

Isolation, Identification and Medium Optimization for Tyrosinase Production by a Newly Isolated *Bacillus subtilis* NA2 Strain

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ARTICLE INFO

Article history:

Received on: 05/05/2018

Accepted on: 10/07/2018

Available online: 30/09/2018

Key words:

Isolation, Identification, *Bacillus*, Tyrosinase, Production, Yield coefficient.

ABSTRACT

Tyrosinase (EC 1.14.18.1, monophenol, O-diphenol: oxygen oxidoreductase) is a copper-metallo-bifunctional enzyme, which catalyzes the O-hydroxylation and oxidation of monophenols to quinones. Tyrosinase plays an important role in the immune defense mechanisms of many prokaryotic and eukaryotic organisms. It also finds many applications in antioxidants and therapeutic fields. In the present work, a newly isolated tyrosinase-producing strain (*Bacillus subtilis* NA2) was identified using 16S rDNA and BLAST analysis. The primary cultivation medium produced tyrosinase at 416.67 U/min/mL with a yield coefficient ($Y_{P/X}$) of 111.75×10^3 U/g cells. Furthermore, the cultivation medium for the production of tyrosinase was optimized. The final optimized cultivation medium composed of (g/L): sucrose, 10; KNO₃, 10; K₂HPO₄, 0.5; MgSO₄·7H₂O, 0.25; tyrosine, 10 and the initial optimized pH of the cultivation medium was 8.5. It has been shown, that the final optimized medium with the optimized pH resulted in an increase of about 39% in tyrosinase production from the initial un-optimized conditions. The new tyrosinase producing strain was identified and its volumetric production was significantly increased after the successful optimization of the production medium.

INTRODUCTION

Tyrosinase (EC 1.14.18.1, monophenol, O-diphenol: oxygen oxidoreductase) is a copper-metalloprotein that is ubiquitously dispensed in nature. Tyrosinase is a bifunctional enzyme and a monooxygenase that catalyzes the O-hydroxylation and subsequent oxidation of monophenols, O-phenols, to quinones (Claus and Decker 2006). Tyrosinase plays an important role in wound healing and the primary immune response of plant life, sponges and many invertebrate (Danial *et al.*, 2018; Decker and Tucek 2000). In human, tyrosinase produces melanin species as a defense against the harmful effects of UV light (Kumar *et al.*, 2011). In plants, it is necessary to synthesize phenolic polymers such as tannins, lignin, and flavonoids. In fungi, this enzyme has crucial importance in virulence and survives reproductive organ differentiation, protection of tissue after infection and spore

formation (Seo *et al.*, 2003). In bacteria, tyrosinase enzyme is the key enzyme in initiating the melanin biosynthesis pathway and has vital roles in their protection. Tyrosinase has been extracted, isolated and purified from various sources such as animals, plants, insects, and microorganisms (Saratale *et al.*, 2011; Xu *et al.*, 2012).

Due to oxidative stress properties, tyrosinase has biotechnological applications for both small phenolic compounds and protein-associated phenolic groups. One of their applications is in the bioremediation of polluted soil and wastewater containing phenolic compounds (Marino *et al.*, 2011). The phenolic molecules are changed by the enzyme to quinones, which are auto-oxidized to become insoluble polymeric compounds and precipitate in water. The enzyme has a significant role in the medical sector, for example, the production of L-DOPA, a favored medication for the treatment of Parkinson's disease (Franciscon *et al.*, 2012). The enzyme is also used in the production of hydroxytyrosol as an estrogenic intermediate (Zhang *et al.*, 2007), food additives (Decker and Tucek 2000), and melanin production for therapeutic applications (Pandey *et al.*, 1989).

Tyrosinase is found in prokaryotic microorganisms, as well as eukaryotic mammals, invertebrates, and plants. Various

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organisms have been used for the production of tyrosinase enzymes on a commercial scale, i.e. bacteria, fungi, and mushrooms, etc. Phytase genes from these organisms were later expressed in *Escherichia coli* and the enzyme production was studied in both batch and fed-batch cultivations (Allouche *et al.*, 2004). *Streptomyces* species isolated from soil were also used for the *in vitro* production of the extracellular tyrosinase (Rao *et al.*, 2013).

Until recently, tyrosinase was produced at a concentration of about 1g/L through the cultivation of the filamentous *Trichoderma reesei* (Caf *et al.*, 2012). Moreover, immobilized tyrosinase enzymes were used for the production of L-DOPA (Shuster and Fishman, 2009). The trade production and extraction of tyrosinase are reported from *Agaricus bisporus* mushroom. Extensive research has been carried out on the production and immobilization of tyrosinase from *A. bisporus*. Recently, Dalfard *et al.* (2006) isolated and characterized a melanogenic soil bacterium capable of producing tyrosinase as well as laccase enzymes, and reported a tyrosinase activity of 2.38 U/mL.

The studies reported here accomplished aimed at (i) isolation, identification of tyrosinase-producing *Bacillus* sp., and (ii) optimization of the culture conditions (nutritional and physical parameters) for increased production of tyrosinase using the isolated *Bacillus* sp.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals/reagents used throughout the work were of high-quality analytical grade and were purchased from Sigma-Aldrich Chemical Company, St. Louis, MO, USA.

Samples collection

One gram of soil (Soil from the potato-planted field, Mansoura Governorate, Egypt) was transferred to 9 mL sterile saline solution (under aseptic conditions), and the tubes were shaken well to get a dilution of 10^{-1} . Several serial dilutions were then made to obtain a proper dilution of 10^{-3} .

An aliquot of 0.1 ml of each dilution was transferred over agar plates containing (yeast extract 0.15%, tryptone 0.15%, NaCl 0.5% and L-Tyrosine 0.1%) and the brown-black colony forming strains as prospective strains were selected to have tyrosinase enzyme. The best of the strains, in terms of brown-black color development due to melanin production, was selected and subjected to enzyme production (Shuster and Fishman, 2009). The plates were incubated at 35°C for 24h.

Isolation of the tyrosinase producing strain

Among 30 different bacterial strains isolated a potent strain which gave a high yield of tyrosinase was chosen for further study. The bacterial strains were routinely grown on nutrient agar medium (Dalfard *et al.*, 2006) at 35°C for 3 days then preserved at -80°C in glycerol.

Identification of tyrosinase producing bacteria

DNA isolation and PCR amplification

An overnight culture of the promising bacteria grown at 35°C was used for the preparation of genomic DNA. The DNA

was isolated using Promega kit for preparation of genomic DNA according to the manufacturer's instructions. PCR amplification of 16S rDNA region was carried out according to Sambrook *et al.* (1989). The 16S rDNA was amplified by polymerase chain reaction (PCR) using primers designed to amplify 1500 bp fragment of the 16S rDNA region. The domain bacteria-specific primer 27F (forward primer) was 5'AGAGTTTGATCMTGGCTCAG3' and universal bacterial primer 1492R (reverse primer) was 5'TACGGYTACCTTGTTACGACTT3' (Edwards *et al.*, 1989).

Culture medium and cultivation conditions

Tyrosinase production was carried out in 250 mL flask contain 50 mL/flask of basal medium of Sharma *et al.* (2006) containing (g/L): casein,10; K_2HPO_4 , 0.5; $MgSO_4 \cdot 7H_2O$, 0.25; tyrosine, 10; (pH 6.5), then sterilized by autoclaving at 121°C. The inoculum was prepared by inoculating the flasks with a loop of the potent isolate and the flasks were then incubated on a rotary shaker at 200 rpm and 37°C (Innova 4080 New Brunswick Scientific CO., NJ, USA).

For the cultivation processes, the flasks were inoculated with 10% (v/v) of the previously prepared vegetative cells. The effect of various carbon sources, such as C4-carbon sources (malic and succinic acids), C6-carbon sources (glucose, fructose, galactose, mannitol, and sorbose), C-12 carbon sources (maltose, sucrose, and lactose), and polysaccharides (starch, dextrin and cellulose) was investigated. Each carbon source was separately autoclaved and individually supplemented to the basal medium in a 10 g/L concentration.

Various organic and inorganic nitrogen sources were individually supplemented in the basal medium as a substitute for casein in order to study their effect on tyrosinase production. The tested organic nitrogen sources (peptone, tryptone soybean, yeast extract, malt extract, and meat extracts) were added to the concentration of 10 g/L. On the other hand, the inorganic nitrogen sources under study (NH_4SO_4 , $(NH_4)_2SO_4$, $NaNO_3$, KNO_3 , and NH_4Cl) were added according to their nitrogen content to be equivalent to that of casein which was used as the control.

Concerning the effect of cultivation pH on growth and tyrosinase production, the broth medium was prepared in 250 mL Erlenmeyer flasks, and different pH values (5.0-10.0) were adjusted using 6 M HCl or 6 M NaOH and then autoclaved. At the end of each experiment, final pH, bacterial growth, total protein content, and tyrosinase activity were determined.

Determination of cell dry weight

Cell dry weight was determined spectrophotometrically at 600 nm according to El-Enshasy and Elsayed (2017). During cultivation, samples of cell growth were taken periodically and the O.D of the bacterial growth was measured after dilution to a range of 0-0.5. Afterward, the sample was filtered using Whatman No.1 filter paper, twice washed, dried overnight at 65°C, and then weighed to get the final cell dry weight. A standard curve, drawn between the cell dry weight and the corresponding optical density, was used to derive the linear cell dry weight equation.

Determination of total protein content

Protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard.

Determination of tyrosinase activity

Tyrosinase activity is assayed by using L-tyrosine and L-DOPA as substrates. The appropriate concentration of the enzyme 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. Dopachrome “colored intermediate” is an intermediate of melanin biosynthesis that is made from *O*-quinones by nonenzymatic oxidation. One international unit (IU) of tyrosinase activity is defined as the amount of enzyme required to oxidize 1 μ mol of L-tyrosine to dopachrome per minute under the above conditions (Danial *et al.* 2018).

Statistical analysis

All experiments performed were repeated for at least three times, and results were given as mean \pm SD. Data were analyzed with the help of SPSS 9.0, and the mean comparison between different evaluated parameters was performed using ANOVA one-way analysis of variance. Statistical significance was defined when $p < 0.05$.

RESULTS AND DISCUSSION

Isolation, identification and molecular characterization of tyrosinase producing strain

In this study, the *B. subtilis* possessing tyrosinase activity (250 U/min/mL in medium culture) was selected for enzyme

production. According to the test, of 16S rDNA (1434 bp) of the bacterial isolate was amplified and sequenced by Genetic Analysis Applied Biosystems. BLAST analysis showed 99% identity of *B. subtilis* with *B. subtilis* DSM10. The phylogram was created using MEGA 7 software (Figure 1). 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 >99% identity of the 16S rRNA gene sequence with the sequence of its closest bacterial relative in the GenBank database (Sambrook *et al.*, 1989). This result was in good agreement with those obtained by Hullo *et al.* (2001) who isolated and studied tyrosinase from *B. subtilis*.

The obtained 16S ribosomal ribonucleic acid (rRNA) sequence was compared with the National Center for Biotechnology Information (NCBI) Gen Bank database using the BLAST program. As a result, the isolated strain was confirmed to exhibit a very high homology with *B. subtilis* subs group (94%) in its 16S rRNA nucleotide sequence (Figure 1). According to the obtained results, isolates were identified as *B. subtilis* strain (NA2).

Optimization of cultivation conditions

The optimization of different cultivation conditions was achieved by cultivating the isolated strain (*B. subtilis* NA2) in the basal cultivation medium, followed by evaluating tyrosinase production, cell dry weight, final pH and total protein content.

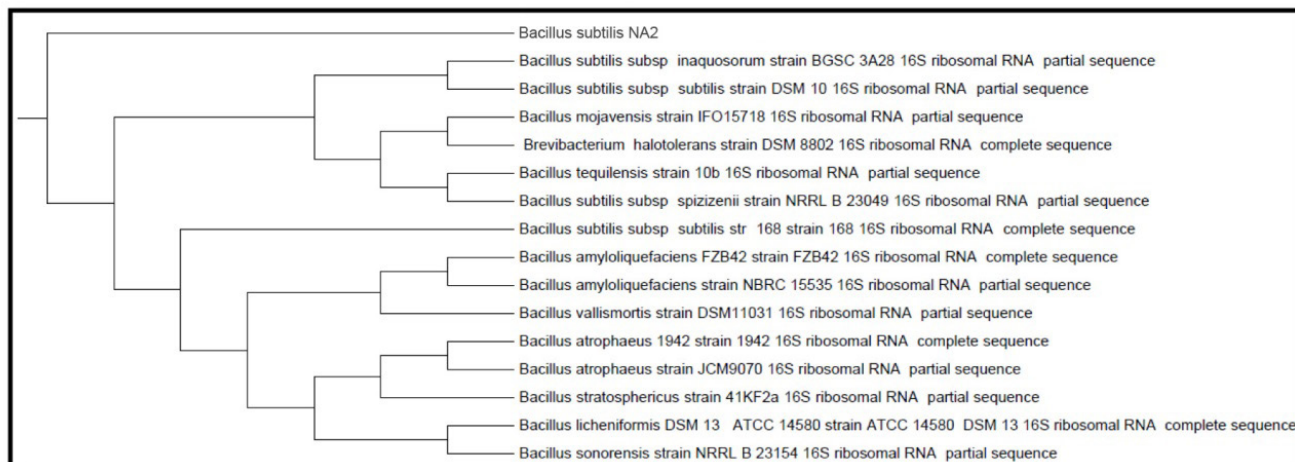


Fig. 1: Neighbor-joining phylogenetic representation of the strains and their closest relatives based on 16S rRNA gene sequences of *B. subtilis* NA2.

Kinetics of cell growth and tyrosinase production

The shake flasks containing the basal un-optimized cultivation medium were inoculated with the propagated cells and were then incubated as previously described. The obtained results (Figure 2) showed that cells grow exponentially with time with a cell growth rate of 0.1011 g/L/h and reached their maximal cell mass of 5.89 g/L after 60 h. After 60 h, cell growth started to slow down and decreased by the end of the cultivation to reach 5.39 g/L. Accordingly, tyrosinase production increased

with cell growth and reached a maximal of 416.67 U/min/mL by 48 h, after which tyrosinase production started to decrease and reached 246.67 U/min/mL at 72 h. During the exponential growth phase, tyrosinase was produced with an average production rate of 11.204 U/mL/h. Similarly, total protein content increased with time to reach a maximal of 844.8 mg/mL after 30 h. The data for yield coefficients showed that the highest tyrosinase production yield ($Y_{P/X}$) of 111.75×10^3 U/g cells was obtained after 36 h of cultivation.

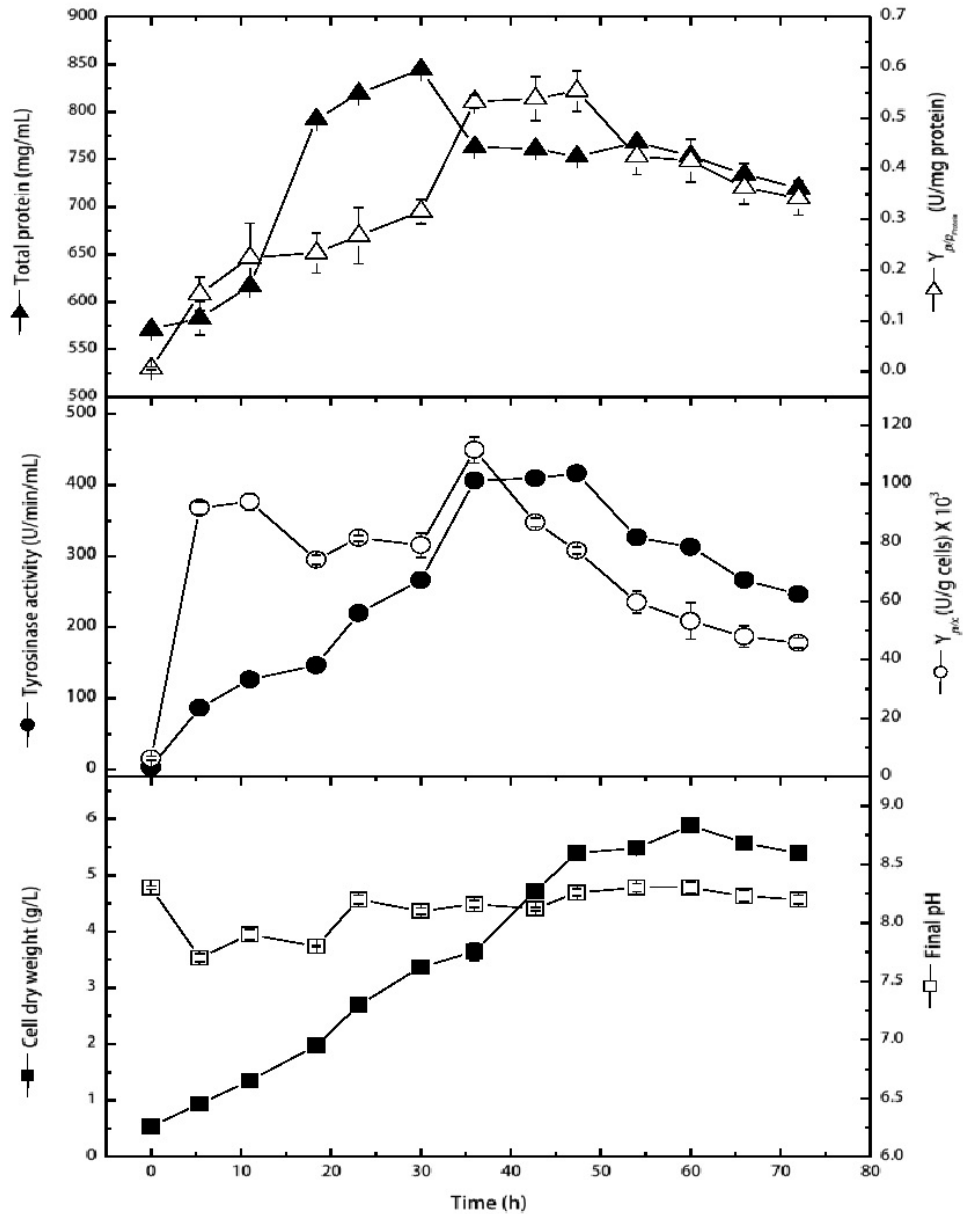


Fig. 2: Kinetics of cell growth and tyrosinase production by *B. subtilis* NA2.

The production of microbial enzymes depends mainly on the availability of suitable environmental conditions, i.e. physiological, nutritional and biochemical requirements. The obtained results can be correlated well with those obtained previously (Surwase *et al.*, 2012a; Valipour and Arikan, 2016). The authors reported the same growth and production profiles for tyrosinase production by *Brevundimonas* sp. SGJ and *B. megaterium* M36, respectively. Moreover, they also reported a similar decline in the production of tyrosinase after reaching the maximal productivity at 18 and 16 h of cultivation and attributed such decline to the proteolytic degradation of the enzyme. Additionally, the decline in tyrosinase production after the stationary phase could be explained on the basis of the depletion of the available nutrients from the cultivation medium as well as the action of protease enzymes during decline phase

(Herwing *et al.*, 2001; El-Enshasy and Elsyayed, 2017). Based on the aforementioned results the following optimization steps were performed, where the cultivation time was adjusted to 36 h.

Effect of different pH values on cell growth and tyrosinase production

The cultivation medium was prepared in different sets with varying pH values. The tested pH ranged from 5.0 to 10.0, and the flasks were then autoclaved, inoculated and incubated. After 36 h, the cultivations were terminated and assayed for cell growth and tyrosinase production parameters. Results illustrated in Figure 3 revealed that the pH range from 5.5-8.0 did not largely affect cell growth, where the mean cell mass ranged from 6.24-6.78 g/L. Furthermore, the highest cell mass of 7.60 g/L was obtained at pH 9.0. On the other hand, increasing the initial pH

of the cultivation medium greatly influenced the production of tyrosinase. Tyrosinase concentration increased gradually with increased initial pH and reached a maximal production of 453.33 U/min/mL at pH 8.5. Further increase in the initial pH resulted in a gradual decrease in the produced tyrosinase enzyme. Additionally,

tyrosinase production yield coefficient based on cell mass ($Y_{p/X}$) and produced protein ($Y_{p/Protein}$) increased with increasing the initial pH value and reached their maximal yields of 62.42×10^3 U/g cells and 0.55 U/mg protein, respectively.

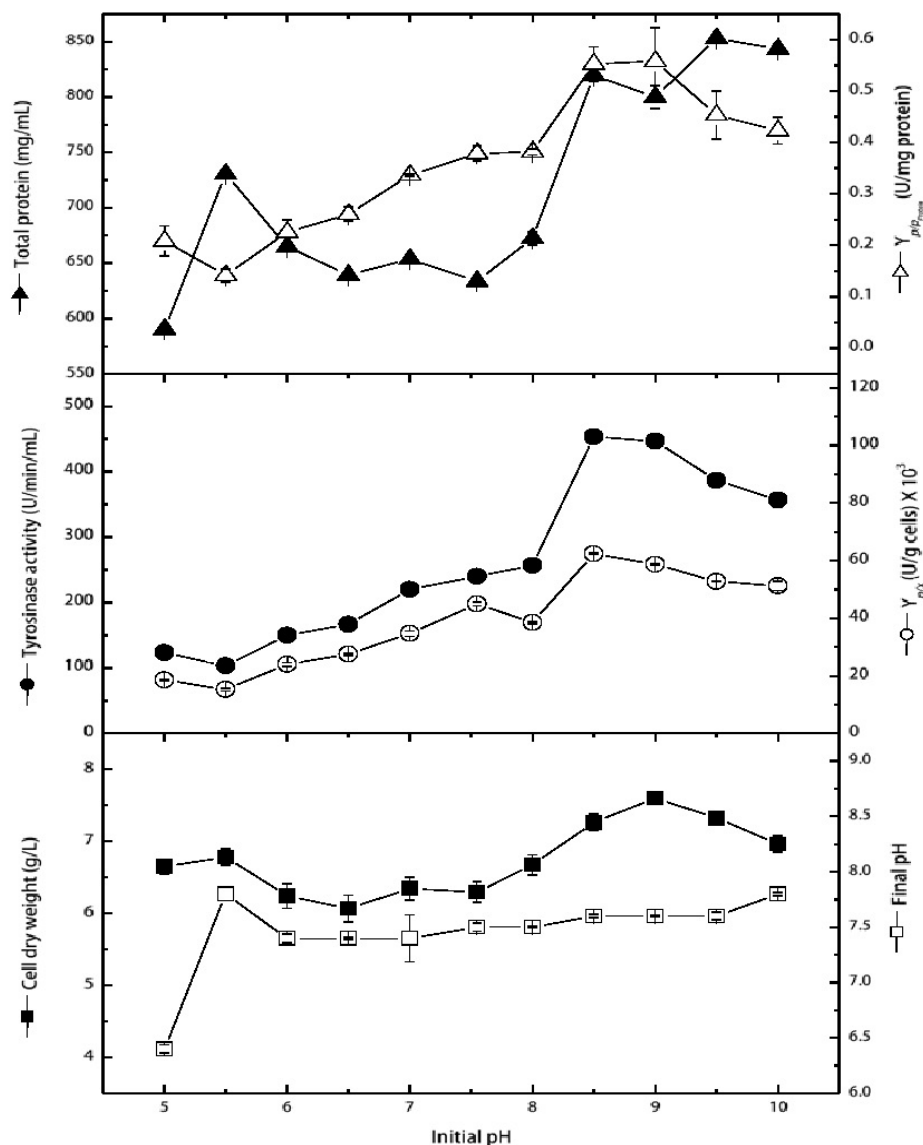


Fig. 3: Effect of different initial pH values on cell growth and tyrosinase production by *B. subtilis* NA2.

Generally, the producing microbe requires the pH of the cultivation medium to be set at the optimal and most favorable pH value. Suitable pH value is not only required for maximal growth, but also for higher enzyme productivity. This may be attributed to the fact that the transfer and uptake of nutrients and products across cell membranes is highly influenced by varying the concentration of hydrogen ions, which may lead to the alteration of spatial and structural geometries of various products (El-Enshasy and Elsayed, 2017). Accordingly, different microorganisms vary in their optimal pH values depending on their different biochemical and nutritional requirements. The obtained results are in good agreement with those of Rao *et al.* (2012), who found that pH

range from 4.5 to 8.5 supported their tyrosinase production by an isolated RSP-T1 strain, with an optimal production pH of 6.5. Moreover, Valipour and Arikan (2015) reported the same trend for their tyrosinase producing *B. sp.* MV29 strain and found that after pH 9.0, the amount of the active enzyme declined significantly due to the change of the shape and configuration of the active site of the enzyme. Additionally, Surwase *et al.* (2012b) found that pH 8.0 was most optimal for the conversion of L-tyrosine to L-DOPA by the action of the tyrosinase produced from *Brevundimonas sp.* SGJ. Collectively, such results can be explained by the fact that pH response is a strain-dependent characteristic. Therefore, the initial pH of 8.5 was chosen for the following optimization steps.

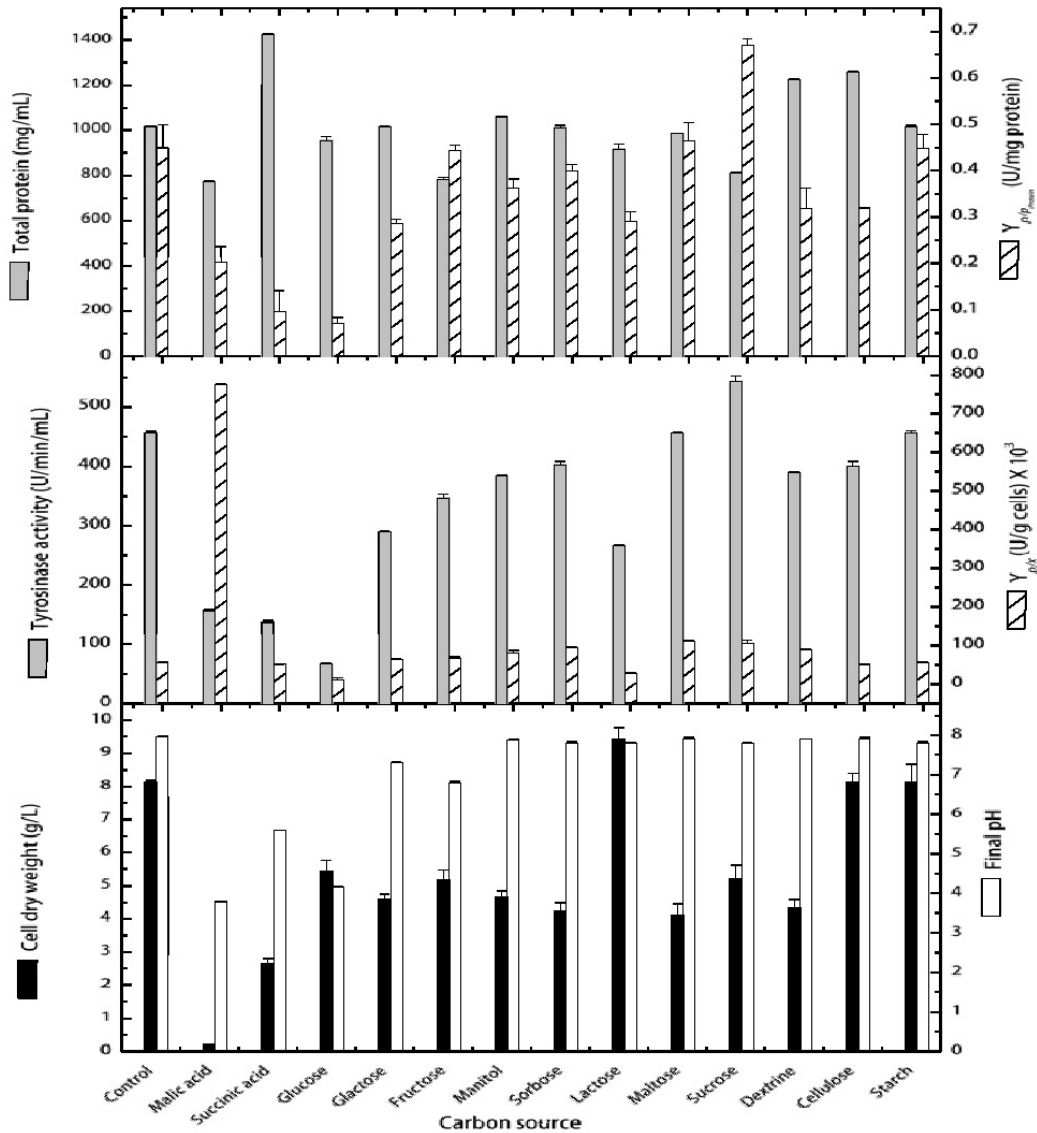


Fig. 4: Effect of different carbon sources on cell growth and tyrosinase production by *B. subtilis* NA2.

Effect of different carbon sources on cell growth and tyrosinase production

The cultivation medium was prepared to contain different carbon sources to evaluate their effect on cell growth and tyrosinase production. Tyrosine was added in 1% concentration as an inducer for the production of tyrosinase and was considered as the control cultivation medium. The other tested media contained 1% of the evaluated carbon sources. The obtained results (Figure 4) showed that the supplementation of the basal medium with either C4 or C6-carbon sources reduced both cell growth as well as tyrosinase production as compared to control cultivation medium containing only tyrosine. The addition of 1% malic acid or succinic acid reduced cell growth by about 97.5 and 67.6%, and tyrosinase production by about 65.7 and 70.1%, respectively. The addition of C6-carbon sources resulted in both moderate and variable cell growth and tyrosinase production; e.g. fructose produced 5.17 g/L and 346.67 U/min/mL, while sorbose produced 4.25 g/L and

403.33 U/min/mL, respectively. These values are lower than those obtained by the control cultivation, where tyrosine was added alone (8.13 g/L and 456.67 U/min/mL, respectively). The addition of C12-carbon sources showed that lactose greatly enhanced cell growth (9.42 g/L), while tyrosinase production (266.67 U/min/mL) was reduced by about 41.61% from control cultivation (456.67 U/min/mL). Maltose addition reduced cell growth by about 50% (4.11 g/L) but gave similar tyrosinase production (456.67 U/min/mL). On the other hand, sucrose was the most suitable carbon source added in terms of tyrosinase production (543.33 U/min/mL) which was 19% higher than the tyrosinase production obtained in control cultivation. In terms of cell dry weight, sucrose gave 5.21 g/L cell dry weight, which was 36% lower than the cell dry weight obtained in the control cultivation. Polysaccharide addition enhanced both cell growth and tyrosinase production, but the production level remained lower or comparable to that of the control (dextrin, 390 U/min/mL; cellulose, 400 U/min/mL; starch,

456 U/min/mL). With the exception of malic acid, results of the tyrosinase production yield coefficient ($Y_{p/X}$) showed that the yield

coefficient was almost doubled when 1% sucrose was added to the cultivation medium in comparison to the control cultivation.

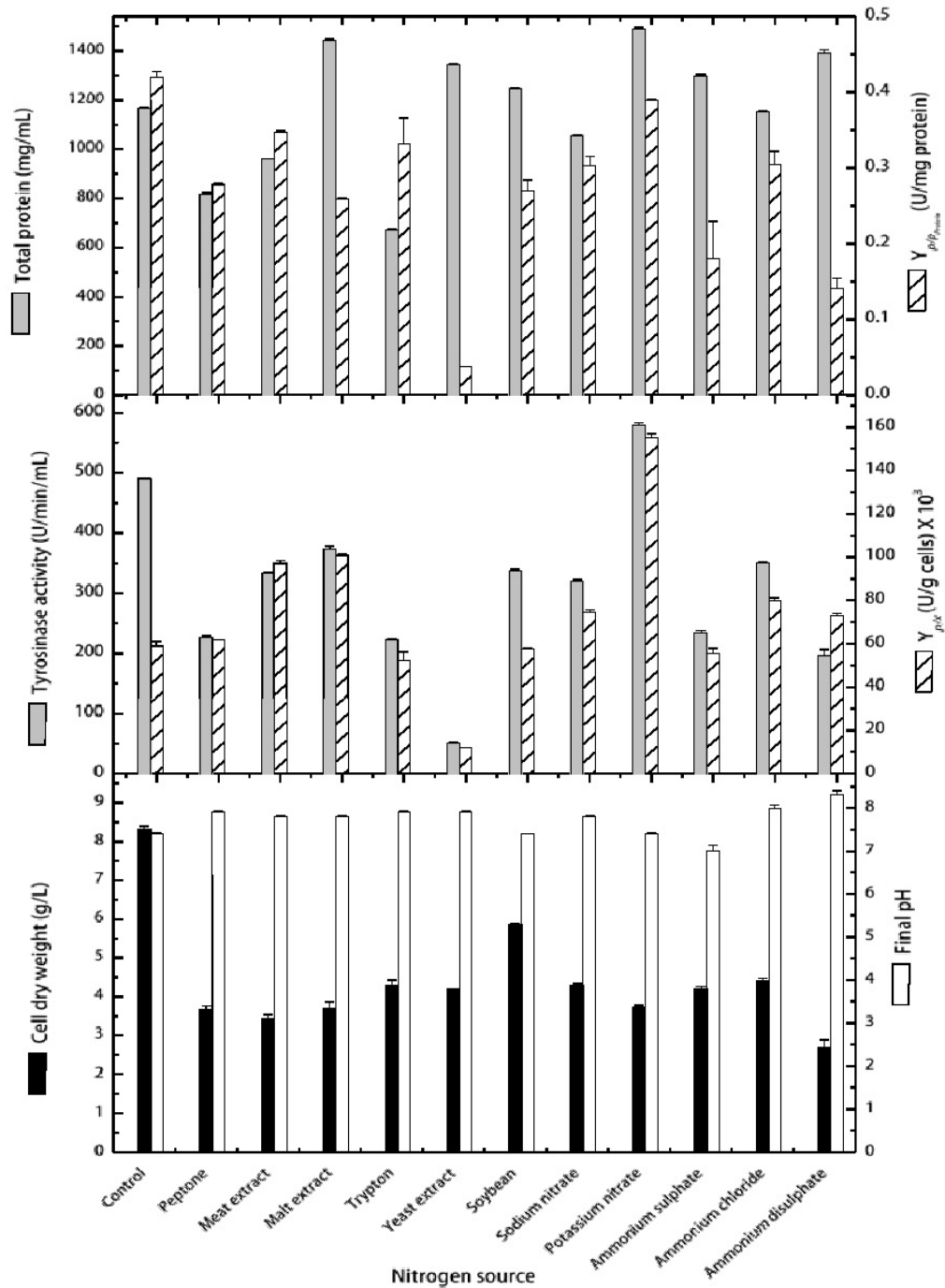


Fig. 5: Effect of different nitrogen sources on cell growth and tyrosinase production by *B. subtilis* NA2.

It can be generally seen that the isolated strain diverge greatly in its affinity towards different carbon sources. Carbon sources have been found to influence both cell growth as well as tyrosinase production depending on the producing strain. Valipour and Arikan (2015) reported that glucose, starch, and maltose increased tyrosinase yield by about 116.6, 110 and 113%, respectively. On the other hand, the yield was decreased to about 66.6 and 83.3 upon using glycerol

and fructose, respectively. Moreover, Chaskes *et al.* (2014) found that fructose was the best carbon source for tyrosinase production by *Cryptococcus gattii*, while Valipour and Arikan (2016) reported that glucose, starch, and other tested C-sources had no noticeable effect on the production of tyrosinase by *B. megaterium* M36. According to our obtained results, 1% sucrose was supplemented to the cultivation medium in the following optimization steps.

Effect of different nitrogen sources on cell growth and tyrosinase production

Different organic and inorganic nitrogen sources were evaluated for their effect as casein substituents on cell growth and tyrosinase production. Different nitrogen sources were prepared to keep the C-N ratio constant among all tested sources. The obtained results (Figure 5) showed that casein (control culture) and soybean nitrogen sources highly supported cell growth and biomass production, which gave highest cell growth values (8.31 and 5.85 g/L, respectively). In terms of tyrosinase production, results showed that potassium nitrate stimulated the highest tyrosinase production, where the maximal activity of 580 U/min/mL was obtained. On the other hand, the control cultivation containing casein gave the second highest activity of 490.67 U/min/mL. However, yeast extract gave the lowest tyrosinase production (50 U/min/mL). Generally, it can be noticed that, with the exception of potassium nitrate and casein, all other tested nitrogen sources gave decreased tyrosinase activities. Their obtained activities were lower by about 36-66% of the highest activity obtained. Furthermore, the highest production of tyrosinase obtained with potassium nitrate was reflected in the obtained yield coefficient. It can be seen that tyrosinase production yield coefficient ($Y_{P/X}$) increased by about 2.6 folds upon using potassium nitrate instead of casein in the cultivation medium 58.96×10^3 and 155.2×10^3 U/g cells for potassium nitrate and casein, respectively. This can be attributed either to the increased productivity of the strain or to

the decreased cell biomass in case of potassium nitrate cultivation.

The producing microorganisms vary distinctly in their behavior to different metabolic substrates. Various reports have mentioned that many tyrosinase producing strains use organic nitrogen sources, mainly tryptone and casein (Valipour and Arikan, 2015; 2016). They reported that tryptone followed by casein are the best nitrogen sources for tyrosinase production by their *Bacillus* sp. MV29 and *B. megaterium* M36 isolates. This can be explained by the fact that organic nitrogen sources provide the growing cells with all necessary vitamins and cofactors required for their metabolic activities. Furthermore, Valipour and Arikan (2015) noticed that using yeast extract above 0.1% in the production medium constantly decreased tyrosinase production. These observations are in good agreement with our results where yeast extract gave the lowest tyrosinase production. On the other hand, our results showed that potassium nitrate as an inorganic nitrogen source resulted in the highest tyrosinase activity among all tested nitrogen sources. These results are in accordance with Ingle and Khobragade (2013) who investigated the production of tyrosinase enzyme from three different soil bacterial isolates. Their results showed for all their three isolated strains, *Rhodococcus ruber*, *Pseudomonas geniculata* C7 and *P. geniculata* C10, that production of tyrosinase increased by about 2.5-2.7 folds for all strains upon using potassium nitrate as a nitrogen source instead of casein hydrolysate. Finally, Table 1 summarizes different cultivation parameters obtained from different optimization steps performed throughout the work.

Table 1. Growth and production parameters of tyrosinase production by *Bacillus subtilis* NA2 under different optimization steps.

Parameter	Growth curve kinetics	Different pH**	Different C-source**	Different N-source**
X_{max} [g/L]	5.89*	7.26	5.21	3.74
dx/dt [g/L/h ⁻¹]	0.18*	n.d.	n.d.	n.d.
P_{max} [U/min/mL]	416.67	453.33	543.33	580.00
Q_p [U/mL/h]	11.204*	n.d.	n.d.	n.d.
Total protein _{max} [mg/mL]	844.8	819.4	811.2	1488.0
$Y_{P/X}$ [U/g cells]	111.75×10^3	62.41×10^3	104.27×10^3	155.19×10^3
$Y_{P/P_{protein}}$ [U/mg produced protein]	0.554	0.553	0.670	0.390

*Data were taken at the end of the exponential growth phase.

**Data taken at the end of the experiment for the optimized component.

n.d.: not determined.

Abbreviations: X_{max} , maximal cell dry weight; dx/dt , cell growth rate; P_{max} , maximal volumetric tyrosinase production; Q_p , average tyrosinase production rate; Total protein_{max}, maximal total protein content; $Y_{P/X}$, $Y_{P/P_{protein}}$, yield coefficients for g cells and mg produced protein, respectively.

CONCLUSION

The present work was designed to isolate and identify a newly tyrosinase producing strain. The most tyrosinase producing isolate was identified by 16S rDNA technology as *B. subtilis* isolate and was designated NA2. Moreover, the isolated strain showed 99% similarity with *B. subtilis* using BLAST analysis. Medium optimization experiments resulted in an increase of about 39.4% in volumetric tyrosinase production when compared with initial tyrosinase production obtained before the optimization of the medium composition. Furthermore, the specific activity of tyrosinase production (U/g cells) was also improved by about 39.6% upon the optimization. This improvement is mainly due to increased cell productivity and not due to microbial cell mass.

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How to cite this article:

Elsayed EA, Danial EN. Isolation, Identification and Medium Optimization for Tyrosinase Production by a Newly Isolated *Bacillus subtilis* NA2 Strain. *J App Pharm Sci*, 2018; 8(09): 093-101.