Oliver H. lowry, (1910-1996), Biochemist who developed the technique for determining the total level of protein in a solution. This method was first proposed in 1951. The total protein concentration is exhibited by a color change of the sample solution in proportion to protein concentration, which can then be measured using colorimetric techniques. His 1951 paper describing the technique is one of the most-highly cited papers ever in the scientific literature, cited over 200,000 times.

Chapter III

PURIFICATION AND CHARACTERIZATION OF AZOREDUCTASE FROM *PSEUDOMONAS OLEOVORANS PAMD_1* BACTERIA

ABSTRACT

An intracellular azoreductase was produced and purified from *Pseudomonas oleovorans PAMD_1* in this chapter. The purification methods including ultrafiltration, ammonium sulfate precipitation and DEAE cellulose-ion exchange chromatography were performed. The enzyme was purified to 9-fold with a recovery of 16 % and specific activity of 26 Umg^{-1}. The enzyme gave a single band on SDS-PAGE with an apparent molecular mass of 29 KDa. The degradation products of the dye produced by the action of azoreductase were analyzed by using HPLC, and FTIR. HPLC method for the determination of dye (Orange II) and its degradation product sulfanilic acid (SA) was carried out with standards and the retention time was found to be 17.49 mins and 2.18 mins respectively. The enzyme had an optimum pH of 7.0 with a maximum activity at 35 °C. The enzyme was almost completely inhibited by Fe^{2+} and considerably by Cu^{2+} and Hg^{2+}. The K_m values of both Orange II and NADH were 0.039 mM and 0.083 mM, respectively.

3.1. INTRODUCTION

In the studies of biological degradation of azo dyes, the effort has been made in order to identify, isolate the purified enzyme responsible for the decolorization. Bacterial degradation of azo dyes is generally feasible only if the azo linkage is first reduced. The reductive cleavage of the azo bond was catalyzed by the azoreductase, the key enzyme of azo dye degradation. Several species of anaerobic bacteria that have azoreductase activity have previously been isolated and studied (Larson, 1976; Zissi, 1996). Generally, azo dyes are resistant to attack by bacteria under aerobic conditions. In contrast, some specialized strains of aerobic bacteria have developed the ability to reduce the azo group by special oxygen-tolerant azoreductases (Kulla, 1981). Therefore the application of enzyme preparations shows considerable benefits over the use of microorganisms. Commercial preparations can be easily standardized, facilitating accurate dosage.

Azoreductases [NAD(P)H: l-(4'-sulfophenylazo)-2-naphthol oxidoreductase] are the enzymes that catalyzes the reductive cleavage of the azo bridge (N=N) in azo dyes to produce colorless amine products (Stolz, 2001). Bacterial azoreductases from different organisms are diverse and variations can exist among the same organism (Ghosh *et al.*, 1992). Classification of azoreductases based on their primary amino acid level is difficult due to low homology. However, a classification scheme based on the secondary and tertiary amino acid analysis has been developed (Abraham, 2007). Based on function, another classification scheme is used in which azoreductases are categorized as either flavin-dependant azoreductases (Chen *et al.*, 2004; Chen *et al.*, 2005; Nakanishi *et al.*, 2001) or flavin-independent azoreductases (Blumel and Stolz, 2003). The flavin-dependent

azoreductases are further organized into three groups; (1) NADH only (Chen *et al.,* 2004; Nakanishi *et al.,* 2001), (2) NADPH only (Chen *et al.,* 2005) or (3) both (Ghosh *et al.,* 1992).

Azoreductase activity has been identified in several bacteria, such as *Xenophilus azovorans KF46* (Blumel *et al.,* 2001), *Pseudomonas luteola* (Hu, 1998), *Rhodococcus* (Heiss *et al.*, 1992), *Shigella dysenteriae Type I* (Ghosh *et al.,* 1992), *Klebsiella pnumoniae RS-13* (Wong and Yuen, 1996) and *Clostridium perfringens* (Rafii *et al.,* 1997). Cloning of genes encoding azoreductase were also carried out from various bacterias, such as *Geobacillus stearothermophilus OY1-2* (Suzuki *et al.,* 2001), *Xenophilus azovorans KF46F* (Blumel *et al.,* 2002) and *Escherichia coli* (Nakanishi *et al.*, 2001).

In the previous chapter, we have standardized various factors influencing the azoreductase production from *P. oleovorans PAMD_1* (Chapter –II). In the present study, we have reported the purification and characterization of a *P. oleovorans PAMD_1* azoreductase and the analysis of the enzymatically decolorized product of Orange II, a commercially important azo dye and widely used in textile and leather industry. To our knowledge, this is the first report of purification and characterization of an azoreductase from *P. oleovorans.*

3.2 MATERIALS AND METHODS

3.2.1 Chemicals and dye

DEAE–cellulose was purchased from Sigma chemicals, USA, Molecular weight marker kit for electrophoretic analysis was obtained from Genei, Bangalore India, NADH, Orange II and other chemicals were purchased from Himedia and SRL, India.

3.2.2 Microorganism, culture media and azoreductase production

The active isolated microbial strain *P. oleovorans PAMD_1* (please refer chapter-I) was used for the production of azoreductase. Azoreductase production was carried out in optimized media (please refer chapter-II). The mineral salt medium used in the degradation study contained (gl^{-1}): K_2HPO_4, 1.73; KH_2PO_4, 0.68; $MgSO_4 \cdot 7H_2O$, 0.1; NaCl, 0.1; $CaCl_2$ 0.1; NH_4NO_3, 1.0; peptone, 16.0; glucose, 1.5 and 5 ml of 0.5 mM NADH. The pH of the medium was adjusted to 7.0. The medium without glucose was sterilized at 121 °C for 20 mins. Glucose was filter sterilized separately and added to the medium. One liter of the production medium was taken in 2 l Erlenmeyer flask containing 200 mg of Orange II inoculated with activated (24 hrs age culture) 2 % inoculum and incubated at 200 rpm in the incubator shaker for 24 hrs at 37 °C.

3.2.3 Crude enzyme preparation

The bacterial cells grown in the culture medium containing dye at a concentration of 200 mgl^{-1} was centrifuged at 10,000 × *g* for 10 mins. The cell pellet was suspended in 100 mM phosphate buffer pH 7.0 and sonicated at 4 °C (30 s, 70 % output, 6×) (Adnane Moutaouakkil *et al.,* 2003). The sonicated solution was centrifuged at 12,000 × *g* for 20 mins and the supernatant was used as a crude enzyme extract.

3.2.4 Azoreductase Assay

The activity of azoreductase was determined based on the procedure described by Zimmermann *et al.*, 1982 with minor modifications. A typical reaction mixture for the standard assay contained in a total volume of 3 ml: 100 mM potassium phosphate pH 7.0, 0.5 mM NADH, 20 μM Orange II and 0.5 ml of enzyme solution.

All solutions were air-saturated. The reaction was started by the addition of NADH after 4 mins of preincubation at 35 °C of a mixture containing all the components except NADH. It was followed in a thermostated cuvette at 35 °C by monitoring the decrease in color intensity at 482 nm. The blank reaction (no protein added) was insignificant. The slope of the initial linear decrease of absorption was used to calculate the azoreductase activity.

One unit (U) of azoreductase is defined as the amount yielding reduction of 1 nmol of Orange II min^{-1} using a molar absorption coefficient of ε=18.2 $mM^{-1}cm^{-1}$.

3.2.5. Decolorization assay

The percentage decolorization of the substrate Orange II was determined at their respective maximum absorption wavelength of 482 nm using a UV-Vis spectrophotometer (Shimadzu UV-2450). The efficiency of color removal was expressed as the percentage ratio of the decolorized dye concentration to that of initial one based on the following equation (Chen *et al.*, 2003).

$$\% \text{ Decolorization} = \frac{\text{Initial absorbance value} - \text{Final absorbance value}}{\text{Initial absorbance value}} \text{ x } 100$$

3.2.6 Protein Estimation

Protein concentration was measured by the method of (Lowry *et al.*, 1951) using BSA as a standard protein.

3.2.7 Purification of Azoreductase

All the purification steps were carried out at 4 °C.

3.2.7.1 Ultrafiltration

The culture supernatant (500 ml) was centrifuged at 21,000 × *g* for 30 mins and filtered through Whatmann No. 1 filter paper to remove fine particles. The filtrate was concentrated by Stirred Ultrafiltration Cell (Sartorius single use filter unit-0.2 µm) through a membrane filter (membrane molecular weight cut off 10 kDa), until a 10-fold concentration was achieved.

3.2.7.2 Ammonium sulphate precipitation

The protein was precipitated from the crude extract by solid $(NH_4)_2SO_4$ in two steps. The first step was the addition of ammonium sulfate up to 40 % (w/v) saturation at 4 °C and centrifugation at 10,000 x *g* for 20 mins. The pellet was then discarded. In the second step, ammonium sulfate was added to the supernatant to a final concentration of 80 % (w/v) saturation at same temperature and the precipitate was collected by centrifugation at 10,000 x *g* for 20 mins and resuspended with 100 mM of phosphate buffer [pH – 7.0] and dialyzed exhaustively against the same buffer for 12 hrs (Valli Nachiyar *et al.*, 2003).

3.2.7.3 Dialysis

The dialysis tube was cut into the required length and placed in 2 % sodium carbonate solution of pH 8.0 and boiled in a hot water bath for 10 mins. The sodium carbonate solution was then decanted. The dialysis bag was rinsed with distilled water 3 times by keeping in boiling water bath for 10 mins. The distilled water was decanted. After the third time, the dialysis bag was boiled in 10 mM EDTA (pH 8.0) in a water bath for 10 mins. The dialysis bag was allowed to cool at room temperature and stored at 4 °C if required water was added to the dialysis bag and checked for

leakage (Sarah *et al.*, 1997). The sample obtained after ammonium sulphate precipitation was poured into the bag and placed in a solution of 100 mM phosphate buffer (7.0) at 4 °C for 12 hrs. The buffer was replaced every 3 hrs.

3.2.7.4 Ion-exchange column chromatography

Ion-exchange chromatography was carried out according to the GeNei teaching kit protocol (Cat No. KT40). The dialyzed enzyme was applied to anion exchange chromotography DEAE- Cellulose column (45 cm × 2 cm) at a flow rate of 6 ml h^{-1} that had been previously equilibrated with 100 mM of phosphate buffer, pH 7.0 and extensively washed with same buffer. After that the enzyme loaded column was washed with 500 ml of the same buffer to remove loosely and unbound sample components. Bound protein was eluted with a linear gradient of 0-1 M NaCl (total volume of 200 ml) in the same buffer. Fractions of 2 ml were collected at the flow rate of 6 $mlhr^{-1}$. Protein containing fractions were assayed for enzyme activity and the active fractions were pooled, dialyzed against same phosphate buffer and preserved.

3.2.8. Molecular weight determination on SDS-PAGE

The SDS-PAGE analysis of purified azoreductase was carried out according to the method of Laemmli, (1970) on 4 % w/v stacking gel and 10 % w/v separating gel. Protein bands were stained with Coomassie brilliant blue R-250. The molecular weight of the enzyme was determined by comparing the electrophoretic mobility of the enzyme with reference (low molecular weight calibration proteins).

3.2.9. Analytical methods

3.2.9.1 UV-Vis Analysis

Degradation pattern of Orange II was analyzed by UV-Vis spectrophotometer (Shimadzu UV-2450). The UV-Vis spectra of dye and azoreductase treated solutions were recorded in the 200-600 nm region. The value of λmax for Orange II and sulfanilic acid (SA) were determined experimentally to be 482 and 248 nm, respectively.

3.2.9.2 HPLC analysis of metabolites

10 ml of the decolorized supernatant was used to analyze the product produced by the action of azoreductase on Orange II. The degradation products were determined by high-performance liquid chromatography (HPLC - Shimadzu). The column used was a 4.6 mm x 25 cm, C-18 reversed phase column with a 5 μm particle size. The sample injected volume was 20 μl. The column was developed at a flow rate of 1.0 $mlmin^{-1}$ with an aqueous solvent system consisting 100 mM phosphate buffer, pH 7.0 over a period of 30 mins. A linear gradient from 20 % methanol (isocratic for the initial 2 mins) increased to 100 % over 10 mins and was then maintained at 100 % concentration for 10 mins with a flow rate of 1.0 $mlmin^{-1}$. The degradation products were monitored at 240 and 485 nm (Alfredo Ruiz-Arias *et al.,* 2010).

3.2.9.3 Fourier transform infrared (FTIR) spectroscopy

Fourier transform infrared spectroscopy analysis was used for investigating the changes in surface functional groups that are involved in decolorization of Orange II azo dye using azoreductase. FTIR analysis was carried out using Perkin-Elmer

Spectrophotometer and changes in % transmission at different wavelengths were observed. The spectra were collected within a scanning range of 4000–400 cm^{-1}.

3.2.10 Characterization of purified Azoreductase

3.2.10.1 Optimization of pH and stability of Azoreductase

The effect of pH on azoreductase activity was determined by incubating the reaction mixture at different pH values ranging from 3 to 9 using different buffer systems (100 mM Citrate buffer for pH 3 to 4.5; 100 mM acetate buffer 5 to 5.5; 100 mM phosphate buffer 6 to 7; 100 mM Tris-HCl buffer 7.5 to 9) at 35 °C. The pH stability was evaluated by incubating the enzyme solution at the above pH for different time period (30 to 240 mins). The residual activity of the enzyme was determined as per the standard assay procedure for the azoreductase activity.

3.2.10.2. Optimization of temperature and stability of Azoreductase

The optimum temperature for enzyme activity was determined by incubating the reaction mixture at different temperatures ranges from 25 to 65 °C with the series increment of 10 °C in 100 mM phosphate buffer, pH 7.0. The thermostability of the enzyme was evaluated after preincubation of the enzyme in the same buffer at different time period (0 to 48 hrs) at various temperatures (25, 35, 45, 55 and 65 °C). The residual activity was determined as per the standard assay procedure for the azoreductase activity.

3.2.10.3. Effect of Salinity

The effect of salinity on purified enzyme activity was determined by incubating the purified enzyme with various concentrations of NaCl (50 to 300 mM). The residual activity was determined as per the standard assay procedure.

3.2.10.4. Effect of Metal ions

Effect of various metal ions on enzyme activity was determined by incubating the purified enzyme with three different concentrations of metal ions (0.5, 2.0 and 10 mM) for 60 mins at room temperature and the residual activity was determined as per the standard assay procedure for the azoreductase activity.

3.2.11. Kinetic studies on Azoreductase

Kinetic values such as Michaelis–Menten constant (K_m) and maximal velocity (V_{max}) for the reduction of the Orange II and for the oxidation of NADH were calculated by varying the concentration of one substrate and keeping the other as constant. K_m and V_{max} were calculated from Lineweaver–Burk double reciprocal plots. The catalytic constant (K_{cat}) for each substrate was determined and the specificity constant (K_{cat}/K_m) was also calculated.

3.3 RESULTS AND DISCUSSION

3.3.1 Purification of Azoreductase

Intracellular azoreductase was purified from *Pseudomonas oleovorans PAMD_1* cell extract by precipitation with $(NH_4)_2SO_4$, followed by dialysis and anion exchange column chromatography on DEAE–cellulose as described in materials and method. The results of purification of azoreductase are summarized in (Table 1).

About 9-fold purification of the enzyme from the initial culture broth was achieved with a recovery of 16 %. Specific activity of the finally purified enzyme was 26 Umg^{-1}. The increase in specific activity may be attributed to the removal of other proteinacious materials as well as low molecular weight substances from the enzyme extract (Aryee *et al.,* 2007). Yan *et al.,* (2004) reported 9.3 % fold purification with a 5.8 % yield for the azoreductase from *E.coli* indicating a higher enzyme concentration in the cell lysate. However, a comparison of activity values from the literature is difficult since most studies are based on the decolorization of different dyes (Table 2).

3.3.2. Molecular weight determination

The SDS-PAGE analysis of the different fractions obtained during the purification showed a progressive enrichment in 29,000 Da protein (Fig 1). The appearance of a single band on SDS-PAGE and a single peak during anion exchange column chromatography suggests that the purified enzyme in its active form is a monomer. Similar values were reported for other microbial azoreductases and all are reported to be monomers (Zimmermann *et al.,* 1984; Mazumdar, *et al.,* 1999).

3.3.3. Spectrophotometer analysis of metabolites

Degradation by-products of Orange II dye were analyzed by considering λmax of the dye and its metabolites. The values of λmax for Orange II and SA (Sulfanilic acid) were determined experimentally to be 482 and 248 nm, respectively. The values of λmax for the metabolites produced by Orange II degradation were taken from literature (Mielgo *et al.,* 2001; Mu *et al.,* 2004). Fig 2a shows the sole peak of absorbance at 482 nm for the dye solution, characterized by nitrogen to nitrogen bond (-N=N-), and the absorption at 482 nm is due to the color of Orange II solution. It is

obvious that the peak at 482 nm disappears completely and no other apparent peaks were detected after the azoreductase treatment in the visible region (Fig 2b), indicating that color removal was practically complete by breakdown in the chromophore group (Herney Ramirez *et al.*, 2005).

3.3.4. HPLC analysis of metabolites

HPLC chromatogram of the extract of the assay mixture without the enzyme exhibits one peak corresponding to Orange II at a retention time of 17.55 mins (Fig 3a). The same retention time was obtained when the Orange II standard was injected. Simultaneously the HPLC chromatogram of the extract of the assay mixture incubated in the presence of the purified enzyme shows the disappearance of the Orange II peak, and the appearance of another peak with retention time 2.18 mins (Fig 3b). The by-product was confirmed as sulfanilic acid (4-aminobenzenesulfonate) on the basis of the retention time of standards and supported by the previous knowledge of orange II degradation.

After Orange II degradation, aromatic amines such as SA: Sulfanilic acid (4-aminobenzenesulfonate) and AN: 1-amino-2-napthol were released. HPLC analysis only detected SA, as AN is a very unstable compound, which was not readily detected (Nortemann *et al.*, 1986; Chamunorwa *et al*, 2008; Davies *et al.*, 2006).

3.3.5 Fourier transform infrared (FTIR) spectra analysis

The FTIR spectra of Orange II and Orange II treated with azoreductase are shown in Fig 4 a & b. The untreated dye shows vibrations located at 1600-1450 cm^{-1} (aromatic C-C stretching), 1613 cm^{-1} (a combination of phenyl ring vibrations with

stretching of the C=N group), 1513 cm^{-1} (N=N bond vibrations or aromatic ring vibrations sensitive to the interaction with the azo-bond, or the bending vibration mode δ-(N–H) of the azo dye), 1401 cm^{-1} (O–H bending vibrations), and 1000–1250 cm^{-1} (S–O stretching and aromatic = C–H bending), representing the characteristic adsorption of Orange II in the infrared band (Stylidi *et al.,* 2004; Lucarelli *et al.,* 2000). In good agreement with the observation from FTIR analysis, the bands at 1613 cm^{-1} and 1513 cm^{-1} provided further support for the coexistence of azo and hydrazone tautomers (Shu-Juan Zhang *et al.,* 2005).

After the azoreductase treatment FTIR spectrum was obviously modified with respect to the aforementioned spectrum of the initial dye. It can be observed that the vibrations between 1723 and 1513 cm^{-1}, which were associated with the azo-chromophore, almost disappeared after treatment of azoreductase, which is in accordance with the results shown in Fig 3b. The strong absorption at 1613 and 1401 cm^{-1} becomes disappeared, which was indicating the degradation of Orange II by azoreductase.

A new absorption peak between 1719 and 1650 cm^{-1} was observed, which may be due to the stretching of C=O in carboxylic groups, aldehydes or ketones (Bauer *et al.,* 2001). The presence of this band indicated the formation of new species, which originated from the fragmentation of the parent Orange II molecule (Stylidi *et al.,* 2004). The strong absorption at 1649 cm^{-1} still remained and other vibrations became very weak or even disappeared. This indicated that the drastic destruction of the aromatic C=N groups (Guoting *et al.,* 2006).

3.3.6 Characterization of Azoreductase

The pH activity profile of purified azoreductase was determined in a wide pH range as described in materials and methods. The purified enzyme showed activity over a broad range of pH (6-8), with optimal activity at pH 7.0 (Fig 5a). These results compare well with azoreductases isolated from *Pseudomonas spp.* (Idaka *et al.*, 1987). This result also supported with azoreductase from *P. aeruginosa* and *Bacillus cereus* which was found to be stable in the pH range of 6-8. (Matsudomi *et al.*, 1977).

However Pseudomonas oleovorans PAMD_1 azoreductase showed a half life of 60 mins at pH 6.0 and at 240 mins it retained around 20 % residual activity (Fig 5b) which indicates its better pH stability than other bacterial species azoreductases. This result resembles a similar enzyme isolated from Bacillus cereus with respect to pH stability (Matsudomi *et al.*, 1977).

Influence of temperature on azoreductase of *P. oleovorans PAMD_1* was shown in Fig 6a. The optimum temperature of enzyme was found to be 35 °C using orange II as substrate, which was quite similar to other azoreductase from *Pseudomonas aeruginosa* (Valli Nachiyar *et al.*, 2003), *Pseudomonas cepacia* (Idaka *et al.*, 1987). Maier *et al.*, (2004) described the azoreductase activity increased linearly in the temperature range of 20 to 45 °C from *Bacillus spp.*, and quite stable at 45 °C, with a half-life of 24 hrs (Fig 6b) and it retained around 30 % residual activity after 40 hrs.

The activity profile of purified azoreductase in various saline concentrations was also determined as described in the materials and methods. The purified enzyme

has capability to retain the activity over a broad range of saline concentration (0 to 150 mM) as shown in Fig 7.

Azoreductase activity appears to be almost unaffected by Mg^{2+}, Mn^{2+}, Ca^{2+} and Zn^{2+} and is inhibited almost completely by Fe^{2+} (Adnane Moutaouakki *et al.*, 2003) and appreciably by Cu^{2+} and Hg^{2+} (Table 3). Similar observations are reported for the azoreductases from *Bacillus cereus* (Matsudomi *et al.*, 1977) and *P.aeruginosa* (Valli Nachiyar *et al.*, 2003). Similarly, azoreductase from *Bacillus strain SF* and *Bacillus cereus* was inhibited by CN^- and by SDS (Matsudomi *et al.*, 1977).

Enzymatic reactions were performed by varying the concentration of one substrate (Orange II or NADH) and fixing the other substrate concentration. The K_m values of both Orange II and NADH were 0.039 mM and 0.083 mM, respectively (Table 4). NADH was found to play an important role in dye degradation as a source of electron donor (Yoo *et al.*, 2001).

3.4 CONCLUSION

In conclusion, we have shown that an azoreductase from *Pseudomonas oleovorans PAMD_1* is responsible for azo dye reduction. Because of the well defined purification procedure and high yield; the azoreductase can be used for the treatment of textile dyeing effluent and other bioremediation processes.

Table 1: Purification profile of Azoreductase from *Pseudomonas oleovorans PAMD_1*

Purification steps	Volume (ml)	Total Protein (mg)	Total activity (U)	Specific activity (Umg^{-1})	Fold Purification	Yield (%)
Crude enzyme	500	987.5	2884	2.92	1	100
Ultrafiltration	50	262.5	2142	8.16	2.79	74.27
Ammonium sulphate precipitation (80 %)	20	209	2060	9.85	3.37	71.43
DEAE-Cellulose	8	18	468	26.00	9.00	16.23

Table 2: Comparative profile of purified azoreductase from various bacterial sources

Azoreductase source	Substrate	Specific activity Umg^{-1}	References
Pseudomonas aeruginosa	Navitan fast blue	22.74	Vallinachyar *et al.*, 2005
Rhodobacter sphaeroides	Methyl red	8.5	Yan Bin *et al.*, 2004
Xenophilus azovorans KF46F	Orange II	10.8	Blumel *et al.*, 2002
Staphylococcus aureus	Orange G	0.14	Chen *et al.*, 2005

Table 3: Effect of metal ions on *Pseudomonas oleovorans PAMD_1* azoreductase

Metal salts	Concentration range (mM)	Activity (% decolorization)	% Activation	% Inhibition
Control	-	72±0.04	-	-
$MgSO_4 \cdot 7H_2O$	0.2	74±0.03	2.7±0.04	-
	0.5	81±0.01	11±0.01	-
	10.0	72±0.01	-	-
$ZnSO_4 \cdot 2H_2O$	0.2	72±0.02	-	-
	0.5	78±0.03	7.6±0.01	-
	10.0	74±0.05	2.7±0.02	-
$CaCl_2 \cdot 2H_2O$	0.2	72±0.07	-	-
	0.5	73±0.09	1.4±0.04	-
	10.0	73±0.02	1.4±0.05	-
$FeSO_4 \cdot 7H_2O$	0.2	54±0.02	-	25±0.03
	0.5	48±0.08	-	33.3±0.06
	10.0	50±0.03	-	30.5±0.05
$CuSO_4 \cdot 5H_2O$	0.2	65±0.01	-	9.7±0.05
	0.5	62±0.01	-	13.8±0.05
	10.0	60±0.07	-	16.6±0.04
$MnSO_4 \cdot 4H_2O$	0.2	61±0.06	-	15.3±0.01
	0.5	65±0.02	-	9.7±0.08
	10.0	62±0.01	-	13.8±0.09
$HgCl_2$	0.2	70±0.02	-	2.7±0.05
	0.5	69±0.06	-	4.2±0.04
	10.0	71±0.04	-	1.4±0.03
$CdCl_2$	0.2	65±0.04	-	9.7±0.01
	0.5	64±0.07	-	11.1±0.02
	10.0	62±0.05	-	13.8±0.03
EDTA	0.2	69±0.02	-	4.2±0.03
	0.5	67±0.05	-	6.9±0.04
	10.0	62±0.05	-	13.8±0.04

Table 4: Kinetic parameters for *Pseudomonas oleovorans PAMD_1* azoreductase

Substrate	**Wave length** *(nm)*	**Mol. Ext. Coefficient (ε)** $M^{-1}\ cm^{-1}$	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m
Orange II	482	18,200	0.039	3.7	94.87
NADH	340	6,220	0.083	6.3	75.90

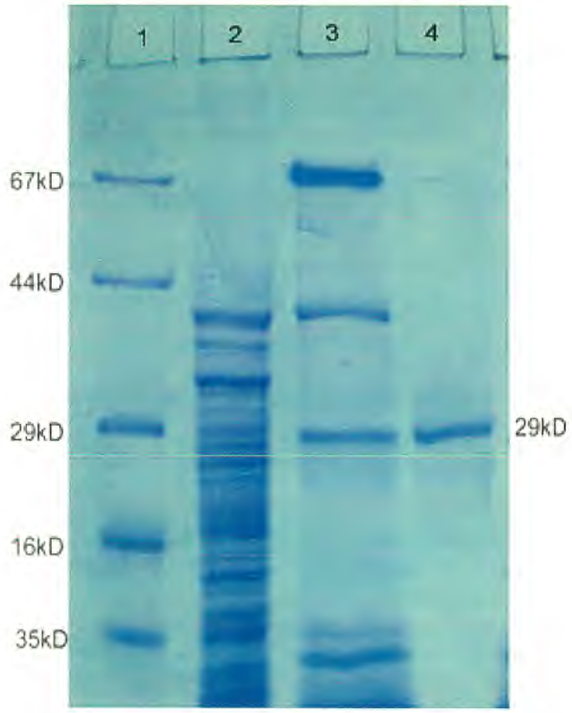


Figure 1: Molecular weight determination of purified azoreductase of *Pseudomonas oleovorans PAMD_1 on* SDS-PAGE. Lane 1- Protein marker; Lane 2 –Crude filtrate; Lane 3- Ammonium sulphate precipitation, Lane 4 – Purified Azoreductase by ion exchange chromatography.

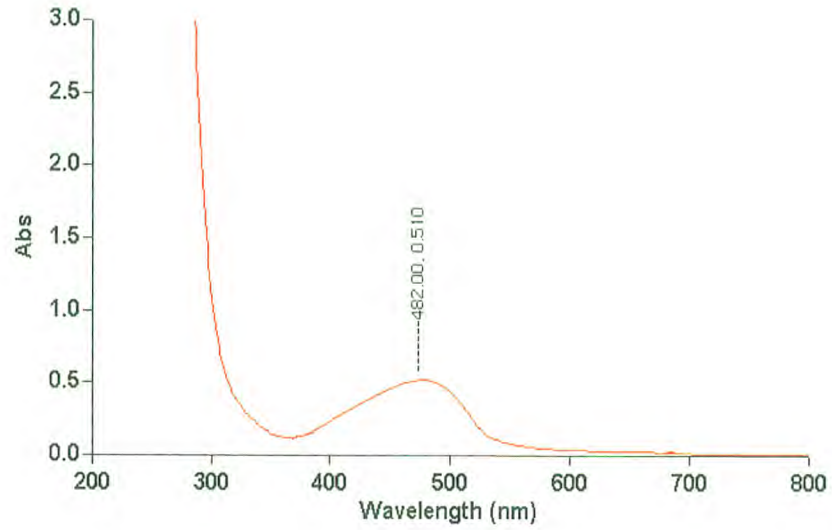


Figure 2(a)

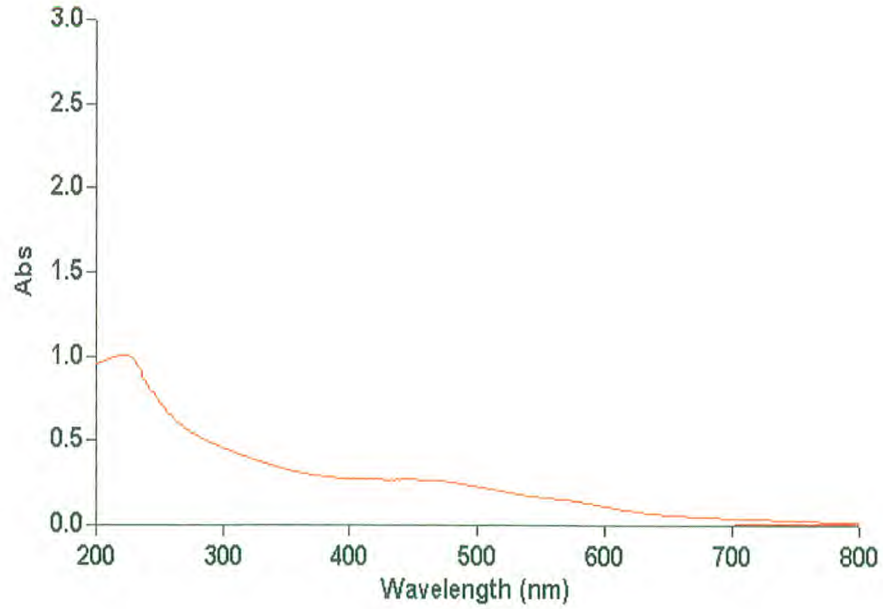
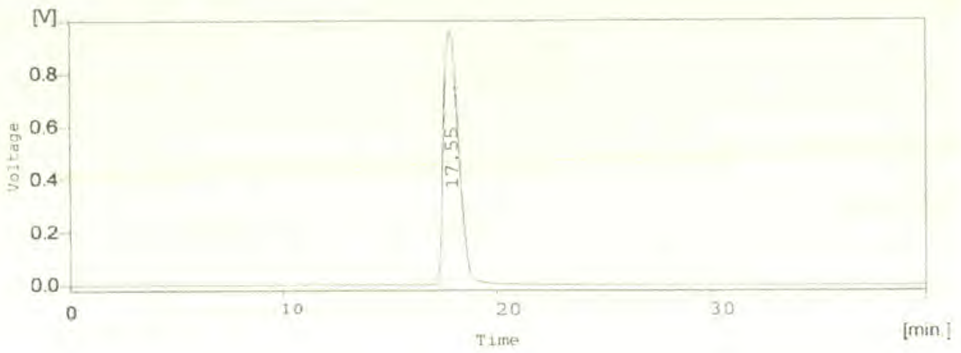


Figure 2(b)

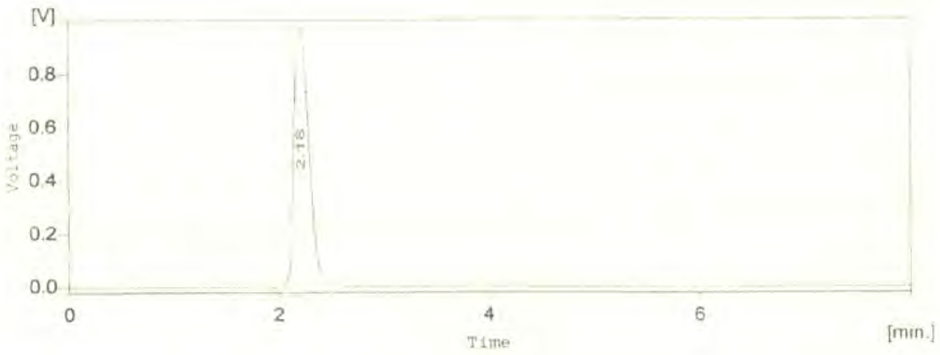
Figure 2: UV-vis absorption spectra of Orange II before (a) and after (b) enzymatic degradation by azoreductase from *Pseudomonas oleovorans PAMD_1.*



Result Table (Uncal - E:\MTSM\Data\DYE-001)

	Reten. Time [min]	Area [mV.s]	Area [%]
1	17.55	9493.858	100.0
	Total	9493.858	100.0

Figure 3(a):



Result Table (Uncal - E:\MTSM\Data\DYE-001)

	Reten. Time [min]	Area [mV.s]	Area [%]
1	2.18	9598.858	100.0
	Total	9598.858	100.0

Figure 3(b):

Figure 3: HPLC chromatogram of the Orange II azo dye (a) without (b) with Azoreductase from *Pseudomonas oleovorans PAMD_1.*

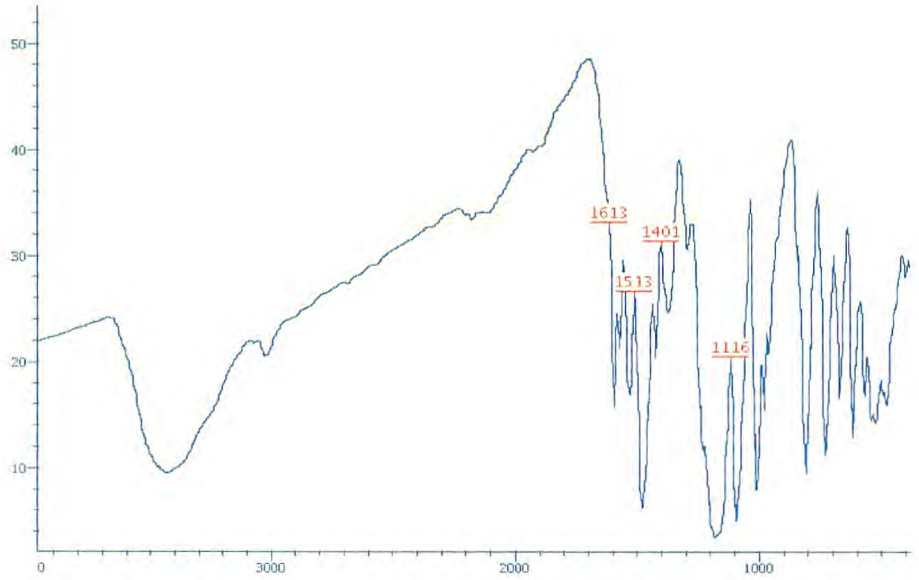


Figure 4(a):

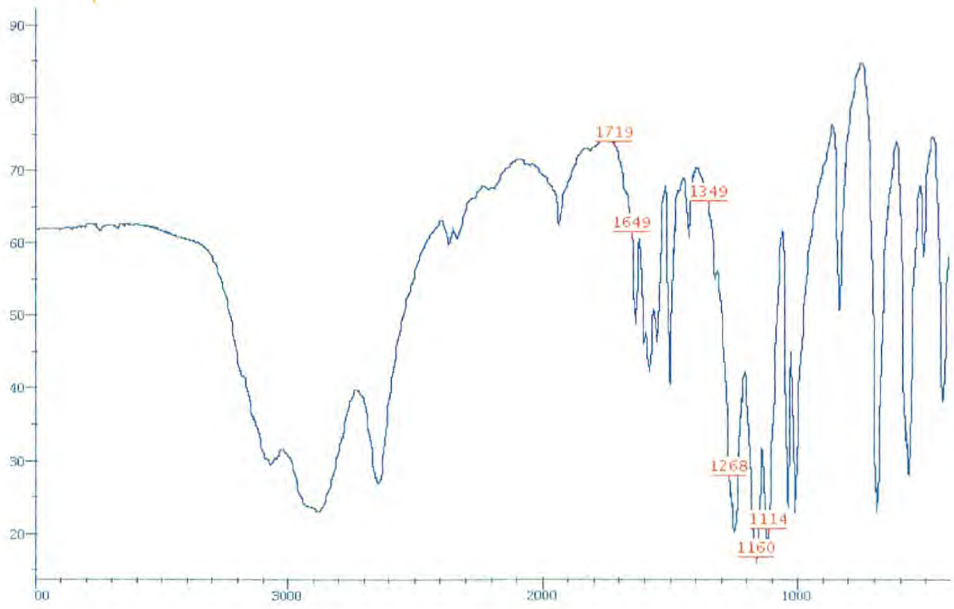


Figure 4(b):

Figure 4: FTIR spectra obtained from the (a) Orange II azo dye (b) Dye with azoreductase from *Pseudomonas oleovorans PAMD_1.*

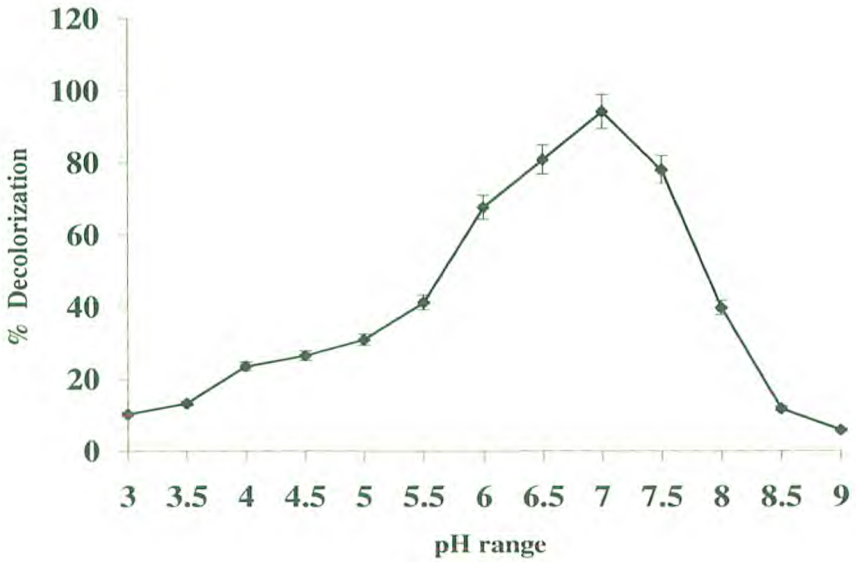


Figure 5 (a): Optimum pH of *Pseudomonas oleovorans PAMD_1* azoreductase

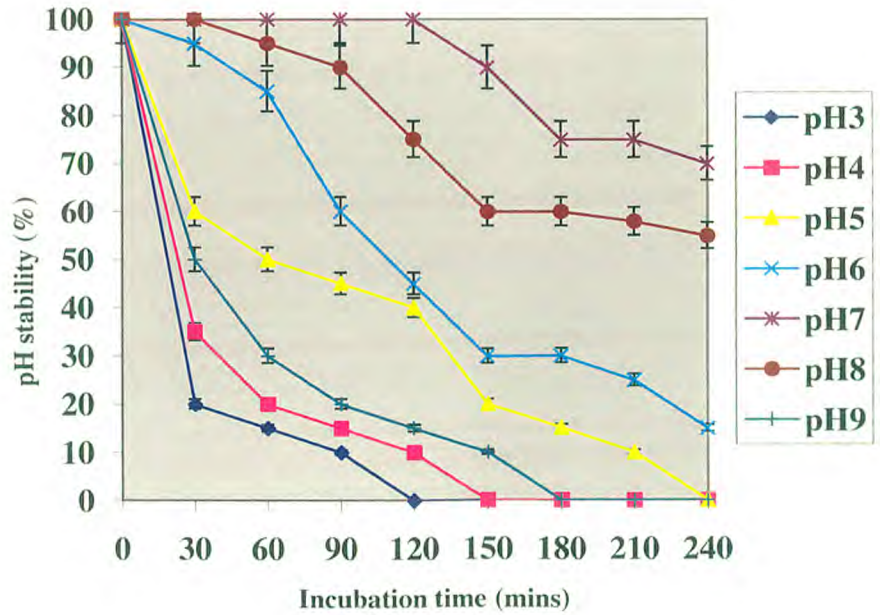


Figure 5 (b): pH stability of *Pseudomonas oleovorans PAMD_1* azoreductase

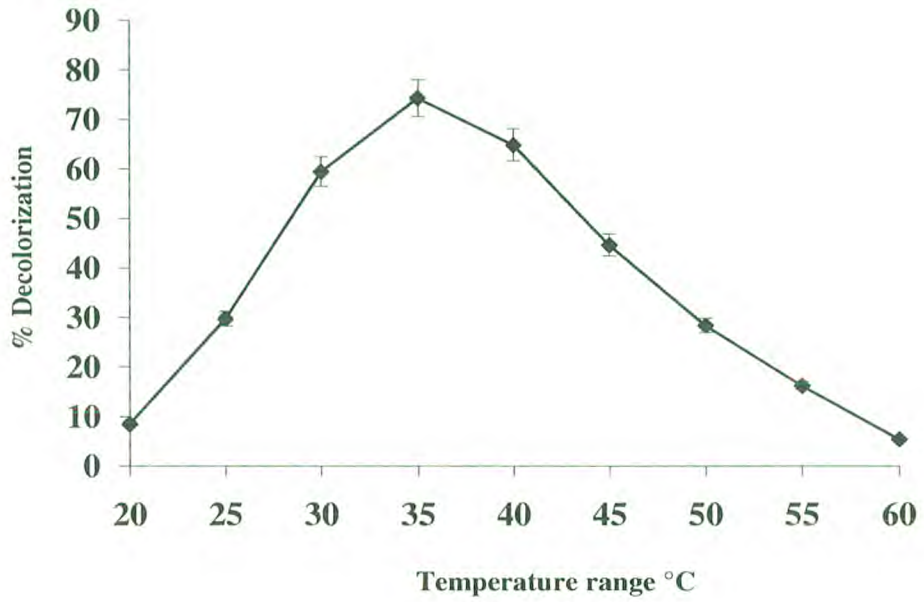


Figure 6 (a): Optimum temperature of *Pseudomonas oleovorans PAMD_1* Azoreductase

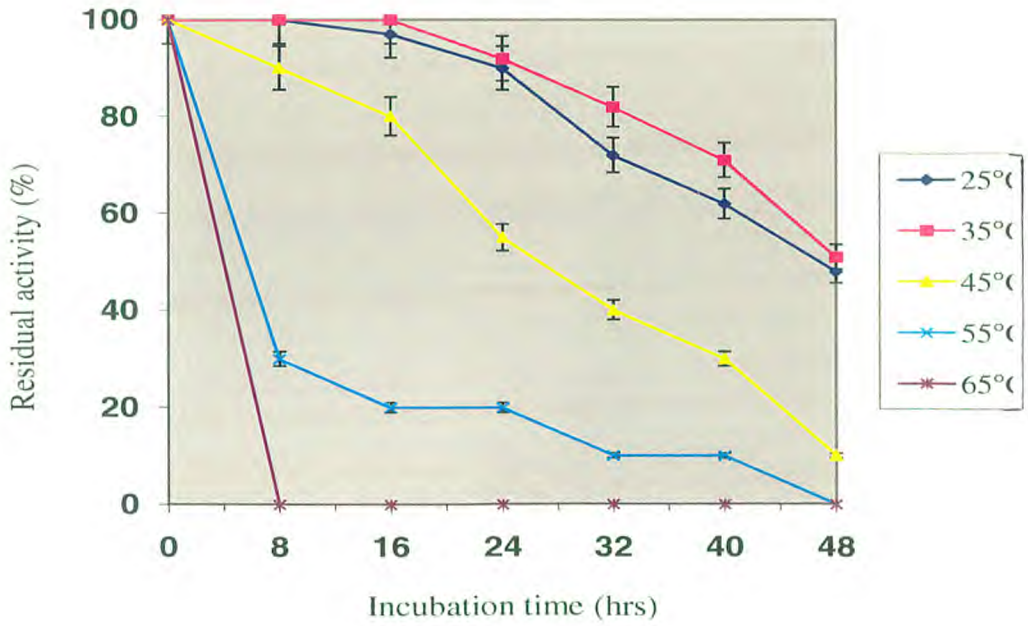


Figure 6 (b): Temperature stability of *Pseudomonas oleovorans PAMD_1* azoreductase

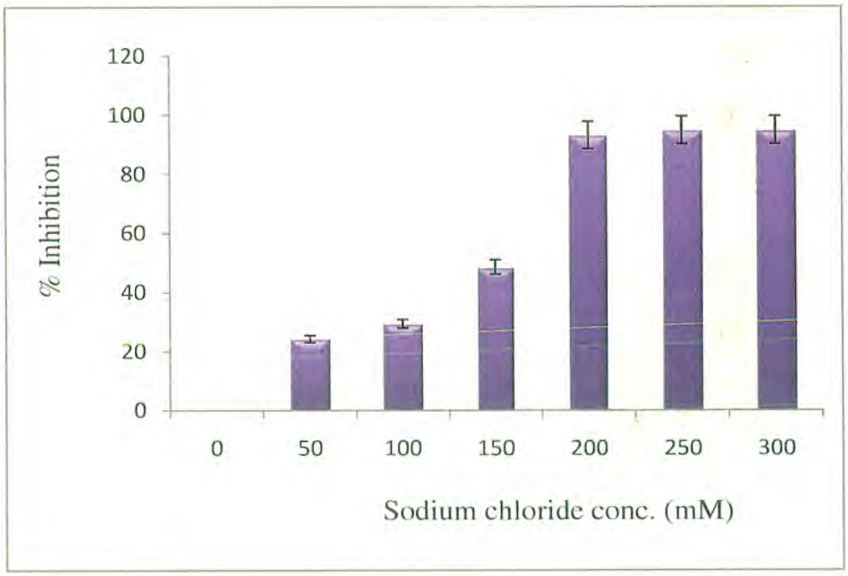


Figure 7: Effect of salinity on *Pseudomonas oleovorans PAMD_1* azoreductase