

Optimization of Tyrosinase Enzyme Production from Native *Bacillus* sp. MV29 Isolate

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Abstract

Tyrosinase is a copper-containing enzyme which catalyzes the conversion of L-tyrosine to L-DOPA and melanin. L-DOPA is a preferred drug for treatment of Parkinson's disease and melanin has many pharmaceutically and therapeutically uses. In this research a native *Bacillus* sp. having tyrosinase enzyme was isolated from soil sample and the best carbon and nitrogen source for the enzyme production was found by analyzing effect of various carbon and nitrogen sources on the yield, also the optimum amount of the sources was selected by testing various amount of them. Various amounts of L-tyrosine and trace element were studied and the optimum amount of them was opted. Enzyme production by the native bacillus sp. was analyzed at different pH and temperature and the optimum pH value and temperature for the enzyme production was selected. By testing the enzyme activity for 56 hour at 4 hours intervals, the optimum incubation time was determined as 72 hour. According to the findings of this research, 40°C as optimum temperature, pH 7.0 as optimum pH, 48h as optimum incubation time was determined as optimum for the enzyme production. The addition of L-tyrosine (5mM) as a substrate to the production medium was highly effective on enzyme production. Consequently, after optimization, 0.7IU tyrosinase enzyme per milliliter of medium culture was obtained.

Key word: L-DOPA, melanin, Parkinson, Tyrosinase, optimization

INTRODUCTION

Tyrosinase (monophenol, o-diphenol: oxygen oxidoreductase, EC 1.14.18.1) is a copper-containing metallo protein that is ubiquitously distributed in nature. Tyrosinases are found in prokaryotic as well as in eukaryotic microorganisms, and in mammals, invertebrates and plants. Tyrosinase is a monooxygenase and a bifunctional enzyme that catalyzes the o-hydroxylation of monophenols and subsequent oxidation of o-diphenols to quinones [9]. the enzyme has an important role in biosynthesis and medical application such as production of L-DOPA, the preferred drug for treatment of Parkinson's disease [42, 34], production of hydroxytyrosol as a food additive [2, 4, 15], production of estrogenic compound [30], production of melanin for therapeutic uses [43].

L-DOPA is a commercially and pharmaceutically important compound, but its production by chemical synthesis is not an economically viable process; therefore, the scientific community is involved in the development of a cost-effective biotechnological process. Hence, the isolation and purification of this enzyme has drawn the attention of the scientific world, as it catalyzes the synthesis L-3,4-dihydroxyphenylalanine (L-DOPA) by the oxidation of tyrosine [24]. melanin ,resulting from the activity of tyrosinase enzyme on l-tyrosine, shows a broad spectrum of biotechnological and biological functions, including antioxidant activity [25], antitumor activity [14], antivenin activity [22], anti-virus [18], liver protecting activity [38]and radio protective [10] etc. Melanins are widely used in medicine, pharmacology, cosmetics and other fields. Also, there is a strong consumer demand for melanin as a natural colourant in food and cosmetics, particularly as a component of photo-protective creams and as substitute for synthetic dyes [13, 25]. Briefly, tyrosinase activity has been clearly shown in some strains, *Bacillus megaterium* [39],

Bacillus thuringiensis [1], *Marinomonasmediterranea* [27], *Pseudomonas putida* F6. [28], *Ralstoniasolanacearum* [21], *Rhizobiummetli*CFN42 [5], *Thermomicrobiumroseum* [23], *Verrucomicrobium spinosum* [17], *Streptomyces* sp. REN-21 [37]. In view of the above, there is an increasing interest for development of the enzyme. So, it was decided to produce a novel bacterial tyrosinase enzyme from native bacterial strain.

MATERIAL AND METHODS

Isolation of tyrosinase-producing bacterial strain:

Soil samples were gathered from different regions of Turkey. Almost 2 g of the soil sample was solved in 10 ml sterile water and subjected to heat –shock for isolating of *bacillus* sp. the isolated bacterial strains were cultured on screening medium (yeast extract 0.15%, tryptone 0.15%, NaCl 0.5% and L-Tyrosine 0.1%) and selected the brown-black colony forming strains as a prospective strains to have tyrosinase enzyme. the best of the strains was selected and subjected to enzyme production and some biochemical identifying tests [39].

Tyrosinase enzyme assay

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. Dopachrom “coloured intermediate” is an intermediate of melanin biosynthesis that is made from o-quinones by nonenzymatic oxidation. The extinction coefficient (ϵ) for the product dopachrome is 3600

L/mol.cm [35]. 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 1 μmol of L-tyrosine to dopachrom per minute under the above conditions, which was calculated using the molar extinction coefficient of dopachrome ($3600 \text{ M}^{-1} \text{ cm}^{-1}$) by the following equation:

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

Optimization of culture conditions for enzyme production

Different carbon sources and nitrogen sources were tested to optimize enzyme production. Also different values of pH, temperature, tyrosine as substrate and incubation time were studied to optimize the enzyme production [40].

Incubation time

In order to optimize incubation time, the isolated strain was cultured in the screening medium and incubated at 37°C ; 175 rpm for 4 days and amount of tyrosinase enzyme in the culture was evaluated at 6 hour intervals by spectrophotometry.

Effect of pH and temperature on tyrosinase enzyme production

To determine the effect of temperature and pH on the growth and enzyme production, experiments were carried out at various temperatures (20°C - 80°C) and pH (5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, and 12.0), and optimum pH and temperature for tyrosinase enzyme production was selected [11].

Effect of different nitrogen sources on enzyme production

For selection of optimum ingredient of the medium, the effect of some nitrogen sources such as peptone, casein, gelatin and ammonium nitrate was studied by using each of these compounds as a nitrogen source instead of tryptone in medium. Then the best nitrogen source was selected. After that by testing various amount of the selected nitrogen source, optimum concentration of the nitrogen source was determined for enzyme production [3].

Effect of different carbon sources on the tyrosinase enzyme production

For this purpose, some carbon sources (starch, glucose, glycerol, maltose and fructose) were incorporated to the media (with optimum nitrogen source) and their effect on enzyme production were evaluated [3].

Effect of substrate and trace element concentration on the tyrosinase enzyme production

In this research by incorporating various concentrations (0.2-3mg/ml) of L-tyrosine to medium, the concentration of L-tyrosine which had maximum effect on enzyme production was selected.

By addition of various concentration of trace element (The trace elements solution contained (% w/v): $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2%), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (0.015%), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.014%), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.026%), $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ (0.023%) and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.061%)) to the medium, the optimum concentration of trace element for enzyme production was found.

Evaluation of L-DOPA produced by the M36 tyrosinase enzyme

The conversion of L-tyrosine to L-DOPA by the native isolated 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. The standard L-tyrosine (0.05%) and L-DOPA (0.1%) were used [36].

RESULTS AND DISCUSSION

Bacillus sp. MV29 isolate was isolated and selected for the tyrosinase enzyme production. The strain was rod-shaped, and Gram (+) (figure 1), Voges-Proskauer(+), Catalase (+), Hemolysis (+), Methyl red (+), Amylase (+). Also, the strain produced blacked- brown color when it was cultured at medium containing L-tyrosine and CuSO_4 (figure 2).



Figure 1. Gram reaction of the *Bacillus* sp. MV29.

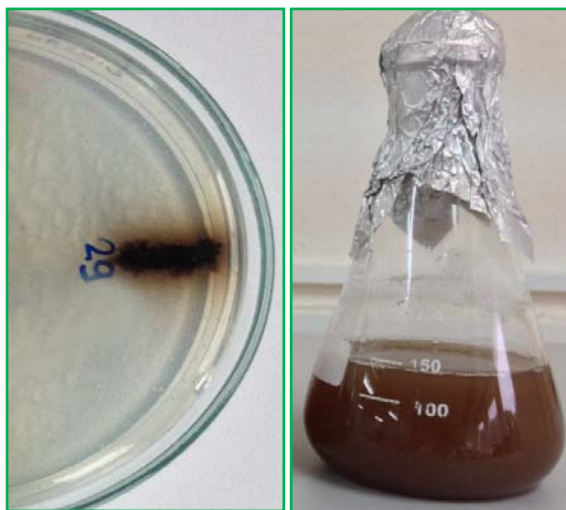


Figure 2. Growth and activity of the *Bacillus* sp. MV29 isolate on the medium containing L-tyrosine and CuSO_4 , after 72 h the medium was black-brown.

Optimization of culture conditions for enzyme production

Effect of incubation time for enzyme production

The *Bacillus* sp. MV29 isolate was cultured on production medium (yeast extract 0.15%, tryptone 0.15%, NaCl 0.5% and L-Tyrosine 0.1%) supplemented with L-

tyrosine 0.05% and CuSo4 (100µM) and incubated at 37°C and 175 rpm for 96 hours. Testing of the enzyme activity at 4 hours intervals shows the strain to produce optimum amount of active enzyme at 72th hour. Incubation beyond 72 h was undesirable as this resulted in decreased enzyme yields. The reason for this might have been due to the denaturation of the enzyme caused by the interaction with other components in the medium and probably due to depletion of nutrients available to microorganism [29].

Effect of pH and temperature on tyrosinase enzyme production

The hydrogen ion concentration of an environment has the maximum influence of the microbial growth and enzyme production. According to the earlier researches, there is an optimum pH range between 6.0 and 7.0 for the bacterial strains growth and enzyme production [20]. The *Bacillus* sp. MV29 produced maximum amount of tyrosinase enzyme at pH, 7.0, in accordance with this finding, Parka and et al. [31] have showed the optimum pH of 7.0 for enzyme production by *Bacillus* sp. After pH (9.0) the amount of active enzyme was dropped drastically. It might be due to the fact that Changes in pH can change the shape of the active site and during high or low pH concentrations result in loss of enzyme activity due to denaturation [39].

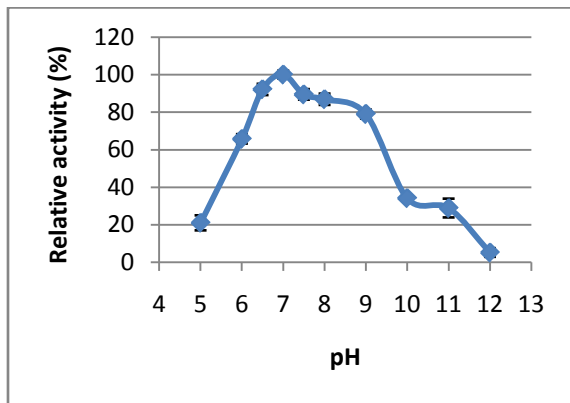


Figure 3. Effect of pH on enzyme production. The *Bacillus* sp. MV29 produced maximum amount of the enzyme at pH, 7.0. the lower and higher pH negatively affected the yield. At pH, 12 and pH, 13 the yield was reached to 21.05% and 5.26% of maximum yield.

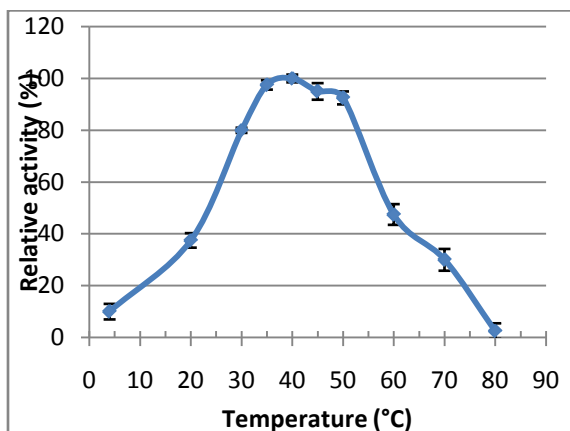


Figure 4. Effect of the temperature on enzyme production by the *Bacillus* sp. MV29. Optimum temperature for enzyme production was determined as 40°C; the enzyme production was dropped off at 5 and 80°C.

The incubation temperature is the next most critical factor that has to be optimized for enzyme production. The optimization of temperature is very important as it determines the velocity of the enzyme reaction. All enzymes have an optimal temperature at which reaction rates go fastest without denaturing the enzyme [41]. Many of the enzyme production by *Bacillus* sp. have been carried at temperature of (30-40 °C). The *Bacillus* sp. MV29 showed maximum tyrosinase enzyme production at 40°C which is in the range *Bacillus* sp. optimum temperature. The results of this paper were in line with the results that showed the optimum temperature of 37 °C for tyrosinase enzyme production from *Bacillus* sp. [11].

Effect of carbon and nitrogen sources on the tyrosinase enzyme production

The medium components play a significant role in enhancing the enzyme production [19]. Also according to the paper published by Surwase et al., the culture medium plays an important role, as the culture medium should provide all the essential nutrients required by the organism for enzyme production [41]. In analysis of the effect of various carbon sources on tyrosinase enzyme production by the *Bacillus* sp. MV29, it was observed that by addition of 0.1% of some carbon sources such as Glucose, Starch, Maltose the yield was increased to 116.6%, 110% and 113.3% respectively, while the Glycerol and Fructose decreased the yield to 66.6 and 83.3 respectively. All of the

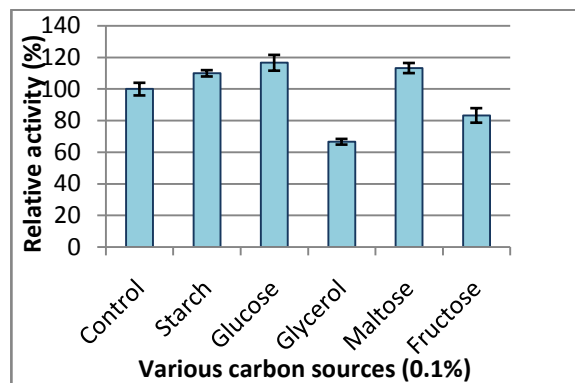


Figure 5. Effect of various carbon sources on tyrosinase enzyme production by the *Bacillus* sp. MV29, the glucose, starch and maltose was positive effect while the glycerol and fructose was negative effect on the tyrosinase enzyme production.

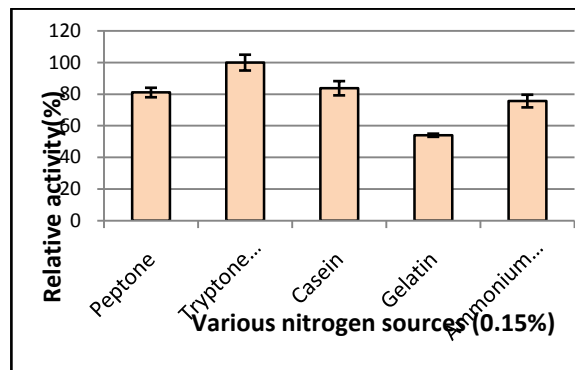


Figure 6. Effect of various nitrogen sources on tyrosinase enzyme production by the *Bacillus* sp. MV29, maximum amount of tyrosinase enzyme production was obtained when the tryptone was used as nitrogen source.

carbon sources more than 0.2% blocked the enzyme production completely. Carreira et al. [6] showed that glucose was the best carbon source for melanin production by yeast while Dastager et al. [12] indicated that starch was the effective carbon source for *Streptomyces* sp. followed by glycerol and fructose. Chaskes et al. [7] had a different opinion: In the case of *Cryptococcus gattii*, the carbon source was fructose. This research showed the tryptone (0.2%) as best nitrogen source for tyrosinase enzyme production, of course, the casein was also good for enzyme production. This can be resulted from that tryptone is an enzymatic digest of casein and both of them have the same composition. More than 0.2% of tryptone decreased the amount of tyrosinase enzyme, for example addition of 0.5% of tryptone to medium decreased the yield about 60%. The presence of yeast extract (0.1) was needed to use along with other compounds of medium. Without yeast extract, the strain didn't produce detectable enzyme. Yeast extract with concentration more than 0.1% steadily decreased the amount of tyrosinase enzyme. The result of this study was similar to findings of Survas et al. [40] who showed the effect of tryptone on tyrosinase production by RSM methodology. Production of melanin using casein as a nitrogen source by *Bacillus thuringiensis* [8] and using tryptone as a nitrogen source by *Bacillus cereus* [43] are in accordance with the result of this research. The effect of yeast extract was significant in this research. It probably is because of vitamins complex in yeast extract. This finding was in contrast with the result of other scientists who showed yeast extract as a non-significant for tyrosinase enzyme production or tyrosinase activity [41].

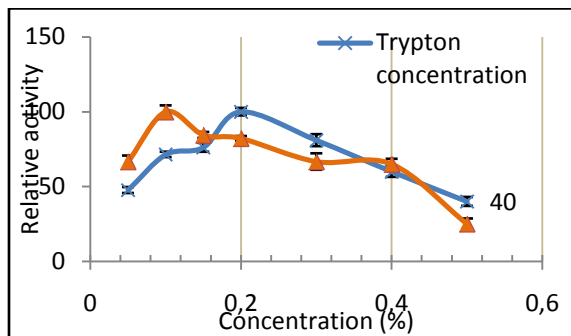


Figure 7. Effect of tryptone and yeast extract concentration on tyrosinase enzyme production by the *Bacillus* sp. MV29, maximum amount of the enzyme was obtained at tryptone, 0.1% and yeast extract 0.2%.

Effect of L-tyrosine and trace element concentration on tyrosinase enzyme production

Due to the some previous reports, supplementation of culture medium by L-tyrosine enhances the production of tyrosinase enzyme by *Bacillus* sp. So in this research by incorporation of various concentrations (0.2-3mg/ml) of L-tyrosine to medium, the concentration of L-tyrosine which had maximum effect on enzyme production was selected. The result of this research pointed at 0.4 mg/ml of L-tyrosine as optimum concentration for enzyme production. This result was in accordance with others findings [40, 41]. Supplemented of medium with 0.1% trace element increased the yield, while concentration more than 0.1% of trace element negatively affected the enzyme production.

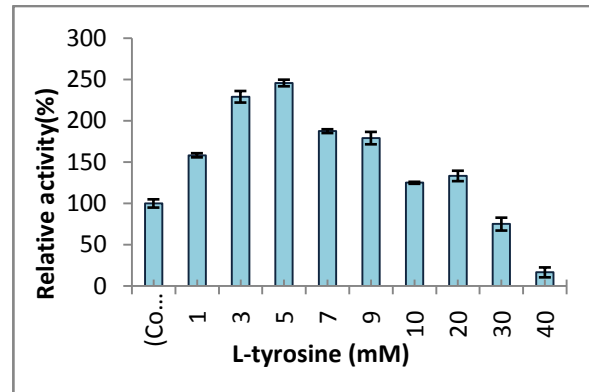


Figure 8. Effect of L-tyrosine concentration on the tyrosinase enzyme production. 5mM was optimum concentration for the enzyme production while the concentration more than 20mM negatively affected the yield.

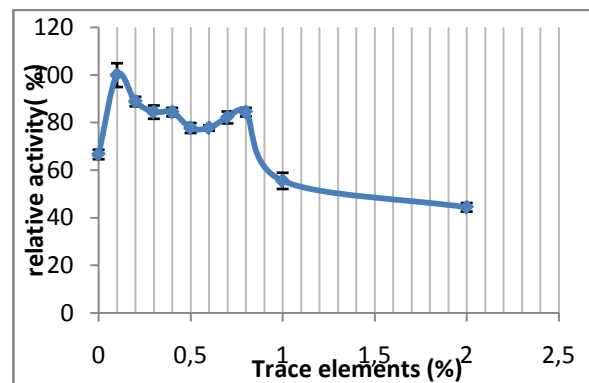


Figure 9. Effect of trace element concentration on the tyrosinase enzyme production. Maximum tyrosinase enzyme was obtained when 0.1% of L-tyrosine was used in medium nevertheless the concentration above 0.1% decreased the yield.

Evaluation of L-DOPA produced by the M36 tyrosinase enzyme

In TLC analysis, the transformation of L-tyrosine to L-DOPA was conspicuously detected. Also in quantitatively assay of L-tyrosine conversion to L-DOPA by using both standard graph of L-tyrosine and L-DOPA, the following result was obtained: After one hour incubation of reaction mixture having 0.744 mg/ml L-tyrosine, the remained L-tyrosine was 0.33mg/ml and obtained L-DOPA was 0.3454 mg/ml.

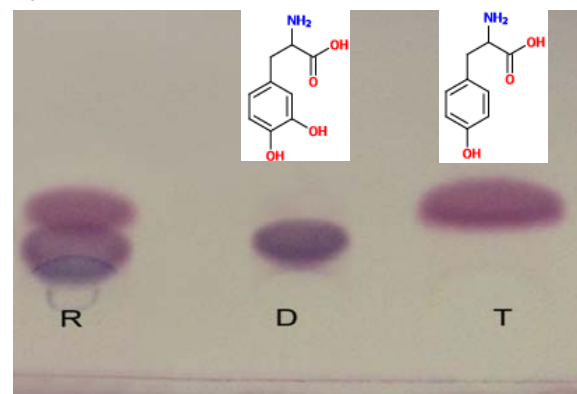


Figure 10. TLC analysis of L-tyrosine conversion to L-DOPA by *Bacillus* sp. M29 isolate. The lane of R represents the reaction solution, the lane D represents L-DOPA and lane T represents L-tyrosine.

CONCLUSION

Furthermore 40°C as optimum temperature, pH 7.0 as optimum pH, 48h as optimum incubation time was determined for the enzyme production. The addition of L-tyrosine (5mM) as a substrate to the production medium was highly effective on enzyme production. Consequently, after optimization, 0.7IU tyrosinase enzyme per milliliter of medium culture was obtained.

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