

Screening of *Streptomyces* and Process Optimization for the Production of Tyrosinase

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The present study explains the extracellular production of tyrosinase by *Streptomyces* in submerged fermentation (SmF). A total of 12 isolates of streptomyces were screened for the qualitative and quantitative synthesis of tyrosinase in STM (starch tyrosine mineral) and TGB (tyrosine gelatin beef extract) media. *Streptomyces* DSV 12 was screened and selected to be an efficient strain for the maximum production (98.1 IU) of tyrosinase in TGB medium. The enhanced production of tyrosinase by process optimization was carried out with physical and nutritional parameters. In Physical parameters the optimum conditions for maximum production (112.4 IU) were pH-7.5, temperature-40°C and aeration-180rpm. Further, enhanced production of tyrosinase was achieved with nutritional, such as carbon and nitrogen sources. Among all nutritional sources tested, glucose (0.5%) has shown higher production (120.7 IU) followed by L-tyrosine (0.5%) with maximum (125.4 IU) production. CuSO₄ (0.06%) was proved to be the best inducer among all metal ions tested for the highest production (158.6 IU) of extracellular tyrosinase.

Key words: Screening, Optimization, Tyrosinase, *Streptomyces*.

Actinomycetes are Gram-positive bacteria showing filamentous highly branched structural features. Numerically, they cover up about 70-80% of total antibiotic products as compared to other genera¹². *Streptomyces* are inexhaustible sources of novel secondary metabolites with a wide range of biological activities that may ultimately find application as anti infective and anti cancer agents or other pharmaceutically useful compounds¹⁴. They are widespread in nature, and play a significant role in the prospective of

biotechnology, because of their significance as producers of vitamins, enzymes, antitumor agents and anti oxidant agents¹¹.

Actinomycetes are characterized by the production of various pigments on different natural or synthetic media. These pigments are usually described in terms of various shades of blue, violet, red, rose, yellow, brown and black. The pigments may be dissolved in to the medium or it may be retained in the mycelium. Actinomycetes had known to be produced various kinds of antibiotics and moreover these antibiotics include many pigments²⁶. The formation of pigment is influenced by the pH of the medium, aeration, temperature of the growth and carbon and nitrogen sources.

Streptomyces is included in the *Streptomycetaceae* family and represents one of the most important genus of the *Actinomycetales* order due to its impressive number of species and

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their practical role. Members of this genus were deeply studied because of their capacity to produce antibiotics and enzymes of industrial importance as glucose isomerase, protease, amylase, xylanase, while their capacity to produce tyrosinase was studied in a lesser extent³. Among the group of *Streptomyces*, many species are characterized by the production of dark-colored melanin pigments that are synthesized by phenol oxidases¹⁶. *Streptomyces* tyrosinases have been isolated from *Streptomyces nigrificans*²⁸ and *Streptomyces glaucescens*²⁰. The first bacterial tyrosinases have been purified from cell extracts of *Streptomyces nigrificans*²⁸ and *Streptomyces glaucescens*²⁰.

Phenol oxidases are a group of copper enzymes which utilize oxygen to catalyze the oxidation of aromatic compounds^[32]. Tyrosinases (E.C. 1.14.18.1) are copper-containing enzymes which are ubiquitously distributed in nature²¹. They are essential for the formation of melanin^[5] and various other functions. Tyrosinase is known to possess both monophenolase activity, hydroxylation of monophenols to *o*-diphenols and diphenolase activity, oxidation of *o*-diphenols to *o*-quinones.

In soil environments, extracellular tyrosinases are probably involved in the polymerization and detoxification of plant phenolic compounds and the formation of humic matter⁷. Presently there is an increasing interest in using tyrosinases in industrial applications: in the environmental technology for the detoxification of phenol-containing waste waters⁷¹ contaminated soils⁸ and as biosensors for the monitoring of phenols; in cosmetic and food industries, because of either undesirable or beneficial oxidative browning reactions²⁵. Tyrosinases are also suggested to be potential tools in treating melanoma²⁷. Furthermore, the role of tyrosinase in neuromelanin production and damage of neurons related to Parkinson's disease has been extensively studied¹².

In the present study an attempt has been made to detect an efficient strain of *Streptomyces* for extracellular production of tyrosinase. Process optimization was also studied aiming at the enhanced production of tyrosinase under submerged condition.

MATERIALS AND METHODS

Screening of *Streptomyces*

The identified prominent isolates of *Streptomyces* available in our research laboratory were screened for the synthesis of tyrosinase on starch tyrosine mineral agar (STMA), where casein was replaced with tyrosine in SCA¹⁷ and Tyrosine-gelatin-beef extract agar (TGBA)^[30]. The rapid streak plate culture method¹⁹ was employed for the screening of isolates.

Quantitative screening of tyrosinase in submerged condition, employing STM and TGB media was carried out as per the modified method prescribed by Konard Lerch and Leopold Ettlinger (1972)²⁰. 1 ml spore suspensions of *Streptomyces* DSV 8, DSV 12 and DSV 17 of 120 h old were inoculated into 250ml Erlenmeyer's flask containing 100 ml of STM and TGB media separately. Flasks were incubated for 144 hours in an orbital shaker with 120 rpm, at 37 °C and initial pH was maintained 7.0. 10ml of fermented broth was withdrawn from both the media for every 24 h, centrifuged at 10000rpm for 15 min. 1 ml of supernatant was collected and subjected for the crude tyrosinase activity.

Estimation of tyrosinase

The quantity of tyrosinase produced was determined by measuring the dopachrome at 475 nm^[6]. Standard reaction mixture containing 0.5 ml 4 mM L-dopa, 0.5 ml 0.1 M sodium phosphate buffer (pH 6.8) and enzyme extract in a total volume of 3 ml, brought to 40°C in BOD incubator for 10 min. The absorbance at 475 nm was monitored continuously for 3 min in Systronics 2201 UV-VIS spectrophotometer. One unit of tyrosinase activity was referred as the amount of enzyme required to catalyze 1 μmol of L-dopa per minute under the above conditions, which was calculated using the molar extinction coefficient of dopachrome 3600 M⁻¹ cm⁻¹¹⁰.

Process optimization

Effect of physico - chemical factors

Effect of pH

To determine the effect of initial pH on tyrosinase production, a set of six conical flasks each containing 100 ml TGB medium in 250ml Erlenmeyer's flask were employed. The pH range regulated was 6.0 to 9.0 with an increment of 0.5^[9]. The desired pH level was adjusted using dilute

(0.1N) NaOH/HCl. Thus prepared flasks were autoclaved and inoculated with 1 ml spore suspension of *Streptomyces* DSV 12. The flasks were then incubated at 37°C in a shaker incubator with 120rpm for 144 hours. 10 ml fermented broth was withdrawn at every 24h for six days, centrifuged for 10000 rpm for 15 min. 1ml supernatant from each flask was subjected for estimation of tyrosinase by molar extinction coefficient of dopachrome 3600 M⁻¹ cm⁻¹ method, as described earlier.

Effect of temperature

To determine the effect of temperature on tyrosinase production, a set of eight conical flasks each containing 100 ml of TGB media in 250ml Erlenmeyer's flask autoclaved and inoculated with 1ml spore suspension of *Streptomyces* DSV 12. The temperature ranging from 30°C to 44°C^[9], with an increment of 2°C were controlled. The flasks were then incubated in shaker incubator for 120rpm and pH 7.5 was maintained for 144 hours. Estimation of tyrosinase was carried out as described earlier.

Effect aeration

To assess the effect of aeration on the production of tyrosinase, seven flasks containing 100ml TGB media in 250ml Erlenmeyer's flask autoclaved and inoculated with 1ml spore suspension of *Streptomyces* DSV 12. The aeration was maintained by agitation ranging from 150 rpm to 210 rpm, with an increment of 10 rpm. The flasks were then incubated in shaker incubator adjusting pH 7.5 and temperature 40°C for 144 hours. Estimation of tyrosinase was carried out as described earlier.

Effect of nutritional factors

Effect of carbon sources

Various carbon sources like glucose, fructose, maltose, starch, glycerol and sucrose were examined for the production of tyrosinase employing various concentrations (0.25% - 1.25%)^[9]. A set of six conical flasks containing 100 ml of TGB medium in 250ml Erlenmeyer's flask was supplemented separately with different carbon sources. Flasks were autoclaved and inoculated with 1ml spore suspension of *Streptomyces* DSV 12. The optimum physico chemical factors such as pH 7.5, temperature 40°C and agitation speed of 180 rpm were maintained. Physico chemical factors revealed that, the activity of tyrosinase was

maximum at 120 h of fermentation period. In view of this, 10 ml fermented broth was withdrawn at 120 h, centrifuged for 10000 rpm for 15 min. 1ml supernatant from each flask was subjected for estimation of tyrosinase as described earlier.

Effect of nitrogen sources

A set of six conical flasks with 100 ml of TGB medium in 250ml Erlenmeyer's flask supplemented with casein, yeast extract, peptone, L-tyrosine, asparagine and phenylalanine at various concentrations (0.1% - 0.5%) were employed^[9]. Where as the optimum carbon source glucose (0.5%) was incorporated into the medium and the optimum physico chemical factors were maintained as described earlier. Estimation of tyrosinase was carried out as described earlier, at 120h.

Effect of metal ions

Metal ions such as MgCl₂, MgSO₄, CuSO₄, MnCl₂ and FeSO₄ with varied concentrations (0.02-0.10%) were examined^[1]. A set of five conical flasks containing 100 ml of TGB medium in 250ml Erlenmeyer's flask was supplemented separately with different nitrogen sources. Flasks were autoclaved and inoculated with 1ml spore suspension of *Streptomyces* DSV 12. The optimum carbon and nitrogen sources were incorporated into assessment media. Optimized physico chemical factors were maintained and performed estimation of tyrosinase as described earlier.

RESULTS AND DISCUSSION

Screening of *Streptomyces*

One modified medium STMA and other conventional medium TGBA were employed for the screening of *streptomyces*, aiming at the synthesis of extracellular tyrosinase. STM agar exhibits degree of coloration or intensity of color (yellowish, brownish and blackish) which is an exclusive conventional method prescribed^[34] and followed for the detection of microorganisms synthesizing tyrosinase. The degree of intensity of color was recorded as poor (+, yellowish), moderate (++, brownish) and higher (+++, blackish) and correlated for the level of synthesis of tyrosinase in Table 1. TGB agar exhibits the degree of catalytic zone (mm) by the potential isolates of *streptomyces* in Table 1. The *streptomyces* showing the maximum tyrosinase activity based on the intensity of color and catalytic zone (mm)

Table 1. Qualitative screening of *Streptomyces* for the synthesis of tyrosinase on STM and TGB agar

S. No	Isolate	Colour intensity	Catalytic zone (mm)
1	DSV 1	Yellow (+)	05
2	DSV 3	Yellow (+)	07
3	DSV 4	Brown (++)	12
4	DSV 8	Yellow (+)	05
5	DSV 12	Dark brown (+++)	18
6	DSV 17	Yellow (+)	06
7	DSV 20	Brown (++)	13
8	DSV 23	Yellow (+)	08
9	DSV 26	Yellow (+)	06
10	DSV 27	Brown (++)	11
11	DSV 30	Brown (++)	12
12	DSV32	Yellow (+)	06

were selected and examined for further studies. Plate 1 and 2 exhibit the high intensity of color on STM agar and catalytic zone along with intensity of color on TGB agar by *Streptomyces* DSV 12. The study significantly reveals that, *Streptomyces* DSV 12 was the most efficient isolate for the synthesis of tyrosinase. To the best of our knowledge, no literature reveals, the synthesis of tyrosinase by accounting the catalytic zone on TGB agar and correlated with the intensity of color, indicating the level of synthesis of tyrosinase. The formation of catalytic zone around the organism could indicate that the synthesis of tyrosinase by *Streptomyces* DSV 12 is extracellular.

Production of tyrosinase

Production of tyrosinase in STM and TGB media by all the three test isolates of *streptomyces* are as shown in Fig.1. However, TGB medium has

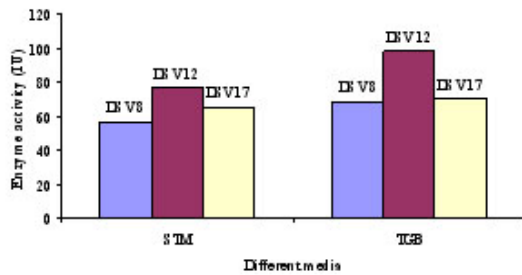


Fig. 1. Quantitative screening of *Streptomyces* for the production of tyrosinase in STM and TGB broth

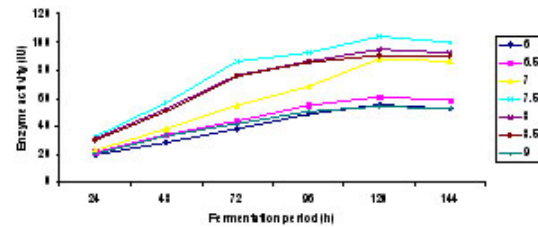


Fig. 2. Effect of pH on the production of tyrosinase by *Streptomyces* DSV 12

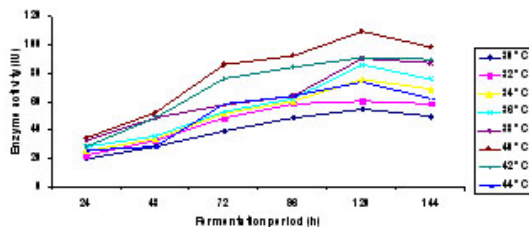


Fig. 3. Effect of temperature on the production of tyrosinase by *Streptomyces* DSV 12

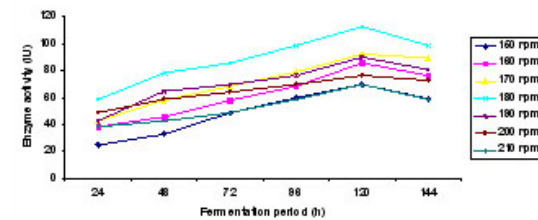


Fig. 4. Effect of aeration on the production of tyrosinase by *Streptomyces* DSV 12

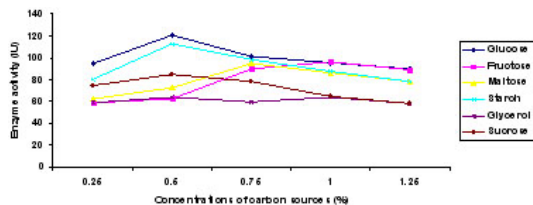


Fig. 5. Effect of carbon sources on the production of tyrosinase by *Streptomyces* DSV 12

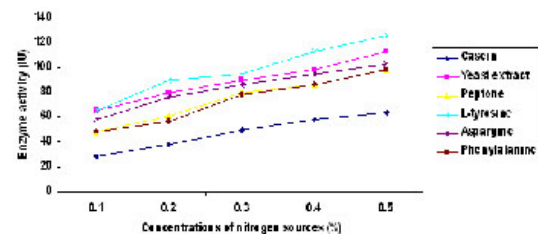


Fig. 6. Effect of nitrogen sources on the production of tyrosinase by *Streptomyces* DSV 12

resulted in more production by *Streptomyces* DSV 8 (68.5 IU/ml), DSV 12 (98.1 IU/ml) and DSV 17 (69.6 IU/ml) of tyrosinase than STM medium by *Streptomyces* DSV 8 (56 IU/ml), DSV 12 (77 IU/ml) and DSV 17 (65 IU/ml). This significantly reveals that, TGB is the better choice of medium, which may be employed to any microorganisms for the synthesis of tyrosinase. Fig 8 shows the quantitative production of tyrosinase in TGB medium. Recent available literature reveals 14.61 U/ml intracellular tyrosinase by *P.sanguineus* and no extracellular activity was observed till 7 days [23], 94 U/ml extracellular tyrosinase by *Bacillus thuringiensis* [22] and 72 U/ml extracellular tyrosinase by actinomycetes [31]. Highly varied range of tyrosinase activities from *Streptomyces* were reported by several researchers. Konard lerch and Leopold ettlinger (1972)[20] reported 24400 units by *Streptomyces glaucescens*, following oxygen consumption method using oxygen electrode and Stephan Phillip et al. (1991) [33] reported 22700 nkat units by *Streptomyces michiganensis*, employing YSI Biological Oxygen Monitor model 5300. Various methods with different principles, type of substrates and their

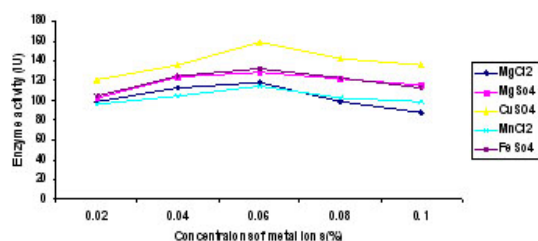


Fig. 7. Effect of metal ions on the production of tyrosinase by *Streptomyces* DSV 12



Fig. 8. Production of tyrosinase in tyrosine gelatin beef extract medium by *Streptomyces* DSV 8, DSV 12 and DSV 17

concentrations employed in the process of enzyme assay would result such higher values. Molar extinction coefficient of Dopachrome is not an uncommon practice being followed by majority of researchers to determine tyrosinase activity. The potential isolate DSV 12 detected in the present investigation was proved to be highly efficient with production of 98.1 IU/ml (TGB medium) tyrosinase.

Process optimization

Variety of physical and nutritional parameters were examined for the enhanced production of tyrosinase. Many fungi [6, 23] and bacteria [22, 2] were known for the production of tyrosinase. To the best of our knowledge, there are no reports available on the process optimization of tyrosinase by actinomycetes in general and streptomyces in particular.

Effect of Physico chemical parameters

The amount of tyrosinase produced at pH 6.0 was 55.6 IU with incubation period of 120 h and gradually increased with increase in pH. Tyrosinase production, was maximum (104.2 IU) at pH 7.5 (Fig.2). A further increase in pH, resulted in a decrease in tyrosinase production, proving the optimum pH as 7.5. Generally, pH 6.5 to 8.0 is the optimum pH range for the growth of *Streptomyces* [18, 24]. At pH 7.5 the growth of the *Streptomyces* DSV 12 was maximum. Naturally, the tyrosinase activity and metabolic rate was much higher. However, pH 5.0 was reported [9] to be optimum for the maximum production (75 IU/ml) of tyrosinase.

The maximum production of tyrosinase (109.4 IU) was obtained at 40°C and it decreased with increase in temperature (Fig. 3). Tyrosinase production was moderate at 42°C. Further, increase in temperature significantly reduced its production.

Thus, the optimum temperature for the production of maximum tyrosinase value resulted at 40°C. However, similarly reveals 30°C as optimum temperature for the maximum production (75 IU/ml) of tyrosinase by *Funalia trogii*.

There was a gradual increase in the tyrosinase production from 150 rpm to 180 rpm. Maximum tyrosinase production (112.4 IU) was resulted at 180 rpm. After 180 rpm the production speed reduced (Fig. 4). At an agitation of < 180 rpm, the tyrosinase production appears to be less due to the inadequate ventilation for conversion of L-tyrosine to dopachrome. The decrease in the tyrosinase production with increased aeration may be because of overgrowth¹⁴ and exhaust of substrate.

Effect of nutritional parameters

Various carbon sources (glucose, fructose, maltose, starch, glycerol and sucrose) were examined for the production of tyrosinase (Fig. 5). Glucose at 0.50% resulted as a better choice of carbon source by yielding maximum production of tyrosinase (120.7 IU). The study also revealed

that starch at 0.50% proved to be the second best choice (112.4 IU) of carbon source when compared to glucose.

Different nitrogen sources casein, yeast extract, peptone, L-tyrosine, L-asparagine and phenylalanine have varied influence on the production of tyrosinase (Fig. 6). The highest production of tyrosinase was achieved by 0.5% L-tyrosine (125.4 IU). The lowest production was recorded by the phenylalanine (40.4 IU).

Effect of metal ions

There was a gradual increase in the tyrosinase production from 0.02% to 0.06% with all metal ions (Fig. 7). Maximum tyrosinase production (158.6 IU) was resulted at 0.06% of CuSO_4 . After 0.06% of metal ions, the production rate was reduced. The study revealed that 0.06% of FeSO_4 (132.6 IU) as the second best choice for the production of tyrosinase production^[1].

Search for newer organisms with higher level production of tyrosinase is ever continuous phenomenon and essential for commercial viability. Screening of potential isolates from natural habitats



Plate 1. Synthesis of tyrosinase on Starch tyrosine mineral agar by *Streptomyces* DSV 12

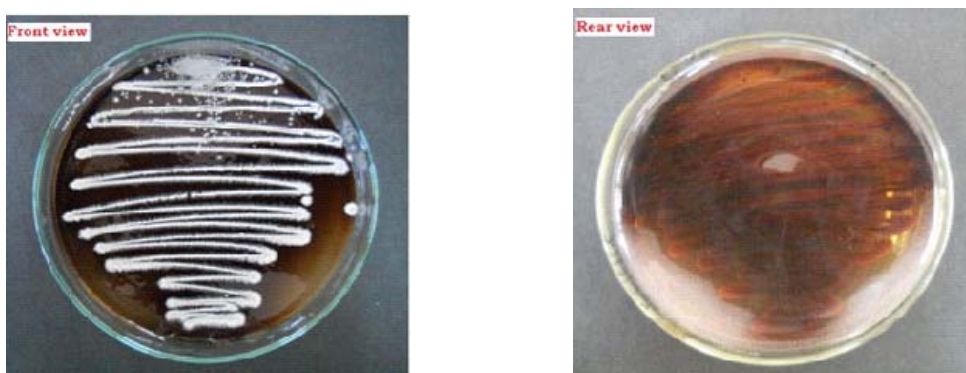


Plate 2. Synthesis of tyrosinase on Tyrosine gelatin beef extract agar by *Streptomyces* DSV 12

is utmost important. Detection of catalytic zone, a novel approach can be a more reliable, accurate and simple method for rapid screening of microorganisms from natural sources for tyrosinase activity. Quite considerable enhancement in the maximum production of extracellular tyrosinase was achieved with all optimal physico chemical and nutritional conditions. The most potential isolate *Streptomyces* DSV12 can be explored further for the large scale industrial production of extracellular tyrosinase with a greater commercial value. TGB medium may be developed further for the much enhanced production of extracellular tyrosinase.

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