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CHARACTERIZATION OF TYROSINASE ENZYME FROM NATIVE *BACILLUS MEGATERIUM* SP. STRAIN M36

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ABSTRACT

Tyrosinase is a type 3 copper-containing enzyme that catalyzes the conversion of l-tyrosine to L-DOPA and finally to melanin. In this study tyrosinase enzyme from native *Bacillus megaterium* sp. strain M36, was produces, characterized and used to produce L-DOPA. The M36 tyrosinase enzyme showed optimum monophenolase and diphenolase activity at pH 7.5 and conserved its maximum activity over than 95 % at pH ranging from 6.5 to 8.0. The M36 tyrosinase enzyme showed optimum monophenolase and diphenolase activity at 40 °C also, the enzyme conserved 100% of its original activity at 4-45 °C. The M36 tyrosinase enzyme was inhibited strongly by β-mercaptoethanol and about 90% by 5mmol of EDTA (a chelating agent). Although the enzyme was activated at the presence of 1mM SDS, it was strongly inhibited at high concentration of SDS (above 15mM). In TLC analysis, the transformation of L-tyrosine to L-DOPA was conspicuously detected.

Keywords: Melanin, monophenolase, diphenolase, TLC

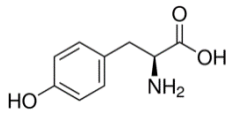
INTRODUCTION

Tyrosinase is a type 3 copper-containing enzyme that has been found widely distributed in microorganisms, plants and animals **(Claus and Decker, 2006).** Tyrosinase catalyses the hydroxylation of monophenol to o-diphenol (monophenolase or cresolase activity) and the oxidation of diphenol to o-quinones (diphenolase or catecholase activity). O-quinones are converted in to melanin by using nonenzymatic steps and molecular oxigene **(Decker and Tuczek, 2000). Howard *et al.*, in 1948**, elucidated the biosynthetic pathway for melanin formation by tyrosinase enzyme. In mammals, tyrosinase catalyzes the biosynthesis of melanin pigments, which contributes to a fundamental part of the skin protection against UV radiation. It is also related to the browning reactions of fruit and vegetables **(Seo *et al.*, 2003)**. Tyrosinases have several biotechnological applications relying on the ability of the enzymes to oxidize both small phenolic molecules and protein-associated phenolic groups, i.e. the side chain of the amino acid tyrosine. Tyrosinase enzyme has very important role in bioremediation **(Marino *et al.*, 201128)**, production of L-DOPA, the preferred drug for treatment of Parkinson's disease and other antioxidants (having crucial application in medical field) **(Xu *et al.*, 2012)**, food industry **(Allouche *et al.*, 2004)**, textile industry **(Franciscon *et al.*, 2012)** and production of melanin **(Kumar *et al.*, 2011)**. Recently, because of increasing application of the tyrosinase enzyme, the interest in the isolation of new tyrosinase enzyme has been increased. Up to present, several tyrosinase enzyme from microbial strains such as *Bacillus thuringiensis* **(El-Shora and Metwally, 2008)**, *Pseudomonas putida* F6 **(McMahon *et al.*, 2007)**, *Ralstonia solanacearum* **(Hernandez-Romero, 2005)**, *Rhizobium etli* **(Pinero *et al.*, 2007)**, *Streptomyces antibioticus* **(Marino *et al.*, 2010)**, *Thermomicrobium roseum* **(Kong *et al.*, 2000)**, *Streptomyces* sp. REN-21**(Ito and Inouye, 2005)**, *Verrucomicrobium spinosum* **(Fairhead and Thony-Meyer, 2010)** have been isolated and characterized. Most of the strains have multicatalytic functions such as peroxidase and laccases in addition to tyrosinase activity, these characteristics make more restrictions for the strains to be used in industrial and pharmaceutical applications **(Dastager *et al.*, 2006)**, any way some strains which produce only tyrosinase enzyme has been isolated from soil samples **(Freddi *et al.*, 2006)**. These strains are appropriate for industrial applications.

The commercial production of tyrosinase enzyme is mostly reported from the common mushroom *Agaricus bisporus*. Extensive research regarding this enzyme has been carried out using this mushroom tyrosinase. The mushroom's tyrosinase enzyme exhibits relatively low pH and temperature stability and its purification is relatively hard, as compared to bacterial tyrosinases **(Seo *et al.*, 2003).** To date, this is the first time that isolation and characterization of a native tyrosinase enzyme from *Bacillus megaterium* strain was carried out.

MATERIAL AND METHODS

In this research all material for making medium were bought from sigma and merck. Also the substrate (l-tyrosine) was bought from sigma. According to its information wrote in sigma, L-tyrosine has the following properties; form: fine crystals and fragments, colour: white, molecular weight: 181.19 g/mol, water solubility: 0.479 g/l at 25 °c, formula: C9H11NO3



Production and partial purification of the m36 tyrosinase enzyme

Culture condition for tyrosinase enzyme production by the *Bacillus megaterium* sp. strain M36had been optimized previously and it was as follow: temperature (36 °C), pH (7.0), incubation time (16 hour), agitation (170rpm) , l-tyrosine (0.4mg/ml), yeast extract (0.05%), tryptone (0.423%), NaCl (3.4%) and CuSo4 (148.4µM). The native *Bacillus* sp.M36 was cultured at optimized culture condition and in order to enzyme extraction, to start with, the cell free extract was prepared then the extract was subjected to ammonium sulfate precipitation and dialysis.

For cell free extract preparation, the medium culture was centrifuged at 6000g for 10 min at 4°C when OD_{530} of medium culture was 1.3, Then the obtained supernatant was stored at +4°C and the pellets were washed twice in ice-cold 50mM potassium phosphate buffer, pH 7.0. After that the pellets were resuspended in 0.1M sodium phosphate pH 7.0 containing an inhibitory bacterial proteases cocktail (1: 4, µl: mg cell mass) and disrupted by sonication. The homogenate was centrifuged at 14000g for 15min. The supernatant achieved both by the previous centrifuge at 6000g and by centrifuge at 14000g were used as a cell free extract **(Lopez-Serrano *et al.*, 2002; McMahon *et al.*, 2007; Michalik *et al.*, 1976)**. The cell free extract was subjected to precipitation with ammonium sulfate (40, 50, 60, 70, 75, 80, 85 and 90% saturation) for 1 hour with gentle stirring. After fractionation with ammonium sulfate, the precipitated proteins are

recovered by centrifugation at 12000g for 30 minute and are dialyzed against 50mM sodium phosphate buffer, pH 6.8 with 0.02% sodium azide, 0.01mM $CuSO_4$. The fractions were tested to tyrosinase activity and active fractions were stored at –20 ° C without loss of activity **(El-Shora and Metwally, 2008)**. Protein contents of the samples were determined by Bradford method using bovine serum albumin (BSA) as the standard **(Kohashi *et al*., 2004).**

Enzyme assay

Tyrosinase activity is assayed by using L-tyrosine and L-DOPA as substrates. The appropriate concentration of the enzyme was determined before the enzyme activity was assayed and an aliquot of the enzyme solution is added to a 0.1M sodium phosphate buffer (pH 6.8) containing 1mM L-tyrosine and L-DOPA , and the formation of dopachrome is monitored by measuring the absorbance at 475 nm **(Rao *et al*., 2013)**. The initial rate is used for the calculation of tyrosinase activity. One international unit (IU) of tyrosinase activity is defined as the amount of enzyme required to oxidize 1µmol of L-tyrosine to dopachrom per minute under the above conditions, which was calculated using the molar extinction coefficient of dopachrome ($3600 M^{-1} cm^{-1}$) by the following equation:

$$\mathrm{IU/ml} \sim \mu\mathrm{mol/\,min\,/ml}) = \frac{\mathrm{absorption/\,min} \cdot \mathrm{assay\ volume\ (ml)} \cdot \mathrm{dilution\ factor} \cdot 10\,000}{\varepsilon_{\mathrm{nm}}(\mathrm{l} \cdot \mathrm{mol}^{-1}\,\mathrm{cm}^{-1}) \cdot 1\,\mathrm{cm} \cdot \mathrm{enzyme\ volume\ (ml)}}$$

Effect of pH and temperature on enzyme activity and stability

For this purpose, 200µl of enzyme solution (protein content, 0.05 mg/ml) was added to 1800µl buffer containing 1mM of L-dopa for diphenolase and 1mm of l-tyrosinae for monophenolase activity and incubated for 45min. The effect of pH on monophenolase activity was investigated by analyzing the activity at different pH values (pH 4, 5, 6, 7, 8, 9, 10, 11 and 12) and for diphenolase activity pH (4-7.5) were tested because L-DOPA spontaneously converted to dopachrome at pH values above 7.5. pH value in which the enzyme showed maximum relative activity was determined as optimum pH for the enzyme activity **(Burhan *et al*., 2003; McMahon *et al*., 2007)**.
Also, the enzyme activity was analyzed at a range of temperatures from 10 to 70°C (10, 20, 30, 40, 50, 60 and 70) and the temperature showing maximum relative activity was determined as an optimum temperature for the enzyme activity. In order to ascertain of the temperature stability, the enzyme solutions in different tubes are incubated at various temperatures in the range from 0°C to 70°C for 2 hour then residual activity is assayed in enzyme assay condition **(Liu *et al*., 2004)**.

Effect of detergents on enzyme activity

To examine the effects of sodium dodecyl sulphate (SDS), ethylene diamine tetraacetic acid (EDTA), Urea, Tween-80, TritonX-100, β-Mercaptoethanol and PMSF are analyzed by incubating enzyme in the presence of these detergents and substrate **(Aygan *et al*., 2009; Caf *et al*., 2012)**.

Kinetic study of M36 tyrosinase enzyme

The initial rate of enzyme reaction for l-tyrosine and L-DOPA was determined at various concentrations. The resulting data was analyzed and the K_m and V_{max} values are calculated by Michaelis–Menten and Hill equation $v_i = \frac{V_{max}[S]}{k_m+[s]}$ and Lineweaver-burk equation $\frac{1}{v_i} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}}$ **(Mc Mahon *et al*., 2007; 45 Zanjani *et al*., 2009)**. After addition of 200µl of enzyme solution (protein content, 0.05mg/ml) to potassium phosphat (50mM, pH, 7) containing various concentrations ranging from 0.02 to 0.8 for tyrosine and 0.06 to 2.0 for L-DOPA, the reaction medium with L-DOPA and with L-tyrosine was incubated at room temperature for 30min and 45 min, respectively. After that the reaction medium with L-tyrosine was diluted 5 times and reaction medium with L-DOPA was diluted 10 time and both of them was subjected to study of OD_{475} by spectrophotometer. The obtained data was used to calculation of velocity.

Electrophoretic study

The enzyme solution was loaded in several well of Non-denaturing PAGE (8% w/v) and after separating protein bands, a single lane of the gel was sliced out of the gel using a clean scalpel. The tyrosinase enzyme related band was stained by placing the gel slice in substrate solution (l-tyrosine (0.1mg/ml) and $CuSO_4$ (50µM) in phosphate buffer (0.1M, pH 7)) for 60 min. The formation of a dark-brown band indicated the position of the tyrosinase enzyme. The remaining lanes of the gel were placed in 50mM phosphate buffer, pH 7.0. Using the activity stained lane as a guide to the location of tyrosinase, the corresponding band was sliced out of the unstained lanes. The gel slice was homogenized and resuspended in a 50mM phosphate buffer and left overnight at 4 ◦C. The gel suspension was centrifuged at 12000 g for 10 min to remove remaining gel fragments and the obtained supernatant was subjected to SDS-PAGE (12%) analysis for determination of the tyrosinase enzyme molecular weight **(Arikan, 2008)**.

Thin layer chromatography analysis of the reaction mixture

The conversion of L-tyrosine to L-DOPA by M36 tyrosinase enzyme was analyzed by thin layer chromatography. For this purpose, phenol-water system (75:25) (w/v) was used as a mobile phase and 3% ninhydrin in n-butanol as spray and staining reagent. Besides of TLC analysis, **(Rani *et al*., 2007; Raval *et al*., 2012)**.

Statistical analysis

All experiments were conducted in three replicates; data generated were subjected to statistical analysis using Microsoft Excel and presented as mean_SE.

RESULT AND DISCUSSION

Preparation of the M36 tyrosinase enzyme

The enzyme was precipitated by ammonium Sulfate 85% and centrifugation at 13000g and dialyzed against 50mM sodium phosphate buffer (pH 6.8 containing 0.02% sodium azide and 0.01mM $CuSO_4$).

Effect of pH and temperature on enzyme activity and stability

The result of this research showed that the M36 tyrosinase enzyme had maximum monophenolase and diphenolase activity at pH, 7.5 (Figure 1). This result was in accordance with tyrosinase enzyme originated from *Streptomyces sp*. REN-21 (pH 7.0) **(Ito and Inouye, 2005)**, *Rhizobium etli* CFN42 (pH 7.5) **(Pinero *et al*. 2007)** and *Pseudomonas putida* F6 (pH 7.0) **(McMahon *et al*., 2007)** notwithstanding, the tyrosinase enzyme from *B. thuringiensis* **(Liu *et al*., 2004)** and *T. roseum* **(Kong *et al*., 2000)** have shown to have maximum activity at 9.0 and 9.5, respectively. The M36 tyrosinase enzyme could conserve its maximum activity over than 95 % at pH (6.5-8.0). Before pH (6.5) and above pH (8.0) the activity and stability of the enzyme was dropped. These findings are similar to the finding of Shuster and Fishman **(2009)**.
The tyrosinase enzymes have two cupper in its active site and each of the two metal atoms; Cu_A and Cu_B, of the active site are coordinated by three conserved histidines which are located in a 'four α-helix bundle' **(Claus and Decker, 2006)**. The α-helix is structured by hydrogen bonds. Generally changing of pH value (extremely basic or acidic) causes changes in the charge of H-bond donor and acceptor groups, it can rearrange the H-bonds and change the conformation/folding of the protein.

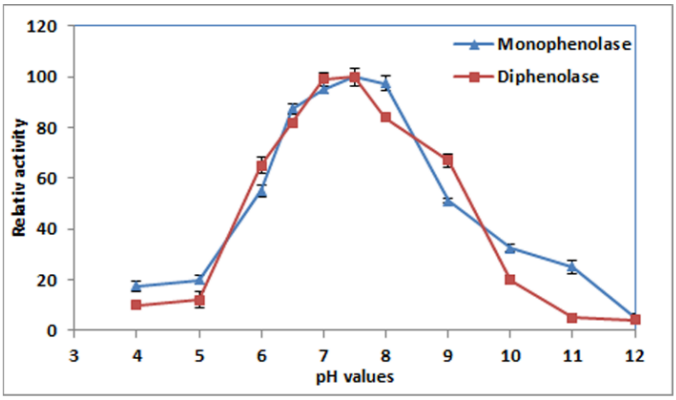


Figure 1 Effect of pH on activity and stability of the *Bacillus megaterium* M36 tyrosinase enzyme (monophenolase). The enzyme showed maximum activity (0.52 IU) at pH=7.5 and 97.5% of its maximum activity at pH=8.0. At pH lower than 6.5 and higher than 8.0 the activity of the enzyme was steeply decreased.

The M36 tyrosinase enzyme showed optimum monophenolase and diphenolase activity at 40 °C also, the enzyme conserved 100% of its original activity at 4-45 °C (Figure 2). The monophenolase and diphenolase activity of the enzyme was deeply decreased at temperature below 30 °C and above 55 °C, probably this result was related to that, the tyrosinase enzyme has mostly composed from α-helix, on the other hand α-helix is more flexible than the others structures. This result was more or less closed to other investigations.
The M36 tyrosinase enzyme showed up to 95% activity at temperature ranges from 35 °C-45°C, in contrast to this, the activity of tyrosinase enzyme from *P. putida* F6 **(McMahon *et al*., 2007)** has been decreased dramatically at temperature above 30°C and the enzyme of *Streptomyces michiganensis* DSM **(Philipp *et al*., 1991)** has showed optimum activity at 33°C. Moreover there is some reported tyrosinase enzymes whit higher optimum temperature. Also the

M36 tyrosinase enzyme was different with the tyrosinase from *Rhizobium etli* CFN42 (50°C) **(Pinero *et al.*, 2007)**, *Bacillus (HR03)* (55°C) **(Dalfard *et al.*, 2006)**, *Bacillus thuringiensis* (75°C) **(El-Shora, Metwally, 2008)** and *Thermomicrobium roseum* (70°C) **(Kong *et al.*, 2000)**. The M36 tyrosinase enzyme conserved its original activity at 45°C, contrary to this *Trichoderma reesei* **(Cura *et al.*, 2010)** tyrosinase started to lose its activity relatively quickly at temperature above 30°C.

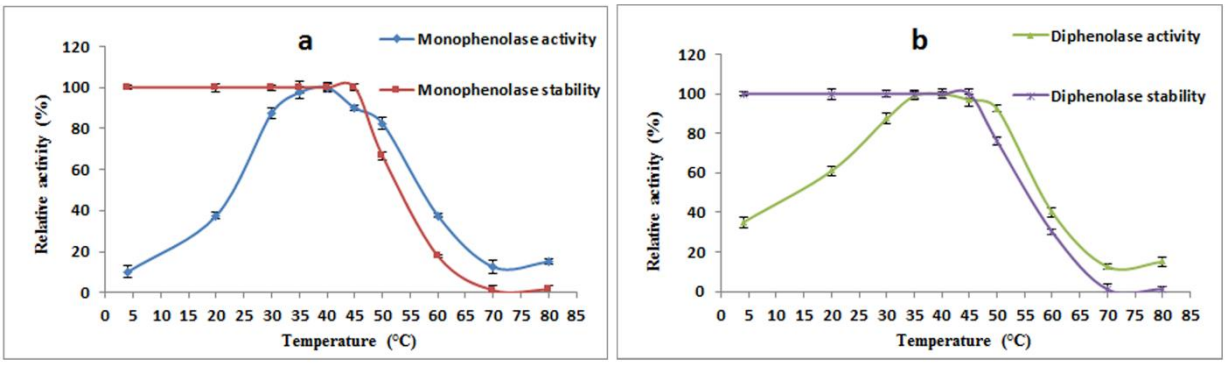


Figure 2 Effect of temperature on a) monophenolase (L-tyrosine as a substrate) and b) diphenolase activity (L-DOPA as a substrate) of the *Bacillus megaterium* M36 tyrosinase enzyme. The enzyme showed maximum monophenolase (0.56IU) and maximum diphenolase activity (0.62IU) at 40°C. Both of the monophenolase and diphenolase activity were conserved 100% at temperature 0-45°C, after that the enzyme loosed its activity.

Effect of detergents on enzyme activity

The M36 tyrosinase enzyme was studied in presence of various inhibitors (Figure 3a). The enzyme was inhibited strongly by β-mercaptoethanol. β-mercaptoethanol is a reducing agent which inhibit dopachrom and melanin synthesis by reducing qinones (an intermediate) to L-DOPA. Similar results were obtained for *Bacillus megaterium* tyrosinase **(Shuster and Fishman, 2009)**, and *Thermomicrobium roseum* tyrosinase **(Kong *et al.*, 2000)** that was completely inhibited by β-mercaptoethanol (1mmol). The M36 tyrosinase was inhibited about 90% by 5mM EDTA (a chelating agent). The agent can inhibit the enzyme by chelating of Cu from its active site. Similarly *Bacills megaterium* tyrosinase was inhibited up to 27% by 1mM EDTA **(Shuster and Fishman, 2009)** and *Bacillus (HR03)* tyrosinase enzyme was partially inhibited by 1mM EDTA **(Dalfard *et al.*, 2006)**. In contrast to the result of this research tyrosinase enzyme from *Bacillus thuringiensis* **(El-Shora and Metwally, 2008)** was activated at high concentration EDTA from 200 to 400mM. Effect of different concentration of SDS (0.2- 30mM) on the M36 tyrosinase enzyme was studied. Although the enzyme was activated at the presence of 1mM SDS, it was strongly inhibited at high concentration of (above 15mM) SDS (Figure 5b). Previously, activating effect of SDS on tyrosinase enzyme from *Xenopus laevis* **(Wittenberg and Triplett, 1985)**, *A. bisporus* **(Espin and Wichers, 1999)**, *Bacillus sp.* **(Dalfard *et al.*, 2006)** and *Bacillus megaterium* **(Shuster and Fishman, 2009)** has been reported which was in agreement with our result. According to the paper published by Gandia-Herrero, although, active site of enzyme is not affected by SDS; a stepwise conformational change affected the enzyme activity by increasing accessibility of its active site to the substrate **(Gandía-Herrero *et al.*, 2005)**.

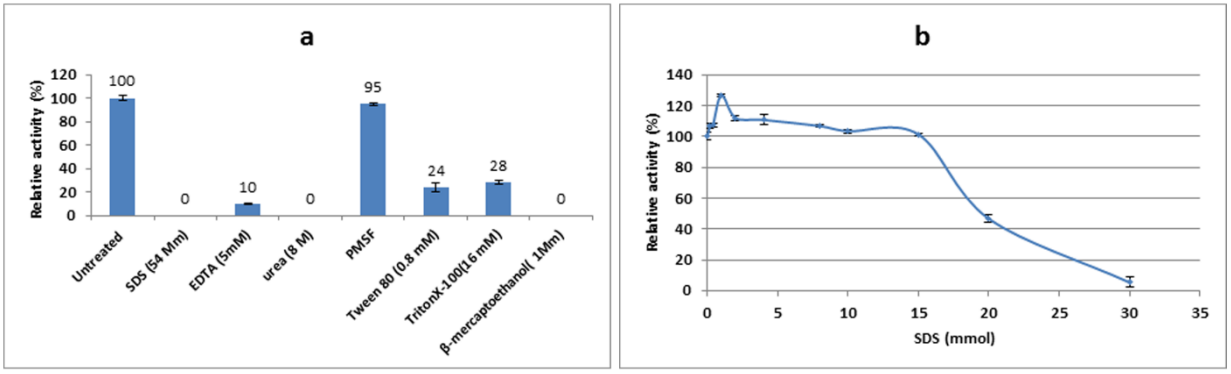


Figure 3 a) Effect of inhibitors on *Bacillus megaterium* M36 tyrosinase activity. The activity of the sample containing the enzyme without any of the additives was considered as control (100%). **b)** Effect of SDS concentration (w/v) on the *Bacillus megaterium* M36 tyrosinase activity. The enzyme showed maximum activity (126.6%) at the presence of 1mM of SDS and its activity was gradually decreased at the SDS concentration more than 1mM, so that it reached to 5.3% at concentration of 30mM. Sample having no SDS in reaction mixture was considered as a control (100%)

Production of L-DOPA from L-tyrosine

In TLC analysis, the transformation of L-tyrosine to L-DOPA was conspicuously detected. Ascorbic acid, used to prevent further oxidation of L-DOPA, did not give interfering spots (Figure 4).

Kinetic study of M36 tyrosinase enzyme

The M36 tyrosinase enzyme was shown to obey Michaelis-Menten kinetics when L-tyrosine and L-DOPA was used as a substrate. The K_m value of M36 tyrosinase for l-tyrosine (0.15mM) was lower than L-DOPA (0.58mM). The obtained V_m was 1.7μM.min^{-1}.ml^{-1}for l-tyrosine and 6.2μM.min^{-1}.ml^{-1} for L-DOPA. K_m value of M36 tyrosinase enzyme was similar to the previously reported K_m values with the l-tyrosine, for example ; 0.2mM for *Agaricus bisporus* **(Selinheimo *et al.* 2009)** and 0.19mM for *Rhizobium etli* CFN42 **(Cabrera-Valladares *et al.*, 2006)**, also it is higher than the value (0.075mM) reported for *Bacillus megaterium* **(Shuster and Fishman, 2009)** and it is less than the values 0.563mM, 1mM, 0.421mm reported for *Bacillus huringiensis* **(El-Shora and Metwally, 2008)** , *Streptomyces* sp. REN-21**(Ito and Inouye, 2005)** and *Verrucomicrobium spinosum* **(Fairhead and Thony-Meyer, 2010)**, respectively. The K_m value of M36 tyrosinase enzyme for L-DOPA was higher than K_m value of tyrosinase from *Agaricus bisporus* (0.17mM) **(Selinheimo *et al.*, 2009)**, *P. putida* F6 (0.33) **(McMahon *et al.*, 2007)** and *Bacillus megaterium* (0.35mM) **(Shuster and Fishman, 2009)** for the same substrate, but it was lower than K_m values of tyrosinase from *Trichoderma reesei* (7.5mM) (Selinheimo *et al.*, 2009), *Rhizobium etli* CFN42 (2.44mM) **(Cabrera-Valladares *et al.*, 2006)**, *Streptomyces antibioticus* (8.9mM) **(Marino *et al.*, 2011)**, *Streptomyces castaneoglobisporus* (8mM) **(Kohashi *et al.*, 2004)** and *Verrucomicrobium spinosum* (7mM) **(Fairhead and Thony-Meyer, 2010)**.

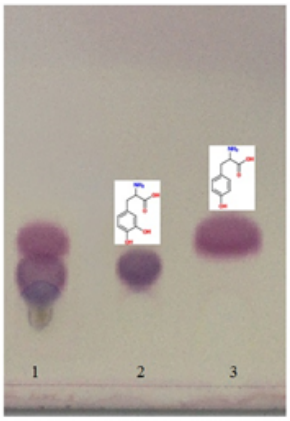


Figure 4 TLC analysis of L-tyrosine conversion to L-DOPA by *Bacillus megaterium* M36 tyrosinase enzyme

Electrophoresis and enzymatic activities in gel

After dialysis, tyrosinase M36 was electrophoresed by using native polyacrylamide gel (8%), after specific staining, a distinct band was detected. by extracting of the tyrosinase enzyme from native gel using the method mentioned in material methods, and the enzyme was subjected to SDS-PAGE (12%) analysis. This analysis showed almost 34kDa bond of the enzyme (Figure 5). This result was similar to the result of Shuster and Fishman **(2009)** who have demonstrated the tyrosinase from *Bacillus megaterium* to be almost 35kDa.

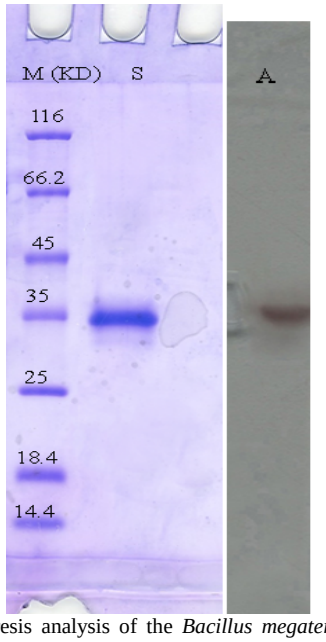


Figure 5 Electrophoresis analysis of the *Bacillus megaterium* M36 tyrosinase Enzyme. Lane (A) shows tyrosinase activity, lane (T) shows the enzyme molecular weight almost 34KDa, almost 15µg of protein was loaded, lane (M) shows protein marker.

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