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Partial Purification and Assay of Azoreductase and Laccase in the Degradation of Reactive Black 5 Dye from *Aeromonas Punctata*

Roopa K. B. and Usha M. S.*
Department of Microbiology, Centre for P.G. Studies, 18/3, 9th Main, 3rd Block, Jayanagar, Jain University, Bangaluru-11

Abstract: A strain of *Aeromonas punctata* isolated from textile industrial effluent was used to carry out decolourisation of Reactive Black 5 dye under aerobic condition by enzymatic activity. Both the crude extract and cell free supernatant of *Aeromonas punctata* was used to determine the enzymatic activity of Azoreductase and Laccase. The cell free supernatant exhibited higher enzymatic activity than the crude extract for the decolourisation of Reactive Black 5 dye. The proteins from the cell free supernatant *Aeromonas punctata* were subjected to partial purification by ammonium sulphate precipitation, dialysis and column chromatography. The molecular mass of purified enzyme determined by SDS-PAGE was found to be 65 kDa and 45 kDa for azoreductase and laccase respectively.
Key words: *Aeromonas punctata*, Azoreductase, Laccase, and Reactive Black 5 dye degradation.

1. Introduction

Synthetic dyes are organic dyes, originally derived from coal-tar derivatives but currently synthesized from benzene and its derivatives. These are widely used in textile dyeing, paper printing, cosmetics and pharmaceuticals [1]. Among the various classes of dyes, reactive dyes are more difficult to remove because of the presence of azo, Nitro and sulpho groups. Azo reactive dyes are the largest class of water soluble synthetic dyes with the greatest variety of colors and structure, making them highly resistant to microbial attack [2, 3].

The reactive azo dyes- containing effluents from these industries have affected serious environment pollution because of the presence of dyes in water is highly visible and effects their transparency and even if the concentration of the dyes is low [4]. Furthermore reactive dyes pose serious health threats to human due to their carcinogenicity and lead to mutagenic and exerts toxic effects [5, 6].

Several reports have clearly demonstrated that azo dyes could be biologically transformed by their extracellular enzyme system such as, laccase [7]. Recently some reports disclosed that azo dyes could also be decolorized by azoreductase under anaerobic conditions to form aromatic amines, which were further biodegraded aerobically via hydroxylation and ring-opening [8,9,10,11,12]. In this present investigation the ability of enzymes to degrade Reactive Black 5 dye from bacterial strain *Aeromonas punctata* was analyzed.

2. Materials and Methods

2.1 Dye and other chemicals: Reactive Black 5 dye and other chemicals were procured from Hi-media Pvt. Ltd., Mumbai, India.

2.1.2 Culture: Enzymatic assay and partial purification of enzymes was carried out using *Aeromonas punctata* with the accession number JN561149. 24 hours old culture of *Aeromonas punctata* was used for the enzymatic assay of enzymes. The culture was subjected to centrifugation at 10,000 rpm for 10 mins and the cell free supernatant was selected to carry out enzymatic assay.

2.1.3 Enzyme Assay: The cell free supernatant was assayed for the presence of enzymes, azoreductase and laccase capable of degrading Reactive Black 5 dye.

2.2 Azoreductase enzyme Assay

Azoreductase activity was assayed by measuring the decrease in optical density at suitable wavelengths of 430 nm [13]. The standard reaction mixture containing 25 mM Tris-HCI buffer (pH 7.4), 25 µM Methyl Red, 100 µM NADH, and suitable amount of enzyme in 3 ml of reaction mixture, was incubated at 30°C. Reaction mixture without Methyl Red was preincubated for 3 min and Methyl Red decolorization was followed by monitoring initial rate of the decrease in absorbance at 430 nm. One unit of enzyme activity was defined as the amount of enzyme required to decolorize 1 µmol of dye per min under the assay conditions. Protein concentration was measured by the Bradford method, using a bovine serum albumin as the standard.

2.2.1 Partial Purification of azoreductase enzyme

The enzyme purification was performed as follows: The crude extract was subjected to ammonium sulphate precipitation at 80% saturation; this was carried out by adding 50.51g of ammonium sulphate to 90 ml of crude extract. The precipitated enzyme was collected by centrifugation at 10,000g for 10 min, and the pellet

was dissolved in 10 ml of phosphate buffer (50 mM, pH 7.0). The solution was then desalted by dialysis against phosphate buffer (50 mM, pH 7.0) overnight [14]. The final step of purification was performed by column chromatography according to Maier *et al.*, [15].

2.3 Laccase Enzyme Assay

Laccase activity was determined by the oxidation of 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) ABTS method [16]. The nonphenolic dye ABTS is oxidized by laccase to the more stable and preferred state of the cation radical. The concentration of the cation radical responsible for the intense blue-green color can be correlated to enzyme activity and is read at 420nm. The assay mixture contained 0.5mM ABTS, 0.1M Sodiumacetate (pH 4.5), and a suitable amount of enzyme. Oxidation of ABTS was monitored by determining the increase in A420 (ε420, 3.6×104 $M^{-1}cm^{-1}$). The reaction mixture contained 0.5 mM substrate (ABTS), 2.8mL of 0.1M sodium acetate buffer of pH 4.5, and 100 µL of culture supernatant and incubated for 5min. Absorbance was read at 420nm in a spectrophotometer against a suitable blank. One unit was defined as the amount of the laccase that oxidized 1 µmol of ABTS substrate per min. Protein concentration was determined by the dye-binding method of Bradford using BSA as standard.

2.3.1Partial Purification of laccase enzyme

To 100 ml of culture filtrate, ammonium sulphate was added to 80% saturation with slow stirring on ice for 2h. The contents were centrifuged at 4°C and the pellets were dissolved in 10mM sodium acetate buffer pH 5.5, dialyzed against the same buffer at 4°C overnight [17]. The final step of purification was performed by column chromatography according to Maier *et al.*,[[15].

2.4 SDS-PAGE

The purified enzyme was loaded onto 12% SDS poly acrylamide gel along with the markers and electrophoresis was done. Protein bands were stained with Coomassie brilliant blue R-250.

3. Result and Discussion

Following ammonium sulphate precipitation, dialysis and column chromatography 8.54 fold purification of azoreductase from the cell free supernatant was obtained.

Table 1 Partial Purification of azoreductase from *Aeromonas* punctata

Purification Step	Protein (mg/ml)	Enzyme Activity (EU/ml)	Specific Activity(EU/mg)	Yield (%)	Fold Purification
Cell free supernatant	38	71.7	1.88	100	1
$(NH_4)_2SO_4$ Precipitation	23	48.2	2.09	67.2	1.11
Dialysis	12	39.6	3.3	55.69	1.75
Column Chromatgraphy	1.63	8.03	5.21	30.02	8.54

During each purification step, specific activity and fold purification increased (Table 1), with an increase yield of 30.02%. Specific activity and fold purification were 5.21 EU/mg and 8.54 respectively.

Table 2 Partial Purification of laccase from *Aeromonas* punctata

Purification Step	Protein (mg/ml)	Enzyme Activity (EU/ml)	Specific Activity(EU/mg)	Yield (%)	Fold Purification
Cell free supernatant	38	300	7.89	100	1
$(NH_4)_2SO_4$ Precipitation	23	217	9.43	72.4	1.19
Dialysis	12	165	13.75	55	1.74
Column Chromatography	1.42	64	6.78	28.24	7.89

During each purification step, specific activity and purification was increased (Table 2), with an increased yield of 28.24%. Specific activity and fold purification were 6.78 EU/mg and 7.89 respectively for laccase.

Protein separation by SDS-PAGE resulted in a band equ.ivalent to molecular mass of 65 kDa approximately for azo reductase and 45 kDa for laccase respectively as shown in figure 1.

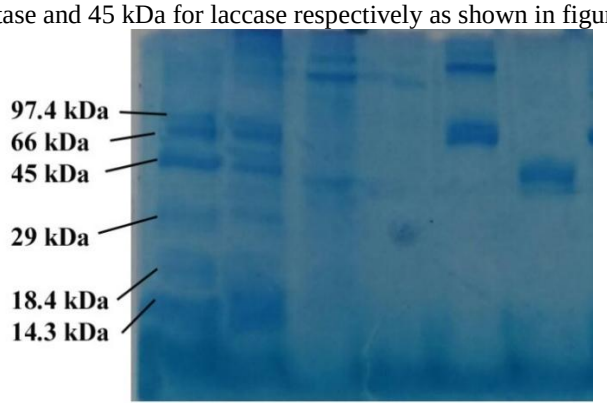

Fig 1: Molecular weight determination of azoreductase and laccase by SDS-PAGE
Lane 1: Molecular Marker, Lane 2: Cell free supernatant, Lane 3: Dialysed sample, Lane 5: Purified sample from column chromatography for azoreductase, Lane 6: Purified sample from column chromatography for laccase

In the present study enzymatic degradation of Reactive black 5 dye from *Aeromonas punctata* was studied using their extracellular enzyme system. The organism has the capability to produce oxidative enzymes capable of decolorizing and degrading Reactive Black 5 dye. Further the proteins were subjected to partial purification by ammonium sulphate precipitation, dialysis and column chromatography the specific activity of partially purified azoreductase and laccase was found to be 5.21 EU/mg, and 6.78 EU/mg, respectively with a fold purification of 8.54 and 7.89 respectively. Following, partially purified proteins were subjected to SDS-PAGE which exhibits a molecular weight of 65 kDa and 45 kDa for azoreductase and laccase respectively.

Nachiyar *et al.,* [18] reported a specific activity of 23 U for Azoreductase purified from *Pseudomonas aeruginosa*. However a comparison of activity values from the literature is difficult since most studies are based on decolourization of different azo dyes. According to Vijaya *et al.,* [19] the specific activity of azoreductase enzyme isolated from *Pseudomonas aeruginosa* was found to be 22.5 U mg^{-1} proteins. Pushkaraj V. P and Das G.D [20] reported that the laccase activity was found to be 16.88 U/ml from newly isolated strain. *Lenzites Sp.* According to Suzuki *et al.,* [12] soil bacteria isolated from azo dye-contaminated wastewater as a skin bacterium, *Staphylococcous aureus* presumably does not produce highly active azoreductase.

4. Conclusion

The present study depicted that the *Aeromonas punctata* isolated from textile industrial effluent was found to have ability to produce laccase and azoreductase capable of degrading Reactive Black 5 dye, exhibited the maximum amount of enzyme activity in the dialysis sample, catalysing the reductive cleavage of azo bond and initiating the azo dye degradation. Hence the assay and partially purified enzymes from *Aeromonas punctata* contribute to our understanding of azo dye degradation and making it possible for the biotechnological application of treating dye containing industrial wastewater.

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