KINETICS OF 2-AMINOPHENOL OXIDATION IN THE PRESENCE OF AN EXTRACELLULAR TYROSINASE FROM *STREPTOMYCES sp*.

Adina Răducan *, **D. Oancea and E. Vissarion**

abstract: A rich tyrosinase extract was obtained from a strain of *Streptomyces* sp. The enzymatic extract was purified by precipitation and gel filtration. The activity was tested on L-DOPA and 2-aminophenol. The kinetics of 2-aminophenol oxidation with air in the presence of this extract was studied and the kinetic parameters were estimated using the Michaelis - Menten model.

Introduction

Tyrosinase (E.C.1.14.18.1) is a monooxygenating enzyme catalysing both the hydroxylation of phenols (monophenolase activity) and the oxidation of diphenols at corresponding quinones (cathecolase activity) [1]. The reaction takes place with the reduction of dioxygen to water. The enzyme can be found in a wide variety of organisms, ranging from prokaryotes to mammals. Although the enzyme is known for over a century, its structure and mechanism of action still remain to be solved. All isolated tyrosinases contain 2 to 4 copper atoms/molecule in the active site, which are usually coordinated to 3 histidine residues, similar to the coordination mode found in hemocyanins [2]. Most of the microbial tyrosinases are located intracellularly, the extraction and purification being quite difficult. Several species of *Streptomyces* genus produces bioactive molecules, including antibiotics, pigments and many extracellular enzymes such as lipases, cellulases and proteases. In addition, this group of actinomycetes is also able ,when are cultivated on organic media, to synthesize and excrete dark pigments (melanins or melanoid pigments) which are considered as an useful criteria in taxonomic studies [3]. Since these strains excrete dark pigments, they are producer of the extracellular tyrosinase; it was extracted and purified from different strains of *Streptomyces sp.* [4,5]. In this paper, some results concerning the extraction, purification of tyrosinase from *Streptomyces MIUG 4.88* and its effect on the kinetics of 2-aminophenol autooxidation are presented.

* Department of Physical Chemistry, Faculty of Chemistry, University of Bucharest, bd. Elisabeta 4-12, sect. 3, 703461, Bucharest, Romania

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Materials and Methods

Materials – Sephadex G-25 was purchased from Pharmacia Fine Chemicals, Sweden, L-DOPA and 2-aminophenol were from Sigma-Aldrich Chemie Gmbh Germany.

Screening of Microorganisms for Tyrosinase Production –117 strains of *Streptomyces sp.* isolated from Romanian soil, being part of the collection of Laboratory of Industrial Microbiology from University "Dunărea de Jos" Galați, Romania, were cultivated on a synthetic medium for melanin formation with the following composition: glycerol 1.5%, L-arginine 0.5%, L-tyrosine 0.1%, L-methionine 0.03%, agar-agar 1.5%, K_2HPO_4 0.05%, $MgSO_4$ 0.02%, $CuSO_4$, $CaCl_2$, $FeSO_4$, $ZnSO_4$ and $MnSO_4$ in bidistillated water. After 5 days at 25^0C, 11% of strains produced a dark brown pigment. By repeating the screening, 4 strains were used in quantitative analysis; these strains were able to synthesize melanins after 1-3 days at 25^0C. After cultivation for 72 hours at 25^0C, the cells were removed by centrifugation (8000 rpm for 20 min) and the supernatant was used as a source for tyrosinase. The strain *Streptomyces MIUG 4.88* was selected due to its higher performances in same cultivation conditions.

Assay for Tyrosinase Activity Using L-DOPA as the Substrate –The enzyme activity was measured spectrophotometrically by the method of Pomerantz [6] with a slight modification. 5mL of enzyme solution was incubated at 30^0C with 25mL L-DOPA 0.05M, in unbuffered system; the conversion of substrate to dopachrome was measured by monitoring the increase of absorbance at 475nm, at which dopachrome shows an absorption maximum. The reaction was followed by using a JASCO V530 spectrophotometer and the enzyme activity was calculated from the initial linear part of A=f(time) curve. One unit of enzyme activity was defined as the amount of enzyme which causes a ΔA at 475nm of 0.01/minute under specified conditions. The concentration of the protein was estimated spectrophotometrically by assuming that $A^{1\%}_{1cm,280nm}$ is 10[4], and the specific activity was calculated as the enzyme activity units per mg protein.

Purification of Tyrosinase –The supernatant was fractionated by ammonium sulphate precipitation at 50% saturation. The precipitate was dissolved in a minimum volume of 10mM sodium phosphate buffer at pH=7.0 and insoluble materials were removed by centrifugation. The supernatant was desalted by gel filtration on a Sephadex G-25 column.

Kinetic Assay Using 2-Aminophenol as the Substrate – The oxidation of 0.0125-0.1M solutions of 2-aminophenol with air was studied in a bubble-air stirred reactor, at constant temperature(30^0C) and initial enzyme concentration. The reaction was followed by measuring the increase of product absorbance at 434nm, by using a JASCO V530 spectrophotometer.

Results and Discussion

Screening of Microorganisms for Tyrosinase Production – From the 117 strains screened, 13 produced a diffusible dark-brown pigments in the medium, and one (MIUG 4.88) was selected as potentially the most efficient producer of tyrosinase in the culture filtrate. The

strain belongs on *Streptomyces* genus, group rectus-flexibilis with green aerian mycelium and compact substrate mycelium.

Purification of Extracellular Tyrosinase – The crude extract was combined with solid ammonium sulphate at 50% saturation and stored at 4^0C for 24 h. The suspension was then centrifuged at 8000 rpm for 30 min. This precipitate can be stored for several month at – 10^0C without loss of its activity. The ammonium-sulphate precipitate was dissolved in 50mL of 10 mM sodium phosphate buffer at pH=7 and centrifuged at 8,000 rpm for 15 min. Then 40 ml of clear supernatant were desalted by passing through a column (2.5 x 40 cm) of Sephadex G-25 previously equilibrated with the same buffer. Finally more than 95% of the contaminants (melanin pigments) were eliminated under these conditions. The activity, using L-DOPA as substrate, was calculated as:

$$A(U/mL) = 1000 \cdot \left(\Delta A_{475} \Big/ \min \right) \quad (1)$$

The concentration of enzyme protein was estimated spectrophotometrically by the method of Warburg ,Christian and Kalckar[7] as:

$$\text{Protein}(mg/mL) = 1.45 \cdot A_{280} - 0.74 \cdot A_{260} \quad (2)$$

The purification procedure is summarized in Table 1.

Table 1. Purification scheme for tyrosinase.

Purification step	Volume (ml)	Total protein (mg/ml)	Activity (U/ml)	Specific activity (U/mg)
Crude extract	500	2.68	21	7.8
50%$(NH_4)_2SO_4$ precipitate	50	0.13	12	92
Sephadex G-25 column	60	0.11	10	90.9

The absorption spectrum of the tyrosinase shows a maximum at at 282 nm and a small shoulder at 290nm (Fig. 1). The shoulder is typical for all tyrosinases investigated so far [1] and is likely to be assigned to oxidized tyrosyl residues.

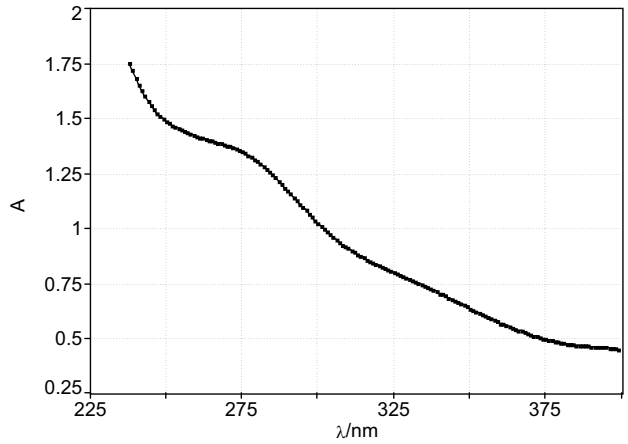

Fig. 1: *Absorption spectrum of Steptomyces MIUG 4.88 tyrosinase (0.1 mg/mL in water).*

Kinetic Study of 2-Aminophenol Oxidation – The oxidation of 2-aminophenol (0.0125÷0.1M) solutions, saturated with air, was studied in a bubble-air stirred reactor, at constant initial enzyme concentration and temperature (298K). The time evolution was observed by measuring the absorbance of product reaction, 2-amino-phenoxazin-3-one, at λ=434nm(ε = 23200 cm^{-1} [8]), where the substrate has no significant absorption. The absorption spectra at different times are presented in Fig. 2, and the integral kinetic curves [P] versus time are presented in Fig. 3.

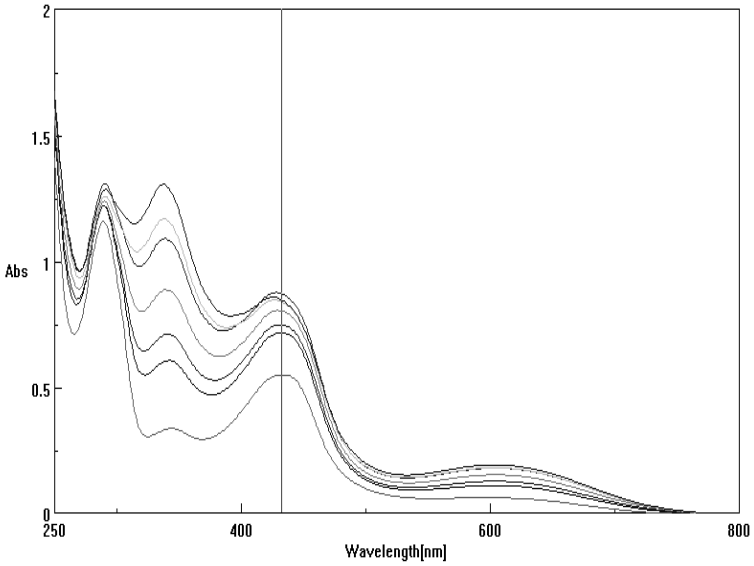

Fig. 2: *UV-VIS spectra of 2-aminophenol oxidation($[S]_0$=0.05M).*

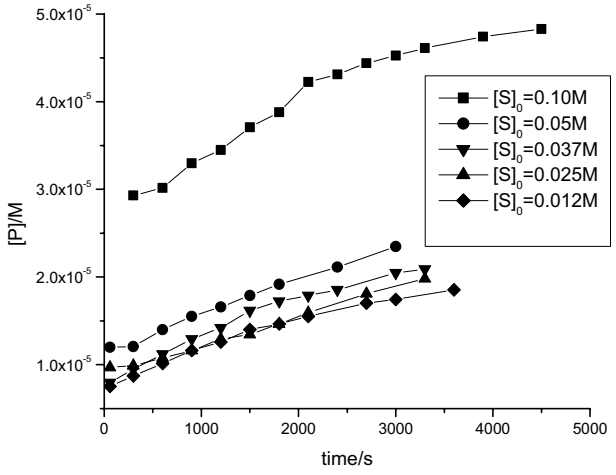

Fig. 3: *Variation of product concentration in time at different initial substrate concentrations.*

The concentration of 2-aminophenol was calculated from the mass balance. From each curve [S]=f(t) the initial rates were calculated as the slope of initial liniar part. The results are presented in table 2.

Table 2. Values of initial rates of 2-aminophenol oxidation

$[S]_0$/M	$v^0\ 10^9$/(M/s)
0.012	2.21
0.025	3.41
0.037	4.08
0.05	5.35
0.10	7.14

The plot of initial rates on initial substrate concentration has a hyperbolic shape, suggesting that the system follows a Michaelis-Menten mechanism. The equation (3) was fitted on the data $v^0=f([S]_0)$, using a nonlinear regression analysis:

$$v^0 = \frac{v_{max}}{1 + K_M / [S]_0} \tag{3}$$

The results are presented in Fig. 4.

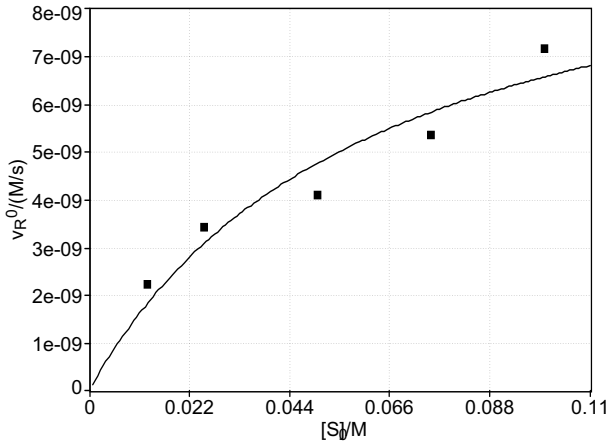

Figure 4. Kinetic parameters, using Michaelis-Menten equation: v_{max}=(1.06±0.28) 10^{-8}M/s; K_M=(62.1±3.4)mM; r=0.9248.

Conclusions

A strain of *Streptomyces* sp. MIUG 4.88 was selected by qualitative and quantitative screening of 117 strains isolated from Romanian soil and found to be the best source of tyrosinase. This strain produced tyrosinase in the culture filtrate which indicates that the enzyme was excreted extracellularly.

The strain produces a dark pigment in the medium; most of the pigment was adsorbed on Sephadex column. The purified enzyme was stable after one month at 4^0C.

The specific activity of tyrosinase for L-DOPA has the same magnitude to other tyrosinases[4,5] isolated from *Streptomyces* sp.

Tyrosinase catalyses the autooxidation of 2-aminophenol to 2-amino-phenoxazin-3-one; the activity for this substrate is quite small as compared to L-DOPA (1.5%). The system follows a Michaelis-Menten kinetics, with a Michaelis constant of 62.1 mM. This value is 10 times greater than those obtained for others substrates, indicating that the specificity of tyrosinase for 2-aminophenol is low.

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