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An alkaliphilic tyrosinase with distinct properties from a novel alkaliphilic bacterium isolated from mangrove ecosystem

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ABSTRACT

Tyrosinase is reported to be a copper containing monophenol monooxygenase which catalyses both o-hydroxylation of monophenols and oxidation of diphenols to quinones. Tyrosine is first ortho-hydroxylated to DOPA by cresolase activity of tyrosinase and DOPA is later oxidized to dopaquinone by the catecholase activity. DOPA is then oxidized to dopachrome and finally polymerized to the black pigment, melanin . The L – dopaquinone is oxidized to form melanin which has antioxidant, antiviral activity and protective effect against the damage caused by ultraviolet radiation.Tyrosinase enzyme was obtained from an obligate alkaliphile isolated from the mangrove ecosystems of Goa, west coast of India. The activity of the tyrosinase was measured under various conditions to characterize it. The optimum pH, thermal stability, reaction time and substrate concentration were determined. The results revealed that the alkaliphilic tyrosinase showed maximum activity at pH 9.0, at 30^0C .

Keywords : pH optimum, stability, obligately alkaliphilic, tyrosinase.

I INTRODUCTION

Tyrosinase is reported to be a copper containing monophenol monooxygenase which catalyses both *o*-hydroxylation of monophenols and oxidation of diphenols to quinones (Lerch 1987). Tyrosine is first *ortho*-hydroxylated to DOPA by cresolase activity of tyrosinase and DOPA is later oxidized to dopaquinone by the catecholase activity. DOPA is then oxidized to dopachrome and finally polymerized to the black pigment, melanin (Trias *et al* 1989 and Kelly *et al* 1990). The L – dopaquinone is oxidized to form melanin which has antioxidant, antiviral activity and protective effect against the damage caused by ultraviolet radiation. (Prota and Thomas 1976 and Liu *et al* 2004).

The pathway of **Tyrosine → DOPA → dopaquinone → dopachrome → melanin** is widely reported in mushrooms, fungi, bacteria and higher organisms including man (Mayer and Hazel 1979, Matoda 1979, Margalith 1992, Shivprasad and Page 1989, Kuo and Alexander 1967). Melanin plays a number of roles such as

in protection of fungi from bacterial enzymes, radiation effects in man etc. (Djordjevic *et al* 1987, Sadasivan and Neyra 1987, Shivprasad and Page 1989, Barnett and Hegeman 1983, Kuo and Alexander 1967).

Tyrosine is also reported to be metabolized by varied pathways such as homogentisate, protocatechuate and homoprotocatechuate pathways (Sparnins and Chapman 1976, Sparnins *et al* 1979, Gerhard Gottschalk 1986).

In our research we have found that an obligately alkaliphilic *bacterium A-131* produced a tyrosinase which was active at high pH. Here, this tyrosinase has been purified and its enzymatic properties have been studied.

II MATERIALS AND METHODS

A-131 was isolated from the mangrove ecosystems of Goa, west coast of India. Standard melanin, tyrosine and L-DOPA were purchased from Sigma. Infra red spectra (IR) were recorded with Shimadzu 1601 FT spectrometer and UV spectra were recorded with Shimadzu 1601 spectrophotometer.

Flavobacterium was grown in Mineral salt medium, pH 10.5 with 0.2% tyrosine as sole source of carbon.

Estimation of tyrosine and DOPA :

(i) Detection of DOPA :

The depletion of tyrosine and the production of the first transformation product DOPA was estimated using Arnow's method (Arnow 1937).

(ii) Detection of the dopa quinone and melanin pigment :

The cell free suspension of 16h culture A-131 grown in 0.2% tyrosine was checked for the presence of dopa quinone using spot tests as the method described by Finley 1974. The detection of black pigment was checked in culture broth grown for more than 24h as per the method described by Kelley *et al* 1990.

Tyrosinase Assay :

A-131 grown in MSM supplemented with 0.2% tyrosine for 24h was pelleted and resuspended in 0.05M phosphate buffer (pH 9.5) to get an absorbance of 4.0 at 540 nm.

(i) Assay of hydroxylase activity :

The assay based on the reaction of L-DOPA resulting from the enzymatic oxidation of L-tyrosine was determined at 280 nm (Marumo and Waite 1986). The reaction mixture in a total volume of 5ml contained 50mM Tris-HCl pH 8.5, 0.5 mM L-tyrosine, 0.5 µM L-DOPA, 2ml enzyme solution and 0.5mM acorbic acid. The mixture was incubated at 42^0C with constant stirring and aeration for 8h. At various times, 0.5 ml of the mixture was withdrawn and diluted with 2ml of distilled water. The reaction was stopped by adding 1ml of 1N HCl and the optical density at 280nm was measured.

(ii) Assay of L-DOPA oxidase activity :

Substrate solution was prepared with DL-DOPA (0.8 mg / ml) in the same buffer as above. Briefly, the reaction mixture contained 2.9 ml of substrate solution taken in a 3ml cuvette, incubated for 5 min at 30°C, followed by addition of 100 µl of cell suspension or cfe. Enzyme activity was determined spectrophotometrically monitoring at 475 nm, the appearance of the dopachrome product of the reaction (Hearing 1987). The change in absorbance

was read at 475nm. The assay was carried out with culture grown in MSM with benzoate (0. 3%), protocatechuate (0.1 %) and glucose (0.2 %) medium.

Substrate Specificity of tyrosinase :

The tyrosinase enzyme was checked for substrate specificity by using various aromatic compounds as substrates and conducting the enzyme assay. The enzyme specific activity was determined. The colour of the reaction mixture was noted visually.

Optimization of factors affecting tyrosinase activity :

Optimization of factors affecting the tyrosinase activity of *Flavobacterium* was done by carrying out the enzyme assay. The enzyme activity was determined under varying conditions of pH (6-12), temperature (20-60°C), reaction time (0-50 mins) and substrate (DOPA) concentration (0.2-1.6 mg/ml).

III RESULTS

Tyrosine and DOPA levels during growth of *A-131* in 0.2% tyrosine

The tyrosine depletion and DOPA formation were estimated using Arnow's method and the amounts were calculated. The tyrosine and DOPA levels at different time intervals during growth of *A-131* in 0.2% tyrosine is shown in Fig. 1. It was noted that as the culture grew in tyrosine, the tyrosine metabolism occurred which could be noted due to the significant drop in the level of tyrosine (Fig. 1). It was also noted that as the tyrosine level decreased, the level of the first transformation product DOPA increased. A maximum concentration of 85 mg / L of DOPA was formed at 24h of growth of the culture in 0.2% tyrosine followed by a decline and the tyrosine level decreased from 2000mg/L to 40mg/L.

Further the qualitative tests were performed on the pink and black coloured pigments formed during tyrosine metabolism by this culture. The tests confirmed the pigments to be dopaquinone and melanin respectively.

Tyrosinase assay

The tyrosinase activity was found to be active in whole cells as well as cell free extract grown in tyrosine, benzoate and glucose as sole substrates. However the tyrosinase activity was higher with cfe as compared to whole cells . Whole cells showed a low tyrosinase activity of 0.23 U/mg as compared to cfe with 0.55 U/mg while an activity of 0.14 and 0.12 U/mg respectively was shown by the cells grown in glucose and benzoate.

Substrate specificity of tyrosinase

The enzyme tyrosinase exhibited highest specific activity of 0.53 U/mg when DOPA was used as the substrate. The reaction mixture in this case turned pink in colour (Table 1). The monophenols such as phenol and cresol failed to oxidise in presence of tyrosinase. The tyrosinase enzyme however showed very low activity towards

cafeic acid, catechol, 2,3 dihydroxybenzoate and protocatechuic acid and the activity was almost negligible with substrates resorcinol, tannic acid and gallic acid (Table 1).

The tyrosinase enzyme of A-131 is seen to prefer substrates tyrosine and DOPA exhibiting maximum specific activities in their presence.

Optimization of factors affecting tyrosinase activity

The factors affecting the tyrosinase activity of A-131 were optimized by carrying out the enzyme assay. The enzyme was seen to be active at an optimum temperature of 30°C where a specific activity of 0.52 U/mg was obtained (Fig. 2a). Maximum activity of 0.48 U/ mg was observed at pH 9.0 and the lowest activity of 0.02 U / mg was obtained at pH 6 (Fig. 2b). The optimum substrate concentration of 0.8 mg / ml and reaction time of 10 mins gave the highest tyrosinase activity of 0.52 U / mg (Fig.2c and 2d).

IV DISCUSSION

In our work, we have identified a tyrosinase from an obligately alkaliphilic *A-131* which produces DOPA and further melanin, providing a new approach for improving studies on tyrosinase production. Studies on the properties of this alkaliphilic tyrosinase are important for understanding the activity of this enzyme under highly alkaline conditions. Tyrosinase is reported to be the most studied catechol oxidase enzyme and is present in humans, mammals, mushrooms and in bacteria and fungi and has multiple forms and molecular weights ranging from 14 to 120 kDa (Mayer and Hazel 1979, Hearing 1987, Makino 1974, Bouchilloux *et al* 1963, Trias *et al* 1989, Mueller *et al* 1996 and Liu *et al* 2004). Secondly, the tyrosinase differed remarkably from the enzymes obtained from other sources in its pH tolerance. It exhibited maximum activity at pH 9.0 and temperature 30^0C. Thirdly, tyrosinase is a copper monooxygenase binding two copper ions. Copper ions were required for the activity of this enzyme. Tyrosinase has been reported to be a copper containing monooxygenase binding two copper ions. Addition of chelating agents could irreversibly inhibit tyrosinase by removing copper ions from the enzyme. However higher concentrations of the chelating agent EDTA (200 – 400 mM) has been reported to activate the *Bacillus thuringiensis* tyrosinase (Liu *et al* 2004). This seems to be the first report wherein EDTA reactivates the enzyme tyrosinase.

Finally although this tyrosinase had low activity towards cafeic acid, catechol, 2,3 dihydroxybenzoate and protocatechuic acid and negligible activity towards resorcinol, tannic acid and gallic acid, It showed maximum activity towards tyrosine and DOPA. Tyrosinase isolated from *Thermomicrobium roseum* is reported to exhibit high substrate specificity towards catechol, chlorogenic acid, L-DOPA and pyrogallol (Kong *et al* 2000) while the heat inducible tyrosinase isolated from *Bacillus thuringiensis* is reported to exhibit specificity towards several compounds like L-DOPA, tyrosine, catechol, 4-methyl catechol, dopamine, 3-4-dihydroxymandelic acid, hydroquinone, 3-4-dihydroxyphenoxy acetic acid and resorcinol (Liu *et al* 2004).

In conclusion we purified tyrosinase from obligate alkaliphile *A-131*, a gram negative bacterium. It appears to be an unique tyrosinase as it is active only at high pH. It will therefore be a useful model for studying the inhibition, stability and mechanism of action of alkaline tyrosinase.

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Fig 1 : Tyrosine depletion and DOPA formation during growth of *Flavobacterium* in 0.2% tyrosine

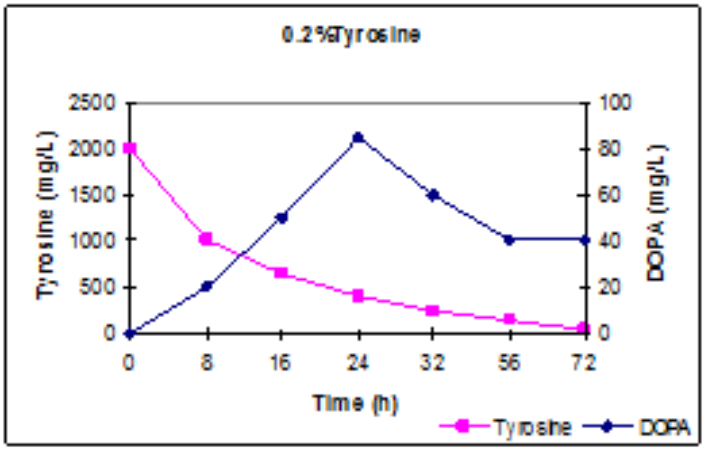

Table 1 : Substrate Specificity of Tyrosinase

Substrate	Specific activity (Enzyme units/mg protein)	Colour of reaction mixture
Phenol	0	-
Cresol	0	-
Tyrosine	0.15	Brown
Cafeic acid	0.1	Reddish brown
DOPA	0.53	Pink
Benzoate	0.1	White
Catechol	0.07	Light brown
2,3,Dihydroxybenzoic acid	0.03	Light blue
Protocatechuic acid	0.009	Pale brown
Resorcinol	0.001	Colourless
Tannic acid	0.08	Light brown
Gallic acid	0.075	Pale brown

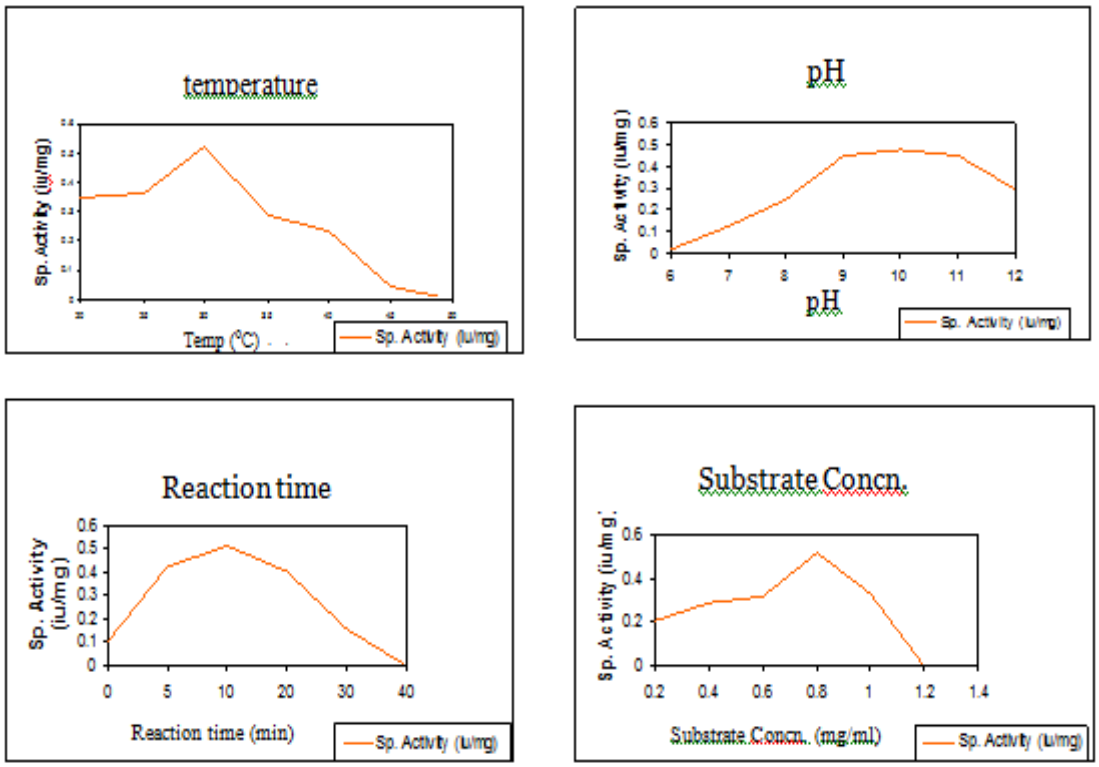

Fig 2 : Optimisation of factors affecting tyrosinase activity:

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