Research Article

Isolation, Purification and Characterization of Oxygen Insensitive Azoreductase from *Pseudomonas aeruginosa* and Biodegradation of Azo Dye - Methyl Red

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Abstract: A *Pseudomonas aeruginosa* was isolated from water sample from Industrial effluent and was tested for decolorization activity against commercially important dye of Methyl red. Percentage dye degradation by the isolated *Pseudomonas aeruginosa* was found to be 90%. The enzyme involved in degradation azoreductase was assayed and purified by anion-exchange chromatography. Total activity of the purified enzyme was 22.5U/mg. The enzyme gave a single band in the SDS–PAGE with a molecular weight of 29 kDa (approximately). The maximal azoreductase activity was observed at pH 7.0 and at 37°C. This activity was NADH dependent. Several metal ions inhibited the purified enzyme including Fe^{2+} and Hg^{2+}.

Keywords: *Pseudomonas aeruginosa*, Decolorization, Azoreductase, Methyl red.

1. Introduction

Thousands of synthetic azo dyes are used in the textile, pharmaceutical, tattooing, cosmetics, food and consumer products (Carliell *et al*., 1995). However, release of residual azo dye into industrial effluents deteriorates the water quality and may cause a significant impact on human health due to mutagenic or carcinogenic effects of some azo dyes or their metabolites (Heiss *et al*., 1992). Methyl red is one of the azo dye which has a large consumption rate in textile industry and it has carcinogenic potential, which belongs to the group-3 class by the International Agency for Research on Cancer (IARC).

Different methods are available for the remediation of dye wastewaters. These include physicochemical methods, like adsorption, chemical oxidation, precipitation, coagulation, filtration, electrolysis, photodegradation, biological and microbiological methods (Churchley, 1994).

Unfortunately, azo dyes present in the wastewater are normally unaffected by conventional treatment processes. Their persistence is mainly due to sulfo and azo groups, which do not occur naturally, making the dyes xenobiotic and recalcitrant to oxidative biodegradation (Kulla *et al*., 1983). The persistence of azo dyes has resulted in several reports, which show that decolorization of azo dyes requires an initial cleavage of azo bonds, after which the resulting aromatic amines can be biodegraded readily under aerobic conditions (van der Zee and Villaverde, 2005). The cleavage of azo bonds is catalyzed by an azoreductase enzyme with the aid of an electron donor. Several bacteria capable of decolorizing azo dyes have been identified and azoreductase enzyme has been isolated and characterized from some of them (Chen, 2006).

The aim of the present investigation was to isolate, purify and characterize an azoreductase enzyme from *Pseudomonas aeruginosa* and its biodegradation activity against methyl red, an Azo dye.

2. Material and Methods

Pseudomonas aeruginosa was isolated from industrial effluent water sample collected from Pallavaram tanners industrial effluent treatment company limited (PTIET) at December 2012 work was carried out in Mohamed Sathak Arts and Science College, Chennai. Diluted sample was taken from 10-5

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dilutions and plated on Luria Bertani agar (g L^{-1} Tryptone 1.0%, Yeast extract 0.5%, Sodium Chloride 1.0%, Methyl red 1%, Agar 15, Distilled water 1000mL) and incubated overnight at 37°C in an incubator.

The isolated *Pseudomonas aeruginosa* strain was tested for decolorization activity against commercially important dye of Methyl red in broth cultures. The flasks containing Mineral salt medium and methyl red (gL^{-1} K$_2$HPO$_4$: 1.73, KH$_2$PO$_4$: 0.68, MgSO$_4$.7H$_2$O: 0.1,

NaCl: 0.1, FeSO$_4$.7H$_2$O: 0.03, NH$_4$NO$_3$: 1.0, Peptone: 1.0, CaCl$_2$.2H$_2$O: 0.02, Glucose: 5.0, Methyl red: 0.1) was inoculated using loop full of isolated bacterial suspension. These flasks were incubated at 30°C for 24 h. Uninoculated flasks served as controls to assess the abiotic decolorization. OD values were measured spectrophotometrically at 620nm to estimate the decolorization process. The rate of decolorization was calculated using the following formula as described by Sani and Banerjee (1999).

$$\text{Decolorization (\%)} = \frac{\text{Initial absorbance} - \text{after decolorization absorbance}}{\text{Initial absorbance}} \times 100$$

Azoreductase activity was assayed by the method of Zimmermann *et al.*, 1982 using methyl red as the dye substrate. The assay mixture contained 2.8ml of 0.1M phosphate buffer (pH 7.0), 0.05ml of 50μM of the dye methyl red, 0.1ml of enzyme solution (0.1ml of distilled water was added for blank) and incubated for 5 minutes at room temperature. 0.05ml of 2mM NADH (redox mediating cofactor) was then added and the decrease in absorbance of assay mixture was read at 430nm (the absorbance maxima of azo dye - methyl red) using a UV spectrophotometer.

The enzyme purification was performed as follows: The culture was grown in mineral salt medium containing 1.0g L^{-1} of methyl red until the dye was completely decolorized. Cells were harvested by centrifugation, washed with 0.1M phosphate buffer (pH 7) and resuspended in the same buffer. Cells were then disrupted by sonication and cell debris was removed by centrifugation at 12,000 for 20 mins at 4°C. The supernatant was used as the crude enzyme source for further study.

The crude enzyme was centrifuged at 10,000 × g for 30 min at 4°C. The cell lysate was fractionated by ammonium sulfate at 40% saturation to remove impurities, followed by 70% saturation to precipitate azoreductase. The precipitated protein was collected by centrifugation at 10,000 × g for 30 min at 4°C, and the pellet was dissolved in 10ml of phosphate buffer (pH 7.4). The solution was desalted by overnight dialysis against phosphate buffer (10mM, pH 7.4).

After centrifugation, the clear supernatant was applied on DEAE- cellulose anion exchanger column (2cm x 45cm), which was equilibrated with 0.1M phosphate buffer with pH: 7.0. The dialysate enzyme was eluted by buffer in the range of 0.1 – 0.3M NaCl in the same buffer. The fractions of 6ml were collected at flow rate 50ml/hr. The fractions were dialyzed individually against 0.1M phosphate buffer. These elutes was then assayed for azoreductase activity and the amount of protein present in it was estimated by Lowry's method. The molecular weight of the enzyme purified was determined by SDS–PAGE, running the

enzyme along with a marker of known molecular weight.

For characterization, the purified enzyme was studied for the effect of temperature, pH and metal ions on its activity. For the effect of temperature, during the azoreductase assay, the different temperatures (12, 25, 37, 40, 55°C) were maintained during the incubation time. Similarly, for the effect of pH, the buffer used in the azoreductase assay was manipulated to get different pH values: acetate (pH: 4.0, 5.0), phosphate (pH: 6.0, 7.0) and Tris (pH: 8.0, 9.0). In the case of metal ions, the enzyme was incubated with 2mM of metal ions such as Mg^{2+}, Ca^{2+}, Zn^{2+}, Fe^{2+} and Hg^{2+} for 30 min at 37°C followed by the measurement of residual activity under the standard assay conditions.

3. Result and Discussion

Using an enrichment culture technique a dye degrading soil bacteria was isolated and identified as *Pseudomonas aeruginosa* on the basis of various physiological and biochemical tests as described in Bergey's manual of determinative bacteriology (1974). The bacterium was assayed for Methyl Red degradation and decolorization.
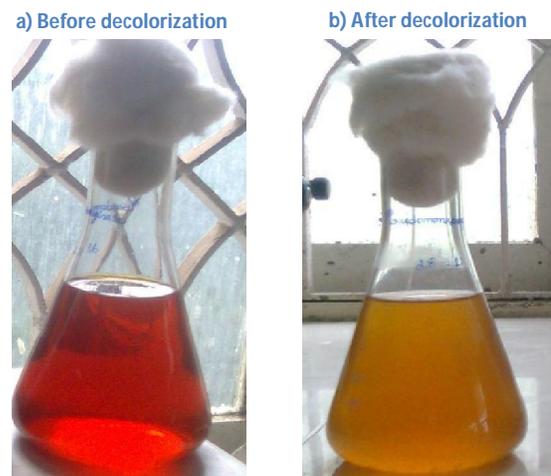

Fig. 1. Decolorizing activity of *Pseudomonas aeruginosa* on mineral salt medium.

Table 1. Determination of Growth and Methyl Dye decolorization.

Time (hrs)	Growth O.D at 430nm	Culture Supernatant O.D at 430nm	% Dye Decolourization
0	0.00	1.00	0%
12	0.203	0.352	65%
24	0.598	0.235	77%
48	0.690	0.162	84%
72	0.701	0.108	90%

Table 2. Purification of Azoreductase from *Pseudomonas aeruginosa*.

Sample	Vol. (ml)	Enzyme Activity (U/ml)	Protein (mg/ml)	Total enzyme activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification Factor (fold)
Crude enzyme	100	0.42	0.30	42	30	1.43	100	1
Ammonium sulphate precipitation	10	1.73	0.43	17.3	4.3	4.02	41.2	2.81
Ion exchange chromatography	6	1.80	0.08	10.8	0.48	22.5	25.7	15.73

The *Pseudomonas aeruginosa* was grown in a mineral salt medium with 100mgL^{-1} of Methyl Red for 72 h, during which the absorbance of Methyl Red decreased significantly, about 65-90 percent removal was observed (Fig. 1 and Table 1). Generally, biological decolorization of the dye solution may take place in two ways: either adsorption on the microbial biomass or biodegradation of the dyes by the cells (Zhou and Zimmermann, 1993). Dye adsorption may be evident from inspection of the bacterial growth; those adsorbing dyes will be deeply colored, whereas those causing degradation will remain colorless. In addition, azo dye decolorization by bacterial species if often initiated by an enzymatic reduction of azo bonds, the presence of oxygen normally inhibits the azo bond reduction activity since aerobic respiration may dominate utilization of NADH; thus impeding the electron transfer from NADH to azo bonds (Chang *et al*., 2011).

Azoreductase assay has been reported that certain azo dyes were decolorized even in the presence of oxygen, showing that an oxygen-insensitive azoreductase was involved in the dye decolorization (Zimmermann *et al*., 1982; Kulla 1981). Crude protein extract obtained from *Pseudomonas aeruginosa* cells was found to decolorize methyl red dye using NADH as electron donor. The specific activity of the azoreductase enzyme was found to be 22.5 U mg^{-1} proteins (Table 2).

The purified enzyme was loaded at 12% SDS polyacrylamide gel and electrophoresis was done. The appearance of single band suggests that the purified azoreductase enzyme, from *Pseudomonas aeruginosa*, in its active form is a monomer and its molecular weight of was estimated to be 29 kDa. The Azoreductase activity of the eluted protein was confirmed again by incubating it with methyl red and NADH.

Changes in the pH resulted in identifying the optimal pH for the activity of the enzyme (Fig. 3). The optimal pH was 7.0, with a higher activity of 1.81 U/mg. Low specific activity was recorded for pH 4.0 (0.65 U/mg), pH 9.0 (0.77 U/mg) and pH 5.0 (0.95 U/mg). Enzymes have been known to operate within the pH of the organism that they are produced in and as a result, the azoreductase purified in this study had an optimal pH that corresponded to the conditions under which the azoreductase were grown. The azoreductase enzymes function optimally in a slightly acidic environment and it can be postulated that the pH dependence of azoreductase reactions is the consequence of the changing degrees of ionization of functional groups in the enzyme as well as the substrate (Zehender, 1988). This is because of the reversible redox reactions carried out by the azoreductase enzymes.
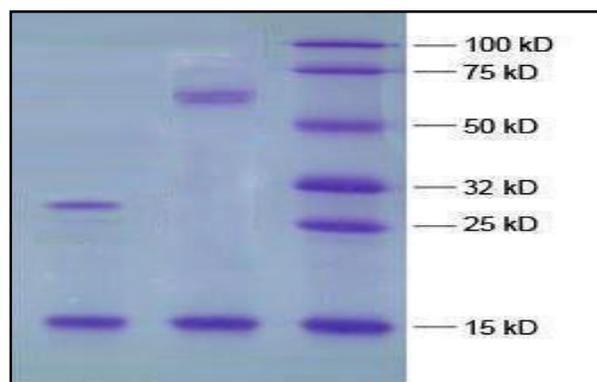

Fig. 2. SDS-PAGE (29 kDa) MONOMER OF AZOREDUCTASE.

The optimum temperature was found to be 37°C with an activity of 1.79U/mg (Fig. 4), which is within the growth temperature of mesophilic bacteria. The temperature curve follows characteristic hyperbolic shapes in which at lower temperatures, the activity is slower, but gradually increases as temperature increases (Fig. 2). This is typical of enzyme kinetics in which rate of enzymatic reaction increases as temperature

increases, this being achieved by an increase in the rate at which enzyme and substrate collide or become in contact with one another (Clark and Switzer, 1977). The downside to this reaction, however, is that at higher temperatures (above 40°C) there is disruption of the quaternary structure of the protein resulting in inactivation and subsequent denaturation of the enzyme.
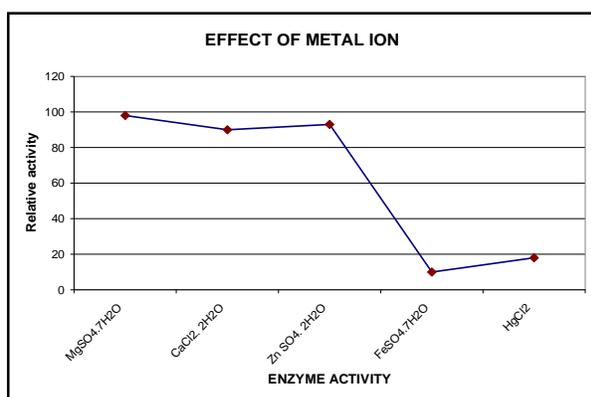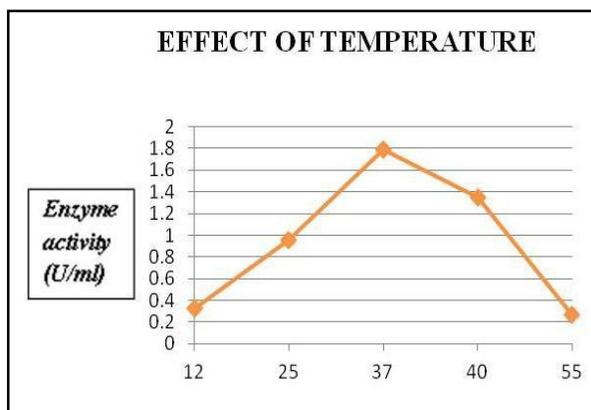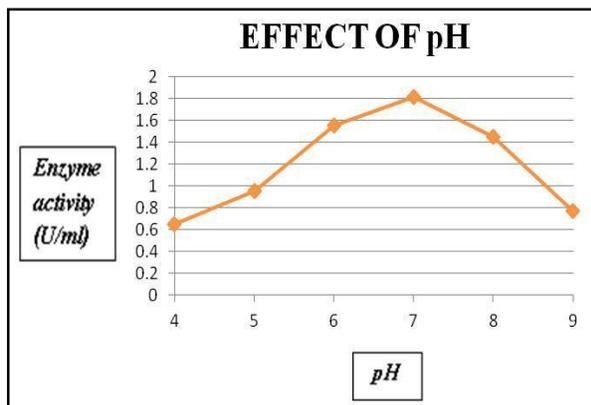
In the case of effect of metal ions, azoreductase activity was almost unaffected by Mg^{2+}, Ca^{2+} and Zn^{2+} and it is inhibited almost completely by Fe^{2+} and appreciably by Hg^{2+} (Fig. 5). The metal ions are general potent inhibitors of enzyme reactions, but they might play a role of cofactors and enhance the activity of

enzymes up to 1mM. This inhibition could be explained in several ways and the following hypothesis can be put forth:

(i)   Excess ions interact with the same amino acid residues in the active center of azoreductase molecules and in this way diminish the binding of a substrate and/or

(ii)   These ions bind to the amino acid residues far from the active center of the enzyme but cause, in the same way, a partial conformational change of the whole azoreductase molecule, altering its catalytic properties which, consequently, results in a decrease of the enzymatic activity (Sharma *et al.*, 2011).

4. Conclusion

The results indicate that *Pseudomonas aeruginosa* isolated from dye contaminated sludge produces an azoreductase enzyme catalyzing the reductive cleavage of the azo bond and initiating the azo dye degradation. This azoreductase differed in several instances from those described previously from other sources (Rashamuse, 2003). The physicochemical properties of the purified azoreductase may open new possibilities for its biotechnological applications and allow the use of *Pseudomonas aeruginosa* in the treatment of azo dyes in industrial effluents. The purification and characterization of azoreductase from *Pseudomonas aeruginosa* contribute to our understanding of azo dye degradation and making it possible for the biotechnological application of treating dye containing industrial wastewater, which will extend the scope of this research work.

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