Tyrosinase production by two *Streptomyces* isolates

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Abstract

The research aims to isolate and identify best two *Streptomyces* spp for tyrosinase production from Egyptian and Libyan soils. Optimization of tyrosinase production by two *Streptomyces* species, identified as *Streptomyces iakyrus* and *Streptomyces echinatus* was studied. The optimum incubation period, temperature and initial pH of medium for organism growth and enzyme yield for *Streptomyces iakyrus* were around 4 days, 30^{o}C and 7.0, respectively, while for *Streptomyces echinatus* were 5 days, 30^{o}C and 8.0, respectively. The maximum tyrosinase production was observed in a medium containing glycerol and tyrosine as carbon source and nitrogen source for the two identified *Streptomyces* species.

Key words: Tyrosinase, *Streptomyces iakyrus* and *Streptomyces echinatus*, Optimization.

إنتاج إنزيم التيروسينيز من عزلتين من الإستربتوميسيس

المستخلص:

يهدف هذا البحث إلى دراسة تأثير ظروف النمو وتركيب الوسط الغذائي على إنتاج إنزيم التيروسينيز من عزلتين من إستربتوميسيس بواسطة بيئة التيروسين الغذائية. أظهرت النتائج أن الظروف المثلى لإنتاج إنزيم التيروسينيز للعزلتين كانت كالأتى: فترة التحضين المثلى هى 4 و5 أيام والأرقام الهيدروجينية كانت 7، 8 لكل من إستربتوميسيس أيكيرس وإستربتوميسيس إيكاناتس على التوالى. وكانت درجة الحرارة 30م° هي المثلى لإنتاج الإنزيم للكائنين. أفضل مصدر كربوني لإنتاج إنزيم التيروسينيز كان مادة الجليسرول للسلالة استربتوميسيس أيكيرس واستربتوميسيس إيكاناتس كمادة كيميائية نقية بتركيز 1.5%. أما مادة التيروسين كانت أحسن مصدر نيتروجيني لإنتاج إنزيم التيروسينيز لكلا الكائنين.

Introduction

The tyrosinase enzymes from various species have been studied for more than a century (Gessard, 1900). In particular it has been known for several decades that bacteria such as Streptomyces when are growing using organic media have the ability to convert L-tyrosine into melanin or melanoid, which are considered as useful criteria in taxonomic studies (Zenova, 1965; Aria and Mikami, 1972). The first biochemical characterisation of a bacterial enzyme was also performed using a tyrosinase from Streptomyces species (Della- Cioppa *et al*., 1998; Matoba *et al*., 2006). The first bacterial tyrosinase have been purified from cell extracts of *Streptomyces nigrifaciens* (Nambudiri and Bhat, 1972), and *Streptomyces glaucescens* (Lerch and Ettlinger,1972).

Tyrosinase (EC 1.14.18.1, monophenol, O-diphenol: oxygen oxidoreductase) is a type 3 copper-containing enzyme that is ubiquitously dispensed in nature; they are found both in prokaryotic as well as in

eukaryotic microbes, in mammals and plants (Claus and Decker, 2006; Surwase *et al*., 2012). Tyrosinase catalyzes the O-hydroxylation and subsequent oxidation of monophenols, O-phenols, to quinones followed by a series of nonenzymatic steps resulting in the formation of melanin (Decker and Tuczek, 2000). 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 Tuczek 2000).

Due to oxidative stress properties, tyrosinases are one of the most important industrial enzymes. They have attracted interest because of the diversity of their applications. Major industrial applications of tyrosinase in the field of the medical science, food industry, and industrial biotechnology such as, phenols, dyes, and waste water management (Duran and Esposito 2000; Girelli *et al*., 2006; Marino *et al*., 2011)), protein cross linking in food technology (Lantto, 2007) and synthesis and bioconversion of diphenol drugs, like L-DOPA (Duran *et al*., 2002; Ali and Qadeer 2002; Franciscon *et al.*, 2012).

The goal of the current effort is to maximize the conditions necessary for two streptomycetes to produce tyrosinase.

Material and Methods

Isolation of streptomycetes

Streptomyces species used in this study were isolated from various soil samples collected from different localities of Egypt and Libya using standard dilution plate method procedure and purified by streak-plating on starch nitrate agar plates (Waksman, 1959). The plates were incubated for a period of seven days at 30°C. Colonies of streptomycetes were selected, isolated, purified and maintained as spore suspensions in 20% (v/v) glycerol at -20°C for subsequent investigation (Hopkins *et al*., 1985).

The medium used for isolation, cultivation and stock maintenance of isolated strains was starch nitrate agar medium (Waksman, 1959). It contained (g/L): soluble starch, 20; KNO_3, 2; K_2HPO_4, 1; NaCl, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.01; $CaCO_3$, 3; agar, 20; and distilled water up to 1L.

Screening for tyrosinase producing actinomycetes strains

A preliminary analysis for tyrosinase activity was conducted using streaked isolates on tyrosine agar (Shinobu, 1958) that has the composition (g/L): $Ca(NO_3)_2$, 2.5; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.3; NaCl, 0.1; $FeSO_4.7H_2O$, 0.01; $CaCl_2$, 0.1 and distilled water up to 1L, and the pH was adjusted to 7.2 with 1.0 N NaOH, and all the plates were incubated at 30°C for 5-7 days. The occurrence of brown pigmented colonies that gradually changed its color to black (melanin formation) was indication of tyrosinase positive organism (Raval *et al.*, 2012).

Growth condition and enzyme production

Two starch-nitrate agar discs of each *Streptomyces* 5-7 old days culture grown at 30ºC were inoculated in flasks containing 50 ml of tyrosine media (Shinobu, 1958). The pH of the media was adjusted to 7.2 using NaOH and HCl (1.0 M) prior to sterilisation. Cultures were incubated statically at 30ºC for 5 days. The enzyme activity of the culture filtrate was assayed.

Tyrosinase assay

the reaction was conducted to perform the tyrosinase enzyme: A. 1.0 ml of 0.5 M phosphate Buffer, pH 6.5 at 25°C, 1.0 ml of 0.001 M L-tyrosine Solution (Prepared in 100 ml in deionized water using L-tyrosine, Free Base, Sigma Prod. No. T-3754.), 0.1 ml enzyme and 0.9 ml of reagent

grade water were added into test tube. The reaction mixture was oxygenated by bubbling 99.9% pure O_2 through a capillary tube for 3 to 5 minutes to reach temperature equilibration and absorbance was recorded at 280 nm by using UV-Vis spectrophotometer (Raval *et al*., 2012).

The enzyme activity was calculated by using the following Formula:

$$\text{Units/ml enzyme} = \frac{(\Delta A280\text{nm/min Test} - \Delta A280\text{nm/min Blank})\ (\text{df})}{(0.001)\ (0.1)}$$

Where, df = Dilution factor

0.001 = The change in A280 nm/minute per unit of tyrosinase at pH 6.5 at 25°C in a 3 ml reaction mix

0.1 = Volume (in milliliters) of enzyme used

Estimation of intracellular protein of the biomass of the streptomycetes

The biomass yield of the selected isolates was recorded. Microbial growth under different growth factors was assayed. After incubation, Cell mass of isolates were collected, then washed three times by distilled water. 1N of NaOH was added with boiling for 20 min in water bath, then cooled. The centrifugation was performed at 4,000 rpm for 5 min, the supernatant was used for measurement of protein content (microbial growth).

Bradford's method for protein content

The protein content was determined by employing Bovine Serum Albumin (BSA) as a standard protein and the Bradford, 1976 technique.

Optimization of culture conditions

Effect of incubation period: To determine the optimum incubation period of the isolates for maximum enzyme production, the supernatants

were collected after 1, 2, 3, 4, 5, 6, 7 and 8 days of incubation and assayed as before. The growth was also recorded.

Effect of initial pH: To determine the optimum medium pH, for maximum enzyme production, selected medium of different pHs (4, 5, 6, 7, 8, 9, and 10) was inoculated with the isolates. The growth and tyrosinase production were assayed.

Effect of temperature: To determine the optimum temperature for enzyme production the culture medium was incubated at 25°C, 30°C, 35°C, 40°C and 45°C, at optimum pH and incubation period. The effect of temperature on growth and tyrosinase production was recorded.

Effect of carbon source: Different carbon sources; carboxymethylcellulose (CMC), starch, glucose, xylose, maltose, sucrose, glycerol and mannitol (15 g/L) was added separately as a sole carbon source. The effects of these carbon sources on the production of tyrosinase, biomass yield, were recorded.

Effect of nitrogen source: For this purpose, a range of different nitrogen sources includes, $(NH_4)_2SO_4$, $NaNO_3$ and KNO_3, were added in equimolecular nitrogen weights equivalent to the nitrogen content of 1 g L^{-1} L-asparagine and 0.5 g L^{-1} tyrosine of tyrosine medium. Other nitrogen source like tyrosine, peptone (14 % N), yeast extract (9 % N) and beef extract (12.5 % N) was added. The effects of these nitrogen sources on the production of tyrosinase, biomass yield, were recorded.

Statistical analysis

Analysis of variance (one-way ANOVA) was used to identify statistically significant differences. All statistical analyses were performed using SPSS 18.0 software (SPSS, 2006).

Results

Screening of tyrosinase producers

Screening of actinomycetes was conducted on tyrosine agar plate as a preliminary study for choosing the best tyrosinase producers. After 7 days of incubation, two isolates out of 8 actinomycetes showed appearance of brown pigmented colonies that gradually changed its color to black (melanin formation) (indication of tyrosinase positive organisms) were selected.

On the basis of the results obtained on tyrosine agar plates, the potent isolates were inoculated in tyrosine broth. After incubation the potent actinobacteria isolates *Streptomyces iakyrus* and *Streptomyces echinatus*, showed tyrosinase enzyme with activity of 4.8 Uml^{-1} min^{-1} and 8.4 Uml^{-1} min^{-1} respectivily.

Based on their morphological, cultural, and biochemical characteristics, The two streptomycetes isolates that were chosen were identified as *Streptomyces iakyrus* and *Streptomyces echinatus* in the preview work (Abou-Dobara *et al*., 2019) as per the International *Streptomyces* Project (Shirling and Gottlieb, 1968a; 1968b; 1969; 1972; Pridham and Tresner, 1974a; 1974b; Bergey's Manual of Systematic Bacteriology, 1989).

Factors affecting the growth and tyrosinase activity

Effect of the incubation period

The time course for the production of tyrosinase activity is shown in Figure 1 for *Streptomyces iakyrus* and *Streptomyces echinatus*. The tyrosinase activity increased significantly ($P< 0.0001$) during the growth of the organism, with the maximum production of enzyme detected after 4 days and 5 days (4.5 U/ml and 2.86 U/ml) and the maximum growth

(11.467 mg/ml and 13.091 mg/ml) for *Streptomyces iakyrus* and *Streptomyces echinatus*, respectively. After 4 and 5 days, tyrosinase activity declined significantly for *Streptomyces iakyrus* and *Streptomyces echinatus*. Tyrosinase production reached the minimum level (0.266 U/ml and 1.9 U/ml) at the end of the incubation period (5 and 7 days) for *Streptomyces iakyrus* and *Streptomyces echinatus*, respectively.

Effect of different initial pH

The optimum pH for tyrosinase production (Figure 2) was 7 and 8 with the maximum level of enzyme activity 4.6 U/ml and 7.533 U/ml for *Streptomyces iakyrus* and *Streptomyces echinatus*, respectively. On the other hand, the maximum growth (20.3326 mg/ml and 21.995 mg/ml) occurred highly significantly ($P< 0.0001$) at pH 8 and pH 10 for *Streptomyces iakyrus* and *Streptomyces echinatus*, respectively. Tyrosinase activity was completely inhibited at pH 8 for *Streptomyces iakyrus*.

Effect of temperature

The effect of temperature on the production of tyrosinase by *Streptomyces iakyrus* and *Streptomyces echinatus* was studied by growing the organisms at different temperatures in the range of 25ºC to 45ºC (Figure 3). The optimum temperature for the enzyme production (4.26 U/ml and 7.733 U/ml) and maximum growth (9.375 g/ml and 13.343 mg/ml) was 30ºC by *Streptomyces iakyrus* and *Streptomyces echinatus*, respectively. Tyrosinase production was gradually highly significantly ($P< 0.0001$) increased with increasing temperature for both *Streptomyces iakyrus* and *Streptomyces echinatus*. Tyrosinase activity was completely inhibited at 45 ºC for the two *Streptomyces* species**.**

Effect of different carbon sources

The effect of a range of carbon sources on the growth and production of tyrosinase by *Streptomyces iakyrus* and *Streptomyces echinatus* was varied (Figure 4). The best carbon source was found to be glycerol for *Streptomyces iakyrus* and *Streptomyces echinatus* enzyme activity (4.33 U/ml and 4.03 U/ml) respectivily.

The arrangement of different carbon sources in descending order according to its effect on tyrosinase production for *Streptomyces iakyrus* was glycerol, mannitol, maltose, sucrose, glucose and xylose with activity varying from 4.33 U/ml to 0.953 U/ml. In contrast, the carbon sources arrangement for *Streptomyces echinatus* was glycerol, mannitol, maltose, CMC, and glucose, with activity ranging from 4.03 U/ml to 1.33U/ml. Tyrosinase activity was completely inhibited when CMC and starch were used for *Streptomyces iakyrus* and xylose, sucrose and starch was used for *Streptomyces echinatus* as the carbon source.

Effect of different nitrogen sources

The nitrogen sources effect on the production of tyrosinase by *Streptomyces iakyrus* and *Streptomyces echinatus* isolates was investigated (Figure 5). The highest tyrosinase activity (4.066 U/ml for *Streptomyces iakyrus* and 4.66 U/ml for *Streptomyces echinatus* respectively) were recorded with high significant values ($P < 0.0001$) when tyrosine was used as the nitrogen source with maximum growth 9.325 mg/ml and 14.845 mg/ml for *Streptomyces iakyrus* and *Streptomyces echinatus* respectivily). The arrangement of different nitrogen sources in descending order according to its effect on tyrosinase production and growth by *Streptomyces iakyrus* was tyrosine (4.066 U/ml and 9.325 mg/ml), yeast extract (0.266 U/ml and 7.186 mg/ ml), beef extract (2.2 U/ml

and 5.689 mg/ml), peptone (3.33U/ml and 2.536 mg/ml). But tyrosinase activity was completely inhibited when sodium nitrate, ammonium sulphate and potassium nitrate were used as the nitrogen source.

The arrangement of different nitrogen sources in descending order according to its effect on tyrosinase production and growth by *Streptomyces echinatus* was as follows: tyrosine (4.66 U/ml and 12.66 mg/ml), peptone (3.66 U/ml and 14.845 mg/ ml), but tyrosinase activity was completely inhibited when beef extract, yeast extract, sodium nitrate, ammonium sulphate and potassium nitrate were used as the nitrogen source.

Discussion

Streptomyces species have always been a source of thousands of bioactive compounds. Enzymes are one of the important products of this unusual group of bacteria. Several other organisms were reported earlier to produce the tyrosinase enzyme such as *Streptomyces* derived tyrosinases are the mainly characterized enzymes of bacterial source (Della-Cioppa *et al*., 1998). *Streptomyces iakyrus* and *Streptomyces echinatus* isolates exhibited the highest ratio of brown colored pigmentation that gradually changed its color to black (melanin formation) on tyrosine agar compared with other isolates, indicating a higher level of tyrosinase activity. The production of brown/ black color pigment indicates the tyrosinase enzyme production (Jones *et al.,* 2007). The production of brown color was due to the production of melanin by oxidative polymerization of phenolic compound by tyrosinase.

On tyrosine broth, after incubation, *Streptomyces iakyrus* and *Streptomyces echinatus* strains showed positive result for tyrosinase (4.8 Uml^{-1} and 8.4 Uml^{-1} respectivily). The tyrosinase enzyme acts as a bi-

functional for the reaction of orthohydroxylation of monophenols (tyrosine) followed oxidation of diphenols (L-DOPA) to orthoquinones (dopachrome). Dopachrome can then be transformed to the brown/ black melanin pigments (Dolashki and Gushterova, 2009).

Tyrosinase production by *Streptomyces iakyrus* and *Streptomyces echinatus* increased during the growth of the cultures in tyrosine medium with the maximum production that detected after 4 and 5 days incubation, respectively. After this period, the activities of the enzymes decreased. 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 (Nusrat and Rahman, 2007).

The observed peaking and troughing of the production of extracellular enzymes might be attributed to: 1- the differences in the timing of induction of separate components of the tyrosinase system; 2- the products of action of one component inducing the synthesis of another; 3- differential inhibition by products of substrate hydrolysis; and 4- differential inactivation by proteases, or variation in the pH during cultivation conditions (Tuohy and Coughlan, 1992; Wang *et al.*, 1993).

The maximum yield of tyrosinase activity was obtained by *Bacillu s* sp.MV29, *Bacillus* sp CGR6 and *Pseudomonas geniculata* C7/ C10 took only 3 days (Valipour and Arikan, 2015; Pradhan and Sarkar, 2017; Ingle and Khobragade, 2015) respectivily.

Temperature and pH values were found to be important parameters that influenced enzyme activities and production (Odeniyi *et al*., 2009). In this study, we found that the optimum pH for tyrosinase production by the *Streptomyces iakyrus* isolate was. This result is considerably similar to

previous results reported by Park and Son (2009); Valipour and Arikan (2015); Valipour and Arikan (2016); Ingle and Khobragade, (2015) who found that optimum tyrosinase enzyme from *Bacillus megaterium* F7-1, *Bacillus* sp. MV29, *Bacillus megaterium* strain M36 and *Pseudomonas geniculata* C7/C10 respectively at pH 7. At pH 8.0 enzyme production declined drastically. This might be due to the inactivation of the enzyme in the alkali medium (Surwase and Jadhav, 2011), In this study maximum enzymatic activity was observed at alkaline pH 8 for *S. 26*. Which was in different with the findings of many other workers. The amount of active enzyme after pH 9.0 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 (Shuster and Fishman, 2009).

The temperature has a great effect on the enzymes activity. 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 (Surwase *et al*., 2012). *Streptomyces iakyrus* and *Streptomyces echinatus* showed a maximum tyrosinase activity at 30°C within an optimum range 25°C-35°C. The optimum temperature recorded for maximum tyrosinase productivity at 30°C for *Pseudomonas geniculate* C7/C1 (Ingle and Khobragade, 2015) and *Funalia trogii* (Majidi and Aksoz, 2013). Valipour and Arikan, (2016) recorded high tyrosinase activity by *Bacillus megaterium* strain M36 at 36°C.

The medium components good play role in enhancing the enzyme production (Gupta *et al*., 2002). Also according to Surwase *et al*., (2012) the culture medium plays an important role, as the culture medium should

provide all the essential nutrients required by the organism for enzyme production. The best carbon source for the tyrosinase activity of the organism was found to be glycerol for *Streptomyces iakyrus* and *Streptomyces echinatus*. Carreira *et al*., (2001); Valipour and Arikan (2015); Pradhan and Sarkar (2017) showed that glucose was the best carbon source for tyrosinase production by yeast, *Bacillus* sp.MV29 and *Bacillus* sp CGR6 while Dastager *et al*., (2006) indicated that starch was the effective carbon source for *Streptomyces* sp. followed by glycerol and fructose. Chaskes *et al*., (2008) had a different opinion: the best carbon source was fructose, by *Cryptococcus gattii*.

In literature, best nitrogen sources differ from one organism to another. The tyrosinase productivity by *Streptomyces iakyrus* and *Streptomyces echinatus* was at maximum level when tyrosine was used as the nitrogen source. This was in difference with the findings of many other workers who found that maximum tyrosinase productivity was obtained by *Bacillus sp.* MV29, *Bacillus cereus* and *Bacillus megaterium* when tryptone was added as an organic nitrogen source to the production medium (Valipour and Arikan (2015); Zhang *et al*., (2007); Valipour and Arikan (2016). *Bacillus* sp CGR6 strain gave maximum tyrosinase productivity when sodium nitrate was added as an organic source to the production medium by Pradhan and Sarkar (2017).

The tyrosinase identified by both isolates would be fully characterized in a future work in order to investigate their usefulness in the industrial purposes.

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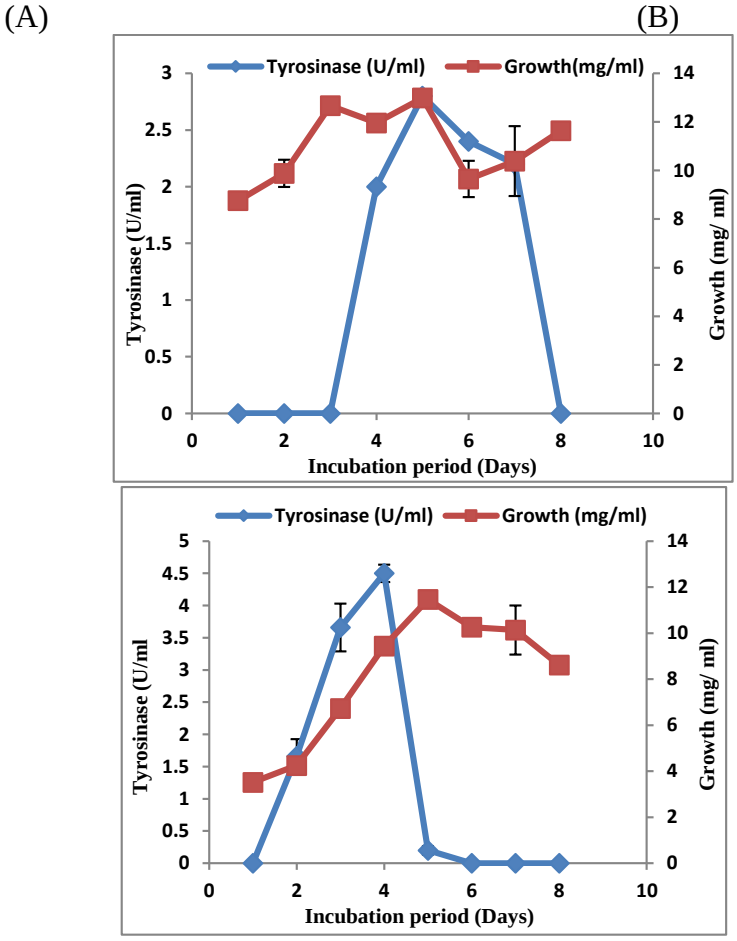

Figure 1. Effect of different incubation periods on tyrosinase production and growth of *Streptomyces echinatus* (A) and *Streptomyces iakyrus* (B).

(A) (B)

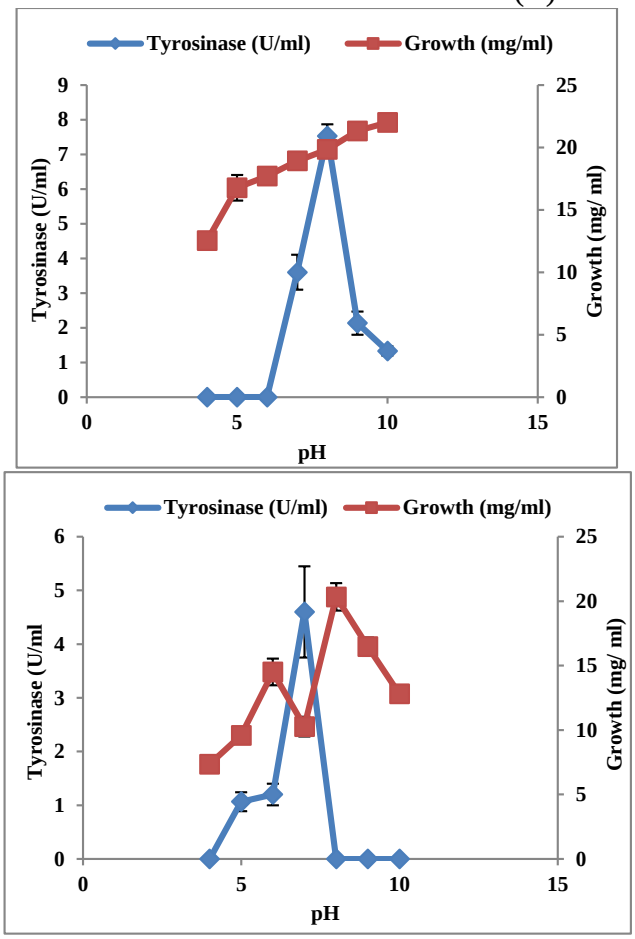

Figure 2. Effect of different pH on the tyrosinase production and growth of *Streptomyces echinatus* (A) and *Streptomyces iakyrus* (B).

(A) (B)

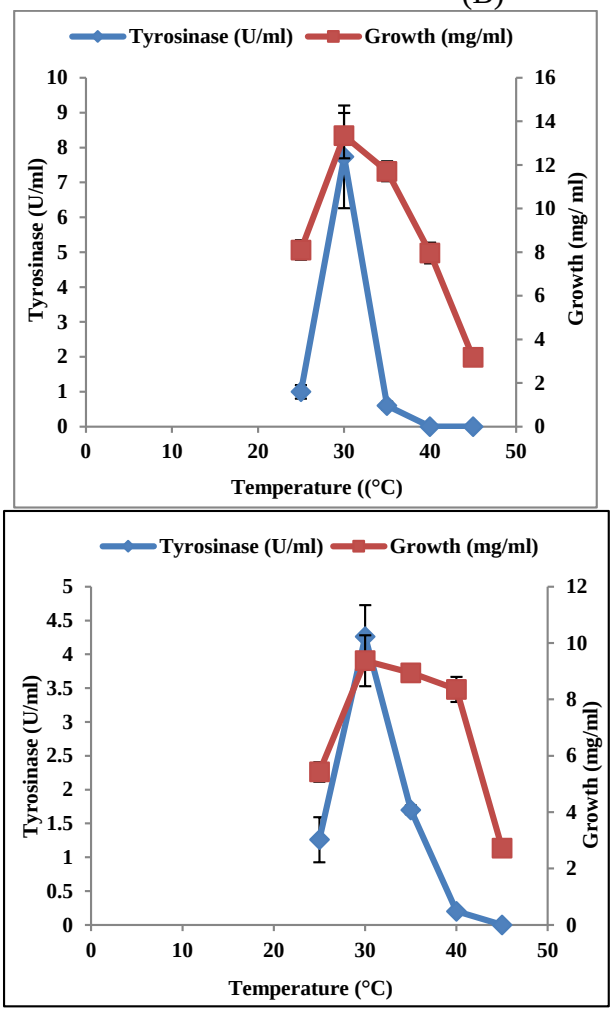

Figure 3. Effect of different temperatures on the tyrosinase production and growth of *Streptomyces echinatus* (A) and *Streptomyces iakyrus* (B).

(A)

(B) (B)

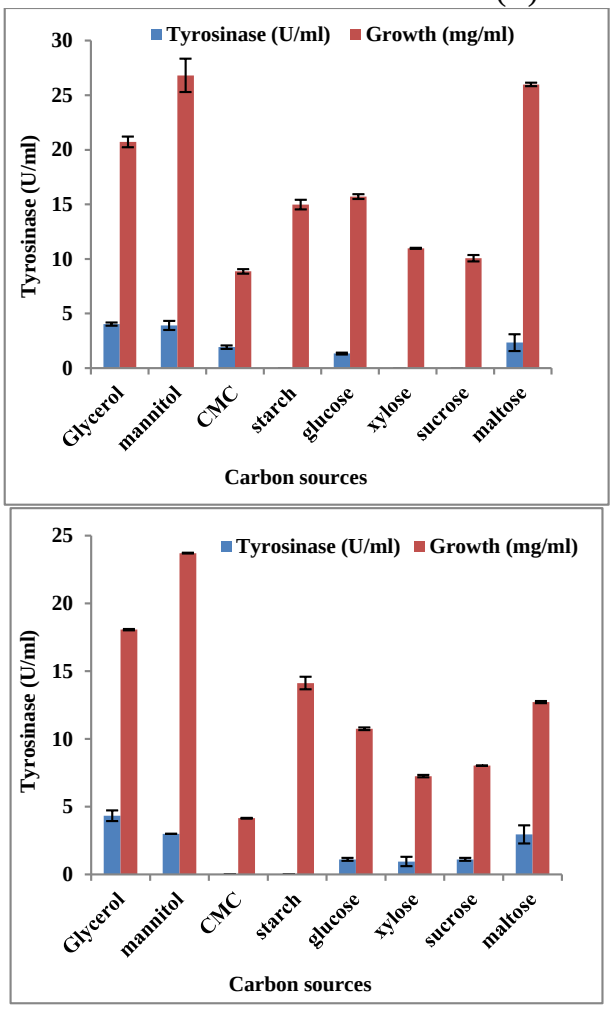

Figure 4. Effect of different carbon sources on the tyrosinase production and growth of *Streptomyces echinatus* (A) and *Streptomyces iakyrus* (B).

(A) (B)

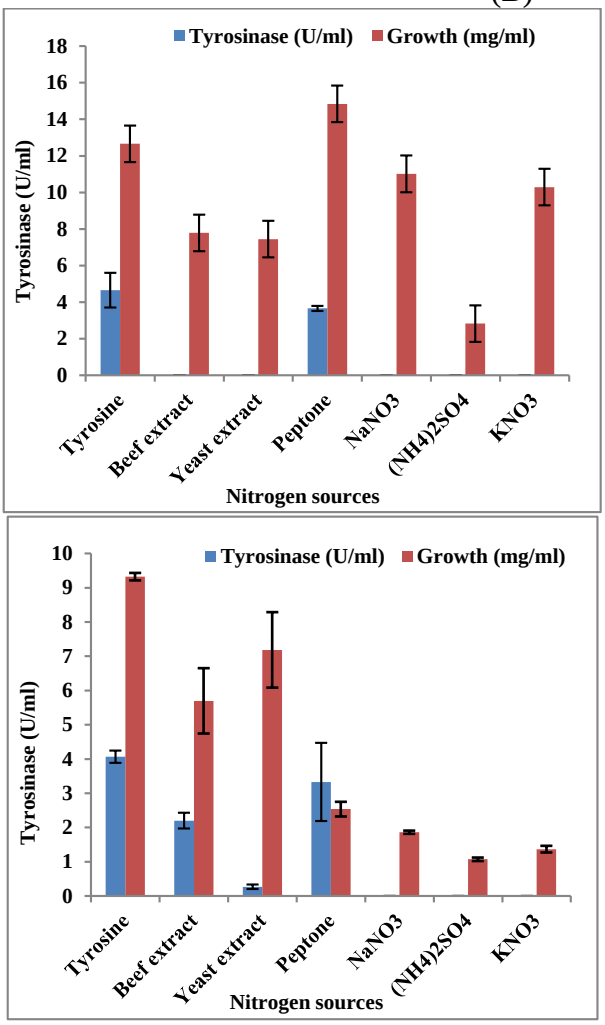

Figure 5. Effect of different nitrogen sources on the tyrosinase production and growth of *Streptomyces echinatus* (A) and *Streptomyces iakyrus* (B).