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INCREASED PRODUCTION OF TYROSINASE FROM *BACILLUS MEGATER IUM* STRAIN M36 BY THE RESPONSE SURFACE METHOD

Ebrahim Valipour* and Burhan Arikan

Biotechnology Department, Institute of Basic and Applied Sciences, Cukuruva University, Adana, Turkey

***Corresponding author:** va.cell@yahoo.com

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Abstract: The bacterial enzyme tyrosinase, with its high oxidizing capacity, can be applied in phenolic biotransformation, pharmaceutical, cosmetics and textile industries. In this research, a native *Bacillus* sp.-producing tyrosinase was isolated from a soil sample. The strain was identified by morphological, biochemical and molecular tests using bioinformatics analysis, and was named *Bacillus megaterium* strain M36. According to the blast analysis of 16S rDNA (1434 bp), the strain showed 99% identity with *Bacillus megaterium* DSM319. The production of tyrosinase from the isolated strain was optimized by classic and response surface methods (RSM). The optimal conditions for tyrosinase production by the strain were determined to be as follow: growth temperature 36°C, pH of medium 7.0, incubation time 16 h, with medium containing 0.4 mg/mL L-tyrosine, 0.05% yeast extract, 0.423% tryptone, 3.4% NaCl and 148.4 μM $CuSO_4$. Results of experiments performed under the optimized condition showed an actual yield of 0.522 IU of enzyme, while the result under the initial conditions using basal medium (before optimization) gave 0.0312 IU of enzyme (16.7-fold increase). SDS-PAGE analysis showed that the tyrosinase enzyme from *Bacillus megaterium* strain M36 is about 34 kDa.

Key words: *Bacillus megaterium*; tyrosinase; 16S rDNA; RSM; optimization

INTRODUCTION

Tyrosinase is a type 3 copper-containing enzyme that has been found widely distributed in microorganisms, plants and animals [1]. Tyrosinase catalyzes the hydroxylation of monophenols to *o*-diphenols and the oxidation of diphenols to *o*-quinones followed by a series of nonenzymatic steps resulting in the formation of melanin [2]. In mammals, tyrosinase catalyzes the biosynthesis of melanin pigments, which provide a fundamental part of the protection of skin against UV radiation. It is also related to the browning reactions of fruit and vegetables [3]. Tyrosinase plays an important role in wound healing and the primary immune response of plants, sponges and many invertebrates [1]. In fungi, this enzyme is of crucial importance in survival and virulence, reproductive organ differentiation, spore formation and tissue protection after injury [4]. In bacteria, tyrosinase enzyme is the key enzyme in initiating the melanin biosynthesis pathway and plays an important protective and survival role. Tyrosinases have several biotechnological applications due to their ability to oxidize both small phenolic molecules and protein-associated phenolic groups, i.e. the side chain of L-tyrosine. One of their applications is in the bioremediation of contaminant soil and wastewater containing phenolic compound [5] and dyes. [6,7]. The phenolic compounds are transformed by the enzyme to quinines, which are auto-oxidized to form insoluble polymeric compounds precipitating in water. Also, the enzyme has an important role in biosynthesis and medical applications such as the production of L-DOPA, the preferred drug in the treatment of Parkinson's disease [8], production of hydroxyl tyrosol as a food additive [9], production of estrogenic compounds [10], production of melanin for therapeutic uses [1], treatment of neurological diseases [11] and production of antibiotic lincomycin [12], cosmetic application as a self-tanning agent [13] and the production of dye [14]. Another use of the enzyme is in food manufacturing, such as the production of theaflavins, a major group of polyphenol compounds

in black tea with strong antioxidant, anticancer and other bioactive properties [15].

Optimization of the culture conditions (nutritional and physical parameters) for enzyme production is of crucial importance. Production efficiency can be increased and the production process can be economized by this approach. Classical optimization was carried out using a one-factor-at-a time method. Today, researchers tend to use static methods, such as response surface methodology (RSM), for enzyme production, which provides important practical information [16]. The objective of this study was to isolate and identify a *Bacillus* sp. producer of tyrosinase and to determine the best conditions for increased production using a *Bacillus* sp. isolate.

MATERIALS AND METHODS

In this study, all components for making growth medium were bought from Sigma-Aldrich (St. Louis, USA) or/and Merck KGaA (Darmstadt, Germany).

Isolation of tyrosinase-producing bacterial strains

Soil samples were gathered from a tomato field in the city of Adana in Turkey. Two g of the soil material were dissolved in 10 mL of sterile water and subjected to heat-shock treatment to preselect/isolate *Bacillus* sp. strains. Then the isolated strains were cultured on T medium (composed of 1.5 g tryptone, 1.5 g yeast extract and 5.0 g NaCl per liter with a final pH of 7.0) supplemented by L-tyrosine (1%) and $CuSO_4$ (100mM), and incubated at 37°C for 72 h. Colonies that formed black or black-brown zone on agar medium containing tyrosine, which formed a clear zone in medium without tyrosine, were selected as candidate colonies possessing tyrosinase activity [12,17,18]. The strain (*Bacillus* sp. M36) producing the largest black-brown zone was cultured in broth medium (the same as the above medium without agar) at 37°C for 72 h with shaking (150 rpm). After that, the amount of tyrosinase activity in the medium culture was evaluated by UV-Vis spectrophotometry at 475nm [13,19]. To determine whether the observed brown color was due to the presence of manganese oxide, small pieces of Whatman paper soaked with 5 M $MnCl_2$ were placed at one end of the plates with *Bacillus* sp. M36 and monitored for one week [14,18,20].

Identification of isolated bacterial strain

Identification of the strain was carried out by studying its morphological and biochemical characteristics according to the methods described in Bergey's Manual of Systematic Bacteriology [21,22]. In order to analyze the 16S rDNA sequence of the isolate, primer pairs (F= GCCTAATACATGCAAGTCGA, R= TACGGYTACCTTGTTACGACT) were designed using the Clone Manager.9 and Oligo.7 programs in such a way that they could amplify (≥1400bp) the 16S rDNA sequence of *Bacillus* sp. The primers were synthesized by Integrated DNA Technologies, Inc. (IDT)". Genomic DNA of the *Bacillus* sp. M36 was extracted and purified using a Wizard® Genomic DNA Purification Kit (Promega) and used as a template to amplify the 16S rDNA sequence by PCR. A DNA thermal cycler (Eppendorf) was used with the following program: initial denaturation at 94°C for 4 min, followed by 30 cycles of melting at 94°C for 30 s, annealing at 50°C for 30 s, elongation at 72°C for 90 s, and a final extension step at 72°C for 5 min. After purification by the Wizard® SV Gel and PCR Clean-Up System, the PCR product was ligated to pGEM®-T Easy Vector and transformed to *E. coli* JM109-competent cells. Transformants with a recombinant vector (containing 16S rDNA fragment) were screened by spreading the transformation mix on LB medium containing ampicillin and X-gal. Then the screened colonies were analyzed by direct colony PCR using the designed primers for amplification. Thereafter, the vector containing a 16S rDNA fragment was extracted and purified by Wizard®Plus SV Minipreps DNA Purification System and sequenced using pUC/M13 Primers, ABI PRISM® BigDye Terminator Cycle Sequencing Kit and Applied Biosystems® Genetic Analysis system with the Sanger sequencing method [23].

Phylogram analysis

The obtained sequence was subjected to nucleotide blast, alignment analysis and construction of a phylogenetic tree by maximum composite likelihood [24] using http://blast.ncbi.nlm.nih.gov/Blast.cgi , http://www.ebi.ac.uk/ and MEGA6 software [25]. The evolutionary distances were computed using the maximum composite likelihood method based on the Tamura-Nei model [26]. A discrete gamma distribution was used to model evolutionary rate differences among sites (2 categories (+G, parameter = 0.2376)). There was a total of 1280 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [25].

Optimization of culture condition for tyrosinase production

In order to optimize production of the enzyme, the culture condition, such as carbon and nitrogen sources, pH values, temperature and incubation time, were analyzed. The initial medium used for screening of bacterial strains showing tyrosinase activity was modified in order to optimize enzyme production. To determine the effect of temperature and pH on the growth and enzyme production, experiments were carried out at various temperatures (20-80°C) and different pH (5-12) (optimal pH and temperature were selected) [17,27]. In order to optimize incubation time, the *Bacillus* sp. M36 strain was cultured at optimum temperature and pH, followed by monitoring of the enzyme activity (by measuring OD_{475}) for 24 h. For the selection of the optimal ingredients of the growth medium, the effect of different nitrogen sources such as peptone, casein, gelatin and ammonium nitrate on enzyme production was studied using each of these compounds as a nitrogen source instead of tryptone. In that way the best nitrogen source was selected. After that, different carbon sources (starch, glucose, glycerol, maltose and fructose) were used (with optimal nitrogen source) and their effect on enzyme production was evaluated. Also, in this study, by incorporating different concentrations of L-tyrosine (0.2-3 mg/mL) in the medium, the concentration with the maximal effect on enzyme production was selected. Finally, the amounts of yeast extract, nitrogen source, NaCl and $CuSo_4$ were optimized according to the Box-Behnken design of the RSM using Design Expert Software (trial version 9) (Table 1). The experimental design of 29 experiments with five central points was formulated and the experiments were conducted in three replicates in 100-mL Erlenmeyer flasks containing growth medium prepared according to the design, inoculated with the *Bacillus megaterium* M36 and incubated for 16 h in a shaker incubator (175 rpm) at 36°C. Production of the enzyme was expressed in international units (IU) of enzyme by measuring the amount of dopachrome by spectrometry at OD_{475} and ANOVA. The IU of the enzyme was calculated using equation 1 [28].

Table 1. The levels of variables chosen for the Box-Behnken optimization experiments.

Variable	unit	Levels (-1)	Levels (0)	Level (+1)
Yeast extract	g/100 mL	0.05	0.525	1
Tryptone	g/100 mL	0.05	0.225	1
NaCl	g/100 mL	1	3	5
$CuSo_4$	µM	50	175	300

Eqn. 1.

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

A second order polynomial model that described the relation between the response and the chosen variables was developed and is provided in the following equation:

Eqn. 2.

$$Y = \alpha_0 + \sum_{j=1}^{k} \alpha_j x_j + \sum_{j=1}^{k} \alpha_{jj} x^2_j + \sum\sum \alpha_{'j}\, x_{'}\, x_j$$

where Y is the response, α_0, α_j, α_{jj}, α_{ij} are the regression coefficients for the intercept, linear, quadratic and interaction effects, respectively, and X_i and X_j are coded independent variables.

Enzyme production and partially purification

After optimization of the culture conditions, the *Bacillus* sp. M36 strain was incubated in the shaker incubator for 16 h; then the cell culture was centrifuged at 6000 g for 10 min at 4°C. The obtained supernatant was centrifuged again under the same conditions (6000 g for 10 min at 4°C) and the obtained cell-free supernatant was stored at 4°C. The pellet was washed twice in 50 mM of ice-cold potassium phosphate buffer, pH 7.0. After that the pellet was resuspended in 0.1 M of sodium phosphate, pH 7.0, containing a bacterial proteases cocktail inhibitor (1 µL/4 mg cell mass) and disrupted by sonication. The homogenate was centrifuged at 14000 g for 15 min. The clear supernatant achieved by the previous centrifugation (at 6000 g) and the supernatant obtained by centrifugation at 14000 g were precipitated with ammonium sulfate (40, 50, 60, 70, 75, 80, 85 and 90% saturation) for 1 h with gentle stirring at 4°C [12,29,30]. After fractionation with ammonium sulfate, the precipitated proteins were dialyzed against 50 mM sodium phosphate buffer, pH 6.8, with 0.02% sodium azide and 0.01 mM CuSO4. The fractions were tested for tyrosinase activity and active fractions were stored at -20°C without loss of activity [31]. The quantity of proteins in the samples was determined by the Bradford method using bovine serum albumin (BSA) as the standard [32].

Electrophoretic study

The active enzyme solution was loaded in 8 wells of nondenaturing PAGE (8% w/v), and after separation of the proteins, each line of the gel was sliced into thin strips using a clean scalpel. By placing each gel slice in substrate solution (0.1 mg/mL l-tyrosine and 50µM $CuSO_4$ in 0.1M phosphate buffer, pH 7) for 60 min, the tyrosinase-related band was visualized as a dark-brown band as a result of tyrosinase activity. The protein band was cut out from the gel slice, homogenized, resuspended in 50 mM phosphate buffer and left overnight at 4°C. The gel suspension was centrifuged at 12000 g for 10 min to remove the remaining gel fragments and the obtained supernatant (containing tyrosinase) was subjected to SDS-PAGE (12%) analysis for determination of the tyrosinase molecular weight using a protein marker (Fermentas) [33].

Table 2. Biochemical and morphological characteristics of *Bacillus megaterium* strain M36.

Characteristic/biochemical test	Observation
α-hemolysis	Negative (-)
β-hemolysis	Negative (-)
Catalase	Positive (+)
VP (Voges-Proskauer)	Negative (-)
Methyl Red	Negative (-)
Citrate	Positive (+)
Starch	Positive (+)
Gelatin	Positive (+)
Growth on medium containing 6.5% of NaCl	Positive (+)
Gram's reaction	Positive (+)
Cell shape	Rod-like
Cell size	About 5 µm

RESULTS AND DISCUSSION

Isolation and identification of *Bacillus* sp. producing tyrosinase

In this study, the *Bacillus* sp. M36 possessing tyrosinase activity (0.05 IU/mL in medium culture) was selected for enzyme production. According to the test using Whatman paper containing $MnCl_2$, the dark-brown pigmentation produced on the plate by *Bacillus* sp. M36 was independent of Mn^{2+}. Morphological and biochemical analysis of the M36 strain showed the characteristics listed in Table 2, indicating that the isolate belongs to the genus *Bacillus*. The 16S rDNA (1434 bp) of *Bacillus* sp. M36 was amplified (Fig. 1) and sequenced by Applied Biosystems® Genetic Analysis system. BLAST analysis showed 99% identity of *Bacillus* sp. M36 with *Bacillus megaterium* DSM319. The phylogram was created using MEGA 7 software (Fig. 2). Previously described criteria were used for this study: identification to the species level was defined as >99% identity of the 16S rRNA gene sequence with the sequence of its closest bacterial relative in the GenBank database, and identification at the genus level was defined as >97% identity of the 16S rRNA

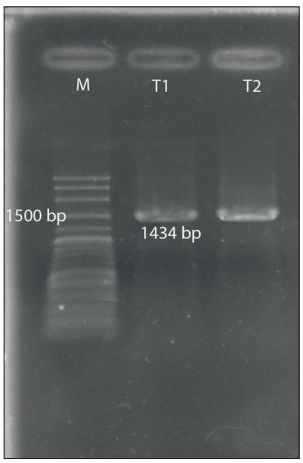

Fig. 1. Amplified 16S rDNA (1434bp) of *Bacillus* sp. M36 in a 0.8% agarose gel, lane M: DNA ladder from Vivantis; lanes T_1 and T_2: amplicons of 16S rDNA of *Bacillus sp.* M36.

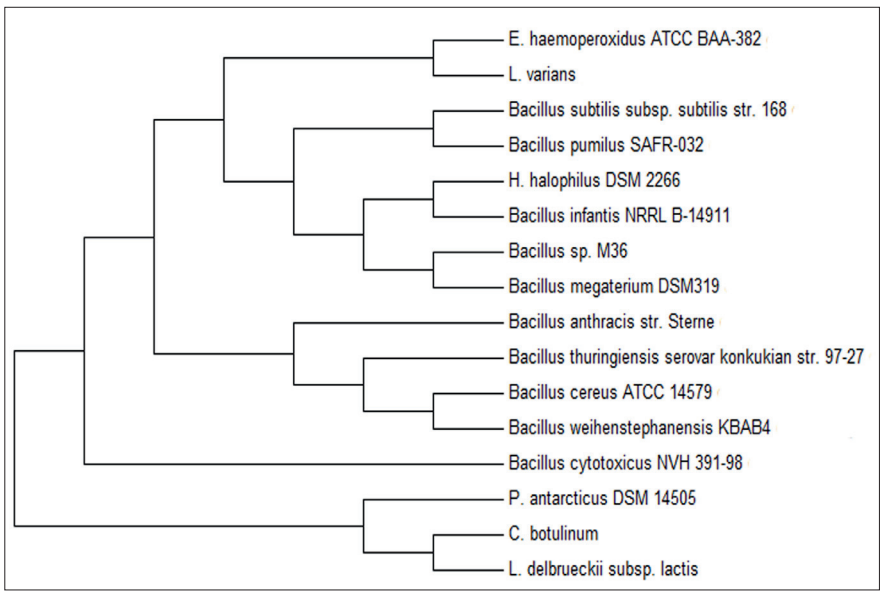

Fig. 2. Maximum composite likelihood tree showing the phylogenetic position of *Bacillus* sp. M36 and some related *Bacillus* species based on partial 16S rRNA gene sequences (1434 nucleotides). Sequence accession numbers are given in parentheses.

gene sequence with the sequence of its closest bacterial relative in the GenBank database [34]. This result matched the results obtained by Shuster and Fishman [20], who isolated and studied tyrosinase from *Bacillus megaterium.*

Optimization of culture condition for enzyme production

Classical optimization

The used pH range demonstrated the impact on the bacterial growth and affected enzyme stability in the medium. According to earlier research findings, the optimal pH range is between 6.0 and 7.0 for the bacterial strain's growth and enzyme production [35,36]. In this study, we found that the optimum pH for tyrosinase production by the *Bacillus megaterium* strain M36 isolate was 70.0 (Fig. 3a). Similarly, Park et al. [37] showed that pH of 7.0 was the optimum for enzyme production by *Bacillus megaterium*. At pH 8.0 enzyme production declined drastically. This might be due to the inactivation of the enzyme in the alkali medium [38]. An important parameter affecting growth and metabolite production by microorganisms is temperature, for which the optimum usually varies from one organism to another [39]. Enzyme production by *Bacillus* sp. has been carried out at different temperatures from 30-40°C. The *Bacillus megaterium* strain M36 showed maximum tyrosinase production at 36°C, which is in the range of optimal temperatures for *Bacillus megaterium* (Fig. 3b) showed by different studies. The yield was very low at temperatures lower and higher than 36°C, so it was decreased by about 50% at 50°C and by about 75% at 10°C. The results of this paper are in line with the results that showed an optimum temperature of 37°C for tyrosinase production by *Bacillus* sp. [17]. The *Bacillus megaterium* strain M36 was cultured in liquid culture medium, incubated at 36°C, and OD_{530} of the culture was monitored for 19 h. The OD_{530} increased until 10^{th} hour of incubation and reached a plateau due to the culture entering a stationary phase, which was not changed until 16^{th} hour. In parallel with OD_{530} analysis, the amount of biomass yield was also monitored for 19 h and showed the same results. After the 16^{th} hour, the OD_{530} was increased exponentially, accompanied by the browning of the medium. As time

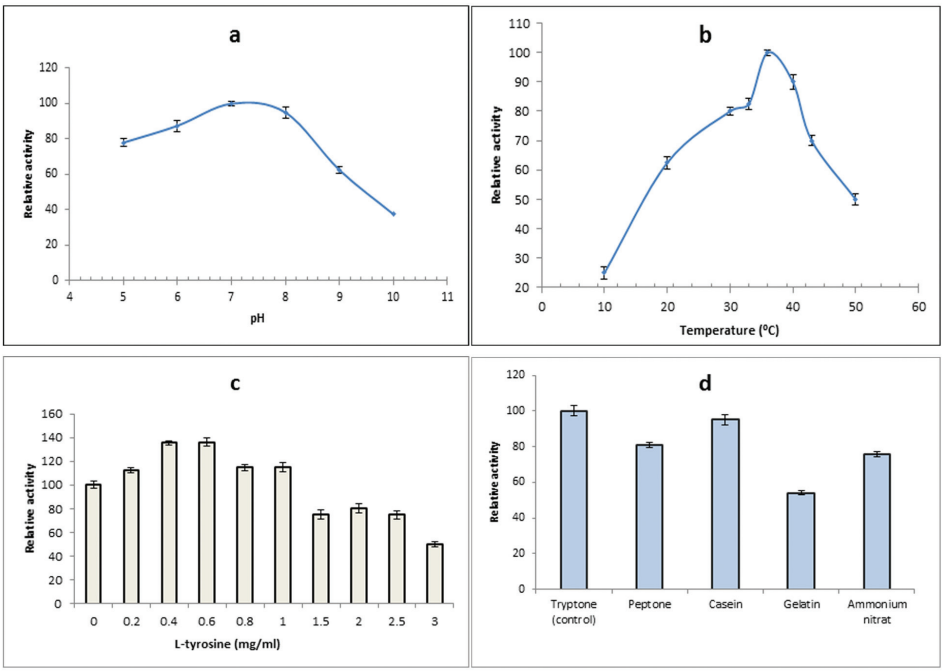

Fig. 3. The effect of different factors on tyrosinase production by *Bacillus megaterium* strain M36. **a)** The effect of pH; **b)** the effect of temperature; **c)** the effect of L-tyrosine concentration; **d)** the effect of various nitrogen sources.

passed by, the medium became black-brown and finally black. The maximum amount of tyrosinase was obtained at the 16th hour of incubation. Although the value of OD_{530} and blackness (production of melanin) of the medium was increased, the amount of active enzyme was decreased after the 16th hour. The decreases can be the result of proteolytic degradation. This result is in correlation with the results published by Surwase et al. [40], who obtained the maximum amount of tyrosinase at the 18th hour of incubation [41]. The positive effect of L-tyrosine on tyrosinase has been proved by many researchers [17,20]. Thus, in this study, by using various concentrations (0.2-3mg/mL) of L-tyrosine in medium, we determined the concentration of L-tyrosine with maximum effect on enzyme production. The obtained result indicated that the concentration of 0.4 mg/mL of L-tyrosine as optimal for enzyme production (Fig. 3c), which is in accordance with findings of other researchers [38,40]. It was found that nitrogen and carbon sources playing a prominent role in the growth and development of the bacteria. Our work showed that among the studied sources tryptone is the best nitrogen source for tyrosinase production. Of course, casein was also good for enzyme production (Fig. 3d). The addition of various carbon sources such as glucose, starch, etc. did not affect enzyme production by the *Bacillus megaterium* strain M36. The results showed that yeast extract should be used along with other compounds of the medium. Without yeast extract, the strain did not produce a detectable amount of enzyme. It is worth noting that when cultured in a mineral salt medium (without yeast extract), as done by Shuster and et al. [20] for 52 h, the *Bacillus megaterium* strain M36 could produce tyrosinase: however, the produced enzyme was less than that produced in the medium mentioned above for 16 h.

Statistical optimization

RSM is one of the statistical techniques to evaluate the interaction between experimental variables and measured responses, and to detect the optimal range of the variables within the design. In this study, the Box-Behnken method was used to optimize the four

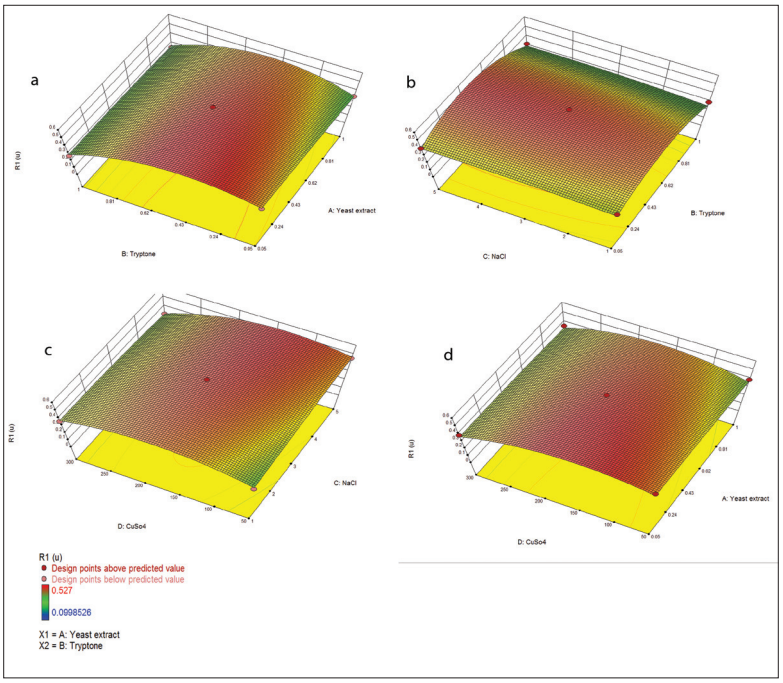

Fig. 4. Response surface plot of the combined effects of yeast extract, tryptone, NaCl and $CuSO_4$ on the tyrosinase enzyme production by *Bacillus megaterium* strain M36.

variables (yeast extract, tryptone, NaCl and CuSo4) to prepare tyrosinase. The F-value of the model (34.10) reveals its significance. The factors with Prob>F value less than 0.0500 were considered significant. In this case, A (yeast extract), B (tryptone), C (NaCl), D ($CuSo_4$), CD, B_2, D_2 were significant factors. An F value more than 0.1000 indicated the model was not significant. The predicted R-squared (0.8709) was very close to the adjusted R-squared (0.9430). The plot between predicted vs. actual was a good tool to study the significance of the suggested model. The responses predicted from the empirical model were in agreement with the observed values in the range of the operating variables. The ratio of 21.353 showed an appropriate signal. A second order polynomial equation given was constructed by using the estimated coefficients (in coded units).

Eqn. 3.

$$\text{Tyrosinase (IU)} = 0.19 - 0.011A - 0.020B + 0.006667 - 0.010D + 0.009\,AB + 0.0065\,AC + 0.007AD + 0.002BC - 0.009250\,BD - 0.016CD - 0.003975A^2 - 0.061B^2 - 0.006475C^2 - 0.045D^2$$

The equation in terms of coded factors was used to make predictions about the response for given levels of each factor. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients. The response surface curves represented in Fig. 4A-D shows the interaction between variants. The mutual effect of yeast extract and tryptone is represented in Fig. 4A. The lower and higher levels of tryptone decreased the amount of tyrosinase, while middle levels enhanced enzyme production. Maximum yield was obtained at a minimal concentration of yeast extract. When the concentration of yeast extract was increased, the enzyme production decreased steadily. The results of this study are similar to the findings of Surwas et al. [40] who showed the effect of tryptone on tyrosinase production using RSM methodology. Production of melanin using casein as a nitrogen source by *Bacillus thuringiensis* [42] and tryptone as a nitrogen source by *Bacillus cereus* [43] are in accordance with the results of our research. The significant effect of yeast extract is most probably because of the vitamin

complex present in yeast extract. This finding is in contrast with the result obtained by other scientists who showed that yeast extract has a nonsignificant effect on tyrosinase production or tyrosinase activity [40]. Fig. 4B shows the effect of NaCl and $CuSO_4$ on tyrosinase production. Increasing the concentrations of NaCl increased the level of enzyme production. The high and low concentrations of $CuSO_4$ had a negative effect on enzyme yield, while the middle concentration had a positive effect. $CuSO_4$ increases tyrosinase activity at lower concentrations because tyrosinase is a copper-containing enzyme. Higher levels of $CuSO_4$ might be toxic to the cells. In earlier reports, 0.25 mM of $CuSO_4$ was shown to be required for *Acremonium rutilum* [44] and 0.15 mM for *Pinguicula grandiflora* tyrosinase activity [45] (Fig. 4).

Validation of the experimental model

The optimum concentration of yeast extract, tryptone, NaCl and $CuSO_4$ predicted by the model were 0.05%, 0.423%, 3.4% and 148.4 μM, respectively. Also, the maximum amount of enzyme that can be produced under the optimum conditions was predicted to be 0.527 IU. According to the results, the optimum culture condition for tyrosinase production by the *Bacillus megaterium* strain M36 was obtained as following: temperature 36°C, pH 7.0, incubation time 16 h, 0.4 mg/mL l-tyrosine, 0.05% yeast extract, 0.423% tryptone, 3.4% NaCl and 148.4 μM CuSo4. Results of experiments under the optimized condition showed an actual yield of 0.522 IU of enzyme, while the result of the experiment under initial conditions using the basal medium (before optimization) gave 0.0312 IU of enzyme. A close correlation between the actual (0.522 IU) and predicted values (0.527 IU) was observed, which validates this model. This was the first time that the production of 0.527 IU of tyrosinase from native *Bacillus* sp. was reported. Shuster and Fishman [20] reported that the extracellular liquid of the *Bacillus megaterium* culture showed tyrosinase activity. They studied the enzyme by cloning because the enzyme produced by the native *Bacillus megaterium* was very low. Liu et al. [46] produced tyrosinase (0.62 unit/mL) from *Bacillus thuringiensis* subsp. Kurstaki.

Electrophoresis and enzymatic activities in gels

After dialysis of the ammonium sulfate precipitate sample, tyrosinase M36 was electrophoresed using native polyacrylamide gel (8%). After activity staining, a distinct band was detected. By extracting the tyrosinase from native gel using the method given in Materials and Methods, the enzyme was subjected to SDS-PAGE (12%) analysis. This analysis showed that tyrosinase is an enzyme of about 34 kDa (Fig. 5). This result is similar to that of Shuster and Fishman [33] who have demonstrated the tyrosinase from *Bacillus megaterium* is about 35 kDa.

CONCLUSION

One isolate of *Bacillus* sp. producing tyrosinase was isolated, identified and analyzed. By optimizing the culture conditions, the yield of 0.03 IU enzyme was increased to 0.522 IU. After optimization, the amount of enzyme considerably increased, indicating that culture growing conditions can greatly affect the production of the enzyme.

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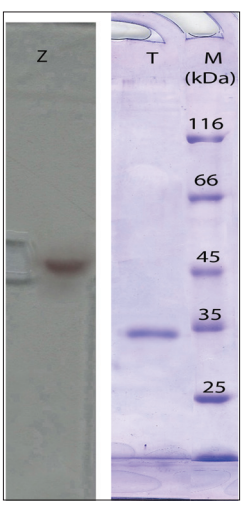

Fig. 5. Electrophoretic analysis of the tyrosinase M36. Lane Z shows tyrosinase activity, lane T shows the enzyme molecular weight about 34 kDa, almost 15 μg of protein was loaded, lane M shows the protein standard.

Conflict of interest disclosure: There is no conflict of interest associated with the present manuscript.

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