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EXTRACTION AND PARTIAL PURIFICATION OF THE TYROSINASE FROM *BREVUNDIMONAS DIMINUTA PV24* ISOLATED FROM AHMEDNAGAR REGION

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Abstract–Tyrosinase is a type of oxidoreductase enzyme which is found ubiquitously in all life form. There are various industrial applications of the tyrosinase enzyme such as production of melanin, L-Dihydroxyphenyl alanine(DOPA), cross linking agent, environmental cleanup. Due to structural complexity eukaryotic tyrosinases are unable to extract and studied. Bacterial tyrosinases are monomeric and can easily be purified. In the current study, the tyrosinase from the *Brevundimonas diminuta PV 24* was partially purified by salting out, dialysis and size exclusion chromatographic technique. Enzyme was fractionated by 50% to 90% Ammonium sulphate saturation. The precipitate was dialyzed against 100 mM potassium phosphate buffer to remove the salts. Biogel P60 was used as gel matrix. A total of 32 elutions were collected and the enzyme was found to be in 6 to 12 elusions. The techniques enhanced the final purification up to 55 folds with increased activity from 45.03 U/ml to 149 U/ml. The stability of the enzyme was also determined and it concluded that the enzyme is stable at pH 7 and temperature 37 ^{0}C.

INTRODUCTION

Tyrosinase is a kind of Cu-containing oxidoreductase that has two different functions for phenolic substrates: it may oxidize diphenols to quinones (a catecholase activity) or ortho-hydroxylate monophenols to diphenols (a cresolase activity). Tyrosinase activity is found broadly throughout all living forms, including microbes and animals. After being isolated, tyrosinases' characteristics and roles were thoroughly investigated. They are primarily involved in the manufacture of melanins and other polyphenolic substances and can be found in entire cells as well as tissues from fruits, vegetables, and mushrooms. The most well studied bacterial enzymes are streptomyces tyrosinases (della-Cioppa *et al*., 1998). Bacterial tyrosinase is an extracellular enzyme that plays a role in the synthesis of melanin. Other species including *Bacillus thuringiensis, Pseudomonas putida, Marinomonas mediterranea, Rhizobium, Symbiobacterium thermophilum, Pseudomonas maltophilia, Pseudomonas putida, Streptomyces castaneo globisporus, Ralstonia solanacearum*, and *Verrucomicrobium spinosum* have also been reported to contain the enzyme (Liu *et al*., 2005; Claus and Decker, 2006; McMahon *et al*., 2007).

Tyrosinase is used as a potential prodrug for treating melanoma where patients were successfully treated via tyrosinase activity (Morrison *et al*., 1985; Jordan *et al*., 1999). L-Dihydroxy phenylalanine is a naturally occurring nutritional supplement and psychoactive substance found in some kinds of food and herbs (e.g., Mucuna pruriens or velvet bean) and is generated from the amino acid L-tyrosine in the mammalian body and brain (Ali and Qadeer, 2002). The essential enzyme for the manufacture of melanin is known to be tyrosinase. The most significant pigment, melanin, is produced by a physiological process known as melanogenesis in membrane-bound subcellular organelles known as melanosomes, which are always present in black; melanocytes are the skin's dendritic cells, and the activity of these cells primarily determines the color of the skin and hair. During development, melanocytes are generated in the neural crest and go

to the hair matrices and the basal layer of the epidermis. Through their ability to absorb UV rays from sunlight and eliminate reactive oxygen species, they are essential in shielding the skin from harmful UV light (Kim and Uyama, 2005; Summers, 2006).

In order to acquire the most specific activity with the best recovery of the initial activity, purification aims to isolate particular enzymes from a crude extract of cells that contains many other undesirable components. The stability, activity, and selectivity of the enzyme can all be impacted by contaminants in the enzyme solution, which can therefore have an impact on the quality and yield of the finished product. Enzymes that have been purified have increased stability, activity, and specificity, rendering them more productive and useful in industrial operations. (Dalfard *et al.,* 2006).

Tyrosinase from Symbiobacterium thermophilum was isolated by Suzuki *et al.* via the S300 gel. (Suzuki *et al*., 1992). An affinity chromatography Nickel(II) column was used to purify a tyrosinase-producing gene that was cloned from *Bacillus megaterium* in *E. coli*. (Shuster and Fishman, 2009). It was also noted that gel filtration (Sephadex 100) was used to purify fungal tyrosinase (Zaidi *et al*., 2014). A recent study described the use of cellulose-coated magnetic nanoparticles in the purification of *Pseudomonas* Tyrosinase (El-Aziz *et al*., 2023).

In our investigation, we have extracted and partially purified the enzyme Tyrosinase from the newly isolated bacterial strain PV 24 from Ahmednagar region.

MATERIALS AND METHOD

Materials

All the including The Dialysis bag, Biogel P 60, were purchased from Himedia (India) and the tyrosinase producing bacterial strain *PV 24* was isolated from the Kapurwadi mud sample, Dist Ahmednagar (Now Ahilyanagar). The sample was serially diluted and streaked on the Sterile Tyrosinase agar (supplemented with 0.1% L-Tyrosine).

Production of the enzyme

Tyrosinase was produced in a 250 ml Erlenmeyer flask containing 100 ml of the baseline medium, which included 0.1% tyrosine, 0.5% NaCl, 0.15% peptone, and 0.15% yeast extract. The shaker incubator was used for five days of production, with a temperature of 37 ^{0}C and a rotation speed of 150 rpm.

Enzyme Assay

L-tyrosine and L-DOPA are used as substrates in the measurement of tyrosinase activity. Prior to measuring the enzyme activity, the proper concentration of the enzyme was established. An aliquot of the enzyme solution is then added to a 0.1M potassium phosphate buffer (pH 6.8) that contains 1mM L-tyrosine and L-DOPA. The formation of dopachrome is then tracked by measuring the absorbance at 475 nm. Dopachrome, also known as the "colored intermediate," is a byproduct of nonenzymatic oxidation of o-quinones that is used in the synthesis of melanin. For the product dopachrome, the extinction coefficient ([ˆ) is 3600 L/mol.cm. Tyrosinase activity is calculated using the initial rate. The amount of enzyme needed to oxidise 1 ìmol of L-tyrosine to dopachrome per minute at the specified conditions is known as one international unit (IU) of tyrosinase activity. This value was determined using the molar extinction coefficient of dopachrome (3600 M^{-1} cm^{-1}). (Lerner and Fitzpatrick, 1950; Valýpour and Arýkan, 2015). Protein concentration was determined by Folin Lowry method (Lowry, 1951).

Ammonium sulfate precipitation

After the fermentation the production medium was subjected to the centrifugation at 10,000 rpm for 10 minutes. Supernatant was collected in the separate vessel and it was subjected to ammonium sulphate precipitation. (Duong-Ly K C and Gabelli S B 2014).Two fractions of the ammonium were collected 0 to 50% and 50 to 90%. Amount of the ammonium sulphate was calculated and measured. Ammonium sulphate was added at cold condition during stirring with the help of the magnetic stirrer and kept overnight for precipitation of protein (Green and Hughes, 1955).

Membrane Dialysis

Precipitate was collected and resuspended in the 50 mM Potassium phosphate buffer (pH 6.5). The suspension was subjected to membrane dialysis with membrane dialysis tube (membrane-50, Himedia) against 5mM Potassium phosphate buffer. Enzyme activity and specific activity was determined (McPhie, 1971).

Size exclusion chromatography

1 ml of dialyzed fraction was added to the chromatography column which consists of Biogel

P60™ matrix. The column was prepared with 50mM Potassium buffer solution having pH 6.8. In all 24 Elutions were collected and protein concentration was determined (Duong-Ly Gabelli, 2014)

Stability of enzyme

The purified enzyme was evaluated for the stability at various pH and temperature. The pH at which the enzyme was evaluated were 3, 5, 7, 9, 11. For the pH range 3 to 5 citrate buffer was used. And for pH 7 potassium phosphate buffer was used. For pH 9 and 11 Tris buffer was used. The temperatures were 15, 25, 35, 45, 55, 65.

RESULTS AND DISCUSSION

Ammonium sulfate precipitation

Ammonium sulphate precipitation was a crucial purification step that partially purified the enzyme. After two salt precipitation fractions were separated, the fraction containing 50 to 90% ammonium sulphate had the highest activity, with an enzyme activity of 67.16 U/ml and 2.93-fold purification. (Table 1).

Reports suggested that the tyrosinase enzyme from the mushroom could be fractionated in between the range of 30% to 80% (Mueller *et al*., 1996; Lee *et al*., 1997). Whereas bacterial tyrosinases were also fractionated in the range of 40% to 80% of the Ammonium sulphate saturation. (Nambudiri *et al*., 1972; Dalfard *et al*., 2006; Zaidi *et al*., 2014).

Dialysis

The dialysis was performed in order to extract salt ions from the protein precipitate. With its molecular weight reduced to 12 to 14 kDa, membrane 50 (Himedia) has the ability to exclude salts from the enzyme preparation process. Following dialysis, the activity was 98.16 U/ml and the purification fold was 7.24.

Size Exclusion Chromatography

In comparison to other fractions, elution numbers 7, 8, 9, 10, and 11 displayed the highest protein content following the dialysed fraction's passage through the chromatography column. Following elution collection, lyophilization was used to concentrate the fractions exhibiting a positive enzyme reaction for additional research. Following chromatography, the fold purification was 55.04, and the enzyme activity was 149 U/ml. Apart from the biogel many other chromatographic columns like Hydroxy apatite column (Bouchilloux, *et al.*, 1963), DEAE cellulose (Dolashki *et al.*, 2012) were also reported to be used. Use of the Affinity column Ni-NTA agarose resin (Wang *et al*., 2021) was also reported to purify the enzyme.

Table 1 Purification of tyrosinase isolated from the strain *Brevundimonas diminuta PV 24*

Fraction	Volume ml	Total protein mg	Total Enzyme U	Specific Activity	Fold purification	% yield
Cell free extract	100	159	4305	27.07	1	100
Ammonium sulphate Precipitation	100	84	6716	79.15	2.93	156.00
Dialysis	10	5	981.6	196.32	7.24	22.80
Size exclusion chromatography (Biogel P-60)	1	0.1	149	1490	55.04	3.46

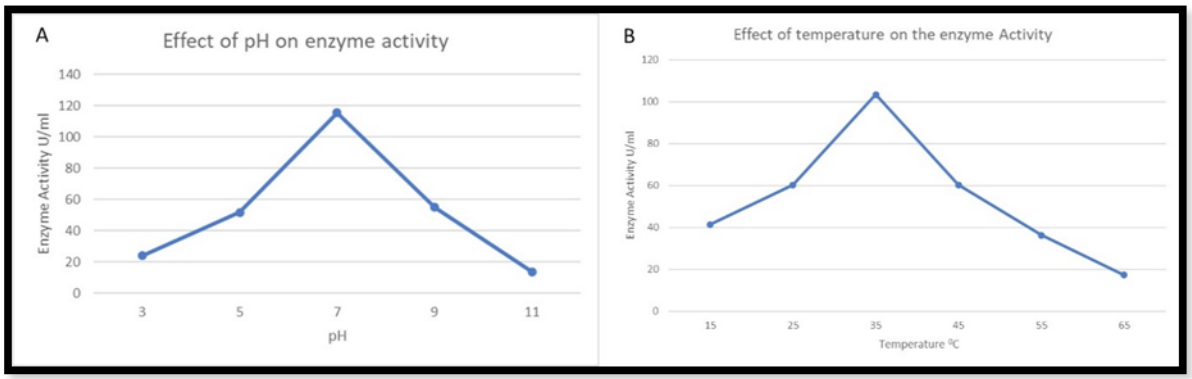

Fig. 1. Effect of [A] pH and [B] Temperature on the enzyme Activity.

Effect of pH and temperature on the enzyme activity

Since enzymes are extremely sensitive to pH and temperature, the activity of the enzyme was measured at different pH and temperature levels in order to verify the stability of the enzyme. At pH 7, it was discovered that the enzyme had the maximum activity, measuring 115.38 U/ml. (Figure 1A). Additionally, at 35^0C, it displayed the maximum activity of 103.33 U/ml. (Figure 1B).

CONCLUSION

The current study sheds the light on the partial purification of the tyrosinase enzyme from the newly isolated bacterium *Brevundimonas diminuta PV 24*. After the salt precipitation, dialysis and finally size exclusion chromatography the purification fold of the enzyme was 55.04 with the subsequent increase of the enzyme activity of 149U/ml. Study was also carried out about the stability of enzyme at different pH and temperature. It was found that enzyme was highly active at pH 7 and at 35^0C.

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Conflict of Interest: Non

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