuration might not be compatible with its substrate. However, in a pH range other than optimum, the enzyme shows activity depending upon its type.

Effect of temperature on azoreductase activity

The effect of temperature on the activity of azoreductase from ED and PA was studied in a temperature range of 20 to 55 °C. In our study, we found that the optimum temperature for the azoreductases of both cultures was 30 °C with maximum enzyme activities of 292.36±13.6 and 152.75±10.1 U ml^{-1} for ED and PA respectively (Figure 2).

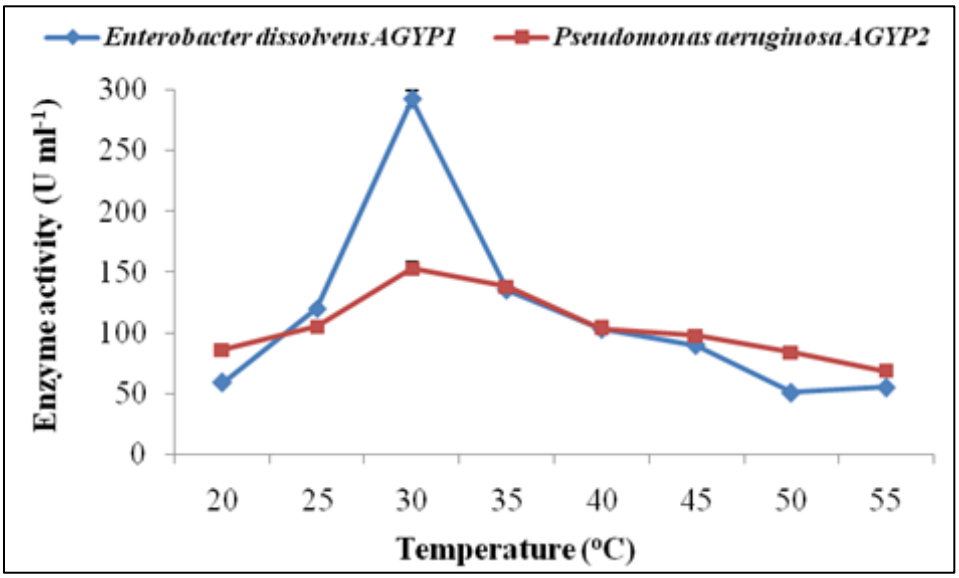

Figure 2. Temperature optima of bacterial azoreductase

As the temperature rose, there was a fall in enzyme activity. For instance, azoreductase activity decreased by 18.74% (ED) and 44.84% (PA) at 55 °C compared to 30 °C. Nevertheless, azoreductase from both bacterial cultures was quite stable and active in a broad range of temperatures, suggesting its temperature tolerance. A variable degree of temperature optima of azoreductase has been documented in previous literatures. For instance, at 35 and 45 °C, respectively, the azoreductases from *Pseudomonas cepacia* [28] and *Pigmentiphaga kullae* K24 [2] showed their peak activities. On the other hand, *Bacillus* sp. azoreductase activity was found to be most effective between 60 and 80 °C [6]. Thermodynamic denaturation of the enzyme protein is connected with decreased enzyme activity at higher temperatures [16].

Effect of metal ions and inhibitors on azoreductase activity

The activities of azoreductase from both bacterial cultures were assessed in the presence of various metal ions and inhibitors. The azoreductase activity in the control was assumed to be 100%, and residual enzyme activity was evaluated after the enzyme was incubated for 15 minutes at 30°C with metal ions and inhibitors (Table 3). Cu^{+2}, Ag^{+2}, Zn^{+2}, and Hg^{+2} significantly inhibited the azoreductase from ED, whereas Fe^{+2}, Mn^{+2}, and Mg^{+2} displayed 50-63% relative activity. However, the enzyme was found to be comparatively stable with Ca^{+2} and retained almost 80% of its activity. Cu^{+2}, Ag^{+2}, Zn^{+2}, and Hg^{+2} caused substantial inhibition of the activity of azoreductase from PA.

Table 3. Influence of metal ions and inhibitors on bacterial azoreductase activity

Metal ions	Concentration (mM)	Residual activity (%)	
		ED	PA
Control	None	100	100
Ca^{+2}	2	80.56	61.23
Cu^{+2}	2	7.08	5.21
Mg^{+2}	2	62.92	75.77
Mn^{+2}	2	51.40	37.46
Hg^{+2}	2	17.72	19.81
Fe^{+2}	2	50.21	56.34
Ag^{+2}	2	8.27	7.92
Zn^{+2}	2	12.99	11.54
EDTA	1	53.42	62.27
	2	47.71	50.51
	5	33.17	24.16
SDS	1	24.53	20.11
	2	3.24	1.02
	5	1.27	ND

Nevertheless, in the presence of Ca^{+2} and Fe^{+2}, the enzyme showed 56-61% inhibition. The activity of azoreductase on the other hand was found to be stable (75%) in the presence of Mg^{+2}. When EDTA and SDS were used in various concentrations, EDTA was found to be a moderate enzyme activity inhibitor. While SDS at a dosage of 5 mM concentration significantly reduced the activity of azoreductase from both bacterial cultures. Our results are consistent with an earlier study in which different metal ions and inhibitors were added, and the azoreductase from *Bacillus subtilis* showed varying degrees of activity [29]. Another study discussed the various effects of metal ions on the activity of the azoreductase enzyme derived from the bacterial flora DDMZ1 [8]. Based on the binding pattern of metal ions to the enzyme protein, both stimulating and inhibitory effect on enzyme activity is observed [30]. Cu^{2+}, Ca^{2+}, Mn^{2+}, and Mg^{2+} were seen to have a stimulating effect on enzyme activity at lower concentrations [31-33].

Effect of electron donors on azoreductase activity

Azoreductase from a variety of bacterial sources is found to be flavoprotein and NADH/NADPH dependent [34-35]. The impact of several electron donor substances on the activity of azoreductase was assessed in our investigation. Among the three electron donors used, the enzyme activity was noticeably higher when NADH was present in the system (Table 4). The presence of NADPH and FAD displayed 7.75 and 2.67% activities of azoreductase respectively from ED compared to NADH. In comparison to NADH, the azoreductase activity of PA was 8.15 and 6.88% with NADPH and FAD, respectively. The above results, therefore, demonstrated NADH as the most suitable electron donor for the catalytic activity of azoreductase. An influence of different concentrations of NADH (0.1-2 mM) was assessed on azoreductase activity. The enzyme activity linearly increased with increment in NADH concentration from 0.1 to 1 mM with maximum enzyme activities of 318.57±4.8 and

169.34±3.4 U ml^{-1} for ED and PA, respectively at 1 mM concentration (Figure 3). The azoreductase activity was negatively impacted by further increases in NADH concentration. At 2 mM concentration, the enzyme activity was 27.41 and 62.12% for ED and PA, respectively compared to 1 mM concentration of NADH. Similar findings have been made in the earlier report, which showed that the

Table 4. Effect of electron donors on bacterial azoreductase activity

Electron donor	Azoreductase activities (U ml^{-1})	
	***Enterobacter Dissolvens* AGYP1**	***Pseudomonas Aeruginosa* AGYP2**
NADH	312.54±13.5	165.87±14.77
NADPH	24.17±7.18	13.44±5.26
FAD	8.34±2.23	11.36±1.66

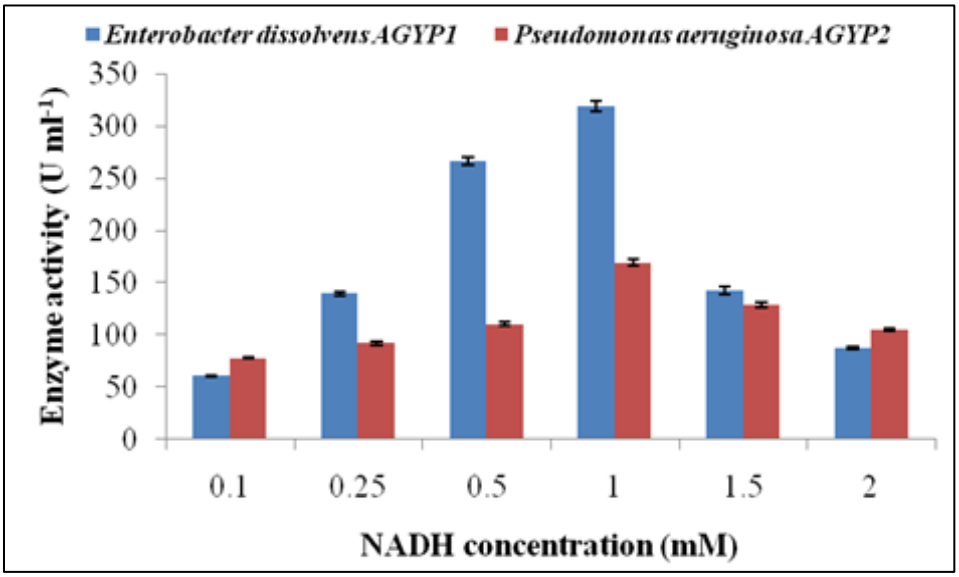

Figure 3. Effect of NADH concentration on bacterial azoreductase

azoreductase from *Enterobacter agglomerans* was NADH dependent for the decolorization of Methyl Red [25]. In another report, the activity of azoreductase from *Enterococcus faecalis*, *Enterococcus avium*, *E. coli*, and *Bacillus cereus* was enhanced with NADH in comparison to NADPH [11]. However, NADPH was found to be an efficient electron donor instead of NADH resulting in 1.6 fold higher activity of *Psuedomonas* sp. KF46 azoreductase using Orange-II and Carboxy Orange-II as substrates [16].

Kinetic study of bacterial azoreductase

The azoreductase activity was carried out at variable concentrations of Methyl Red and 1 mM NADH. The decolorization of dye by azoreductase improved upon the subsequent increase in Methyl Red concentration. The enzyme activity and dye concentration followed a typical Monod-type correlation. Michaelis-Menten double reciprocal model system ($V_{max}[S_{MR}]/(K_m+[S_{MR}])$) was used to establish a relationship between azoreductase activity (V) and initial Methyl Red concentration ($[S_{MR}]$). According to Figure 4A, the apparent K_m and V_{max} values of the azoreductase from ED were 50 µM and 2222 U ml^{-1}, respectively, while those of azoreductase from PA were 250 µM and 1000 U ml^{-1},

respectively (Figure 4B). The specificity constants (V_{max}/K_m) of the azoreductases of both the cultures were determined and found to be 44.44 and 4 U μM^{-1} ml^{-1} for ED and PA respectively. According to the aforementioned results, the partially purified azoreductase enzyme operated effectively across the entire spectrum of Methyl Red concentration. In general, it has been found that the enzyme kinetic parameters differ depending on the source, the kind of substrate employed, and other experimental conditions. The K_m and V_{max} values of *Enterococcus faecalis* azoreductase were 11 µM

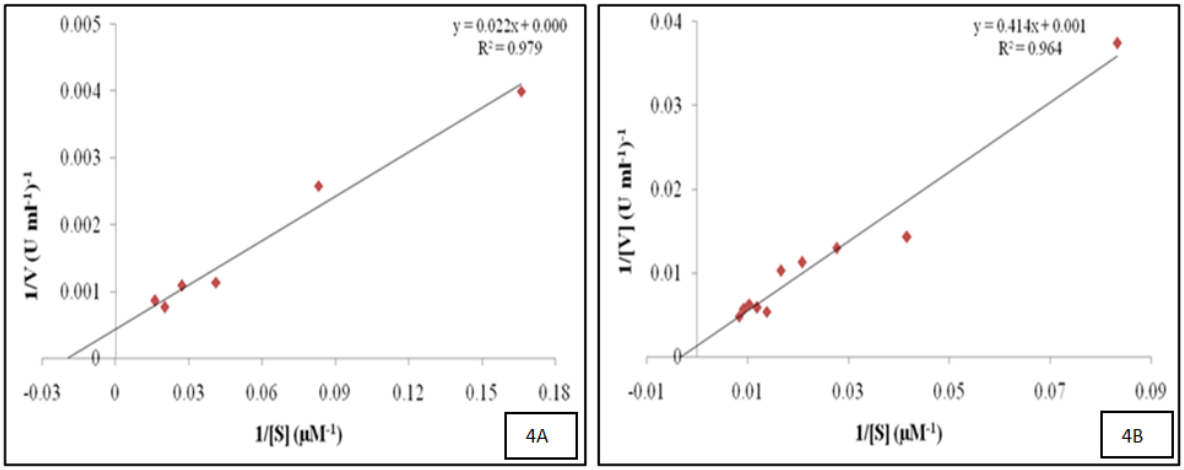

Figure 4. Double reciprocal plots of influence of Methyl Red concentration on the activities of azoreductase from (A) ED, and (B) PA

and 29 U mg^{-1} of protein, respectively using Methyl Red as substrate [34]. Diverse K_m and V_{max} values of azoreductase from *Pseudomonas putida* MET9 were reported for different substrates used [22].

Conclusion

The present study describes the production of azoreductase enzyme from two bacterial isolates ED and PA during biodecolorization Acid Maroon V dye. The enzyme was successfully partially purified after the dialysis process. The azoreductase from both bacterial cultures demonstrated considerable stability with a broad range of substrate, pH, temperature, metal ions, and inhibitors. The azoreductase enzyme activity was increased by adding NADH, the best possible electron donor. The kinetic parameters revealed the effectiveness of azoreductase over a broad range of Methyl Red concentrations. Therefore, the study reveals the potential of azoreductase from ED and PA in the textile dye remediation process.

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