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Production of tyrosinase from *Aspergillus nidulans*

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Tyrosinase (EC 1.14.18.1) was isolated from *Aspergillus nidulans* (AUMC No. 7147). The enzyme was firstly optimized and the highest production was recorded on the 6th day of growth. Seven carbon sources were tested for enzyme production and glucose was the best carbon source followed by lactose, maltose, fructose, mannitol and xylose. The optimal pH and temperature of tyrosinase production were 7.0 and 40°C. Sodium nitrate, urea, peptone, ammonium sulphate, yeast extract, beef extract, malt extract and tyrosine were tested as nitrogen sources for tyrosinase production and tyrosine was the best inducer. The optimal inoculum size and agitation speed were 125 µl and 100 rpm, respectively.

Keywords: Tyrosinase, *Aspergillus nidulans*, Production and Optimization.

INTRODUCTION

Tyrosinase (EC 1.14.18.1) is a multifunctional and rate-limiting enzyme in melanin biosynthetic pathway which is involved in two distinct reactions. Firstly, it catalyzes hydroxylation of tyrosine to L-Dopa and secondly, converts L-Dopa to Dopaquinone by oxidation (El-Shora and Metwally, 2008; El-Shora, and Hegazy, 2014; El-Shora, and El-Sharkawy, 2020). Tyrosinase is copper-containing enzyme which is nearly ubiquitously distributed in all domains of life. This property of tyrosinase can be successfully utilized for the biological treatment of wastewater containing phenols (Mita et al. 2007). Tyrosinase is widely distributed in microorganisms, animals and plants (Matoba et al. 2006). Generally, the structure of tyrosinase can be divided into three domains: central domain, N-terminal domain and transmembrane domain (Van Gelder et al. 1997). The central domain is conserved in all tyrosinases and contains two copper binding sites each of which is bound to three conserved histidine residues (Ismaya et al. 2011). The enzyme occurs in three forms, met-, deoxy- and oxy-tyrosinase during the catalytic reactions Ramsden and Riley, 2014).

Melanogenesis is a biosynthetic pathway for the formation of the brown pigment melanin in the human skin. Tyrosinases are the key enzymes catalyzing the different steps in melanogenesis. Since that time, the role of tyrosinase in melanin production and its melanogenic properties has high focus (Zaidi et al. 2014). Melanin is the essential pigment formed by the dermal cells in the deepest layer of the epidermis known as the basal layer. It is produced by melanocytes. Melanin performs an important role in shielding the skin from the detrimental impacts of the sun's ultraviolet rays (Kim and Uyama, 2005; Bouzaiene et al. 2016). Melanin is a polymorphous biopolymers produced from a complex and multistep oxidative reaction starting from L-tyrosine (Chang, 2009). Thus, the present work aimed to optimize the production of tyrosinase from *Aspergillus nidulans.*

MATERIALS AND METHODS

Experimental organism

Aspergillus nidulans (Eidam) G. Winter (AUMC No. 7147) was obtained from Assiut University Mubasher Mycological Center (AUMMC), Assiut – Egypt – 71516.

Inoculum preparation

Spore suspension of *A. nidulans* was prepared by scraping the surface of 6-day old of sporulating culture in 10 ml of 0.85 % sterile saline solution containing 0.1 % of Tween-80. The spore suspension contained 10^7 spores/ml was counted using hemocytometer and 0.1 ml used as the inoculum.

Production of tyrosinase from *A. nidulans*

Tyrosinase production was carried out using modified tyrosine glucose liquid medium according to Saxena and Sinha, (1981). As triplicate sets of 250 ml Erlenmeyer flasks containing 50 ml of sterilized medium (g/L): tyrosine 5.0; glucose 10; KH_2PO_4 1.0; $MgCl_2.6H_2O$ 0.5; $NaNO_3$ 1.0; $CaCl_2.2H_2O$ 0.1; $FeCl_3.6H_2O$ 0.02 and $ZnCl_2$ 0.02 were used. The medium was adjusted to pH 7.0 using 0.1 N NaOH, then inoculated with 0.1 ml of fresh spore suspension and incubated at 30°C for 7 days. After incubation, fungal mycelium was filtered and separated by centrifugation at 8,000 g for 15 min then the supernatant was used as a crude enzyme.

Assay of tyrosinase

To 0.1 ml culture supernatant 1.0 ml of 0.5 M phosphate buffer (pH 6.5), 1.0 ml of 1 mM tyrosine and 0.9 ml of distilled water were added into test tube. The reaction mixture was oxygenated by bubbling through a capillary tube for 5 min to reach temperature equilibration and absorbance was recorded at 280 nm by using UV-V is spectrophotometer (Raval et al. 2012).

Determination of protein

The protein content was measured using Bovine Serum Albumin (BSA) as a standard protein according to Bradford, (1976). One ml of sample was mixed with 5ml of Bradford reagent (Coomassie brilliant blue G-250) and incubated for 5 min then the absorbance was recorded 595 nm (Raval et al. 2012) and specific activity of tyrosinase was calculated.

Effect of incubation periods on tyrosinase production from *A. nidulans*

Tyrosine-glucose liquid medium was prepared in 250-ml Erlenmeyer flasks each contained 50 ml. After autoclaving the flasks were inoculated with 0.1 ml of spore suspension (10^7 spore/ml) of *A. nidulans.* The inoculated flasks were incubated in static incubator and shaking incubator (130 rpm) with pH 7 at 30°C with different incubation periods (1, 2, 3, 4, 5, 6 and 7 days). Three replicates were used for each period. The cultures were filtered using Whatman no. 1 filter paper and the extracellular activity of tyrosinase was measured as mentioned above.

Effect of initial pH on tyrosinase production from *A. nidulans*

Tyrosine-glucose liquid medium was prepared, inoculated and incubated at the same conditions as above in shaking incubator at 130 rpm for 7 days (optimum incubation period) with different pH values (3, 4, 5, 6, 7, 8 and 9). After incubation the enzyme activity was measured as mentioned above (Swathi and Sridevi, 2015).

Effect of incubation temperature on tyrosinase production from *A. nidulans*

Fifty millimeters of tyrosine-glucose liquid medium were adjusted to pH 7.0 (optimum pH), incubated with 0.1 ml of spore suspension (107 spore/ml) and incubated in a shaker (130 rpm) at different incubation temperatures (10, 20, 30, 40, 50, 60 and 70oC) for 7 days, three replicates were used for each temperature. The extracellular activity of tyrosinase was measured as mentioned above (Swathi and Sridevi, 2015).

Effect of different carbon sources on tyrosinase production from *A. nidulans*

Tyrosine-glucose liquid medium was prepared and glucose was substituted with 1% (w/v) of other carbon source such as glucose, sucrose, maltose, fructose, lactose, mannitol and xylose separately as a sole carbon source with pH 7.0 (optimum pH). Then the flasks were inoculated with 0.1 ml of spore suspension (10^7 spores/ml) and incubated in a shaking incubator (130 rpm) at 40ºC (the optimal) for 7 days. After that, the extracellular tyrosinase activity was measured Khalaf and El-Sayed, 2009).

Effect of different nitrogen sources on tyrosinase production from *A. nidulans*

Tyrosine-glucose liquid medium was prepared with addition of 1% (w/v) from sodium nitrate, peptone, urea, yeast extract, ammonium sulphate, tyrosine, beef extract and malt extract, separately as a sole nitrogen source with 1% (w/v) glucose and adjusted to pH 7.0. The flasks were inoculated and incubated at the same conditions above. The culture was filtered and extracellular enzyme activity was measured (Krishnaveni *et al.* 2009).

Effect of inoculum size on tyrosinase production from *A. nidulans*

The effect of inoculum size on tyrosinase production was tested using various volumes (25, 50, 75, 100, 125, 150, 175 and 200 µl) followed by estimation of extracellular tyrosinase activity.

Effect of different agitation speeds on tyrosinase production from A. nidulans

The effect of agitation speed on tyrosinase production was tested at various speeds (25, 50, 75, 100, 125, 150, 175 and 200 rpm) followed by estimation of extracellular enzyme activity.

RESULTS AND DISCUSSION

Before studying the influence of the various plant extracts on tyrosinase activity it was important to optimize the enzyme production by *A. nidulans* and this optimization was investigated throughout 7 days. The enzyme activity was measured every day and the activity was expressed as units mg^{-1} protein. The results are illustrated in Figure 1. These results indicate that there was continuous increase in the production of tyrosinase up to the 6th day where maximum activity was observed (41.2 units mg^{-1} protein) and after which there was a decline in the enzyme production at the 7th day of growth. Tyrosinase production by *A. nidulans* in the present work was studied throughout 7 days. The optimal production of tyrosinase was recorded on the 6th day. These results are in agreement with that *reported for the enzyme from Fusarium solani (*Odeniyi et al. 2019).

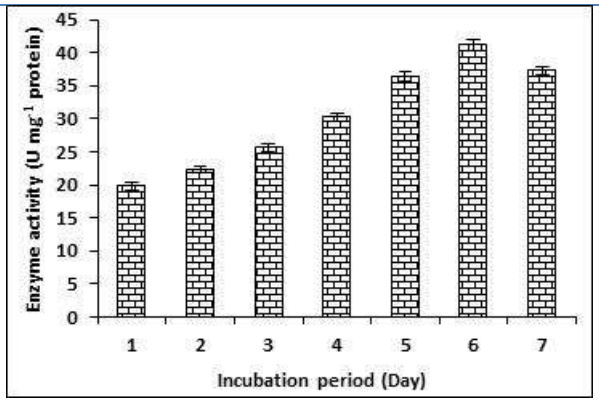

Figure 1: Effect of incubation period on tyrosinase production from *A. nidulans.*

This experiment was carried out to investigate the effect of different carbon sources including glucose, sucrose, maltose, fructose, lactose, mannitol and xylose. The results in Figure 2 reveal that glucose was the best carbon source followed by lactose, maltose, fructose, sucrose, mannitol and xylose. Higher concentration of glucose resulted in tyrosinase inhibition, possibly due to conversion of glucose to gluconic acid which decreases the pH of the medium causing inhibition of the enzyme production (El-Shora et al. 2021). Agarwal *et al.* (2016) reported the constitutive and enhanced production of tyrosinase at 2 % concentration of glucose. Also, lactose yielded considerably high tyrosinase titers, while a sucrose-supplemented medium produced the least tyrosinase by *Fusarium solani* (Odeniyi *et al.* 2019). Moshtaghioun et al. (2017) selected sucrose for the optimization of growth of tyrosinase producing *Neurospora crassa* and based their preference on the presence of invertase in its membrane.

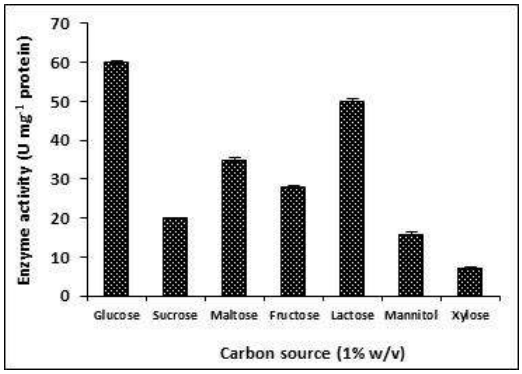

Figure 2: Effect of different carbon sources on the production of tyrosinase from *A. nidulans.*

The influence of pH on the production of tyrosinase was studied at pH 3, 4, 5, 6, 7, 8 and 9. The results in Figure 3 indicate that there was a continuous increase in the tyrosinase production with increasing the pH 3 to pH, 7 which was the optimum one. After pH 7.0 there was continuous decline in tyrosinase production at pH 8 and 9 where the activities were 35 and 15 units mg^{-1} protein. Thus, the optimum pH of tyrosinase production from *A. nidulans* in the present work was 7.0, which is the same pH for tyrosinase production from *Fusarium solani* (Odeniyi et al. 2019). Also, Valipour and Arikan, (2016) reported a maximum yield of tyrosinase production between pH 6.0 and 7.0, which corroborates our observations. However, this contrasts the studies of (Krishnaveni et al. 2009) who reported that the optimum pH of tyrosinase production ranged between 5.0-5.5.

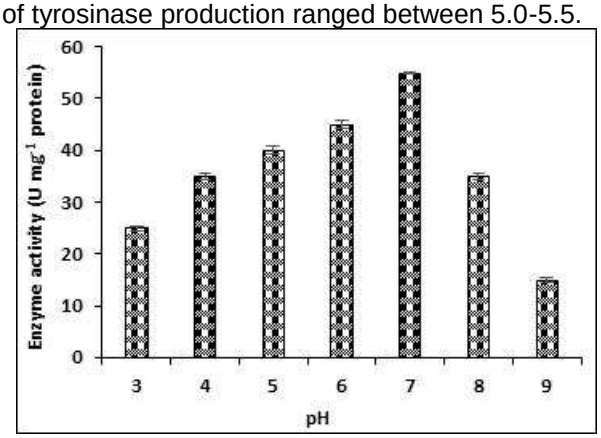

Figure 3: Influence of pH on production of tyrosinase from *A. nidulans.*

The effect of temperature on tyrosinase production from *A. nidulans* was carried out using various incubation temperatures (10, 20, 30, 40, 50, 60 and 70°C). The results in Fig. 4 reveal that the optimal temperature for tyrosinase production was 40°C after which any increase in temperature resulted in reduction of tyrosinase activity. The temperature is an essential factor for production of any enzyme as it is controlling all biochemical reactions of the microbial cells (Pietikäinen *et al.* 2005; El-Shora et al. 2021a).

The optimum temperature in the present work for tyrosinase production from *A. nidulans* was 40°C. At higher temperatures, tyrosinase productivity decreased gradually. It was mentioned that the incubation temperature plays a significant role in cellular activities including protein denaturation, enzyme release and cell viability (El-Shora et al. 2021b). Increasing temperature was observed to cause reduction in the enzyme activity, and this might be attributed to the influence of the temperature of the environment from which the fungi were isolated on their physiology. Earlier studies have shown temperature maxima for fungal tyrosinase production ranged between 25°C and 35°C (Valipour and Arikan, 2016). Generally, it could be assumed that the susceptibility of tyrosinase to inactivation at higher temperatures might be a result of its molecular structure. However, further studies should be conducted to ascertain this claim. Consequently, their efficiencies in applications requiring higher temperature regimes might be inconsequential.

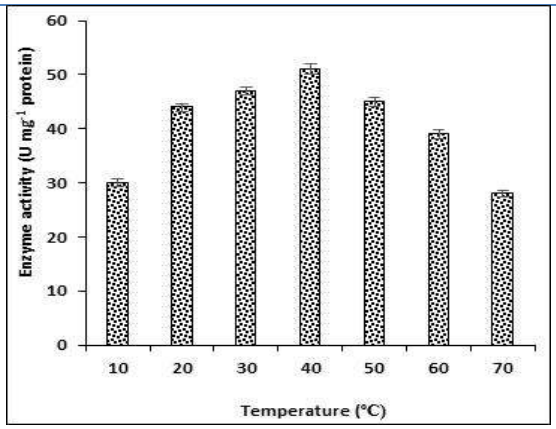

Figure 4: Effect of temperature on the production of tyrosinase from *A. nidulans.*

This experiment aimed to study the influence of various nitrogen sources on tyrosinase production. The tested nitrogen sources were sodium nitrate, peptone, urea, yeast extract, ammonium sulphate, tyrosine, beef extract and malt extract. Each compound was tested at 1% w/v. The results are illustrated in Figure 5. These results indicate that tyrosine was the best followed by sodium nitrate. The least effective nitrogen source was beef extract. The other nitrogen sources offered appreciable activities of tyrosinase activities. These results indicate that tyrosine was the best nitrogen source for tyrosinase from *A. nidulans*. However, nitrate was the best nitrogen source for tyrosinase production from *Fusarium solani* (Odeniyi et al. 2019). Majidi et al. (2013) reported that maximum enzyme production was obtained when sodium nitrate was used as nitrogen source, but they concluded that best nitrogen source might differ from one fungus to another. This could be due to the repressive effects of amino acids and analogues which might be present in the production of tyrosinase by some fungi (Faccio et al. 2012). The metabolism of the organism is important because the carbon and nitrogen sources supply the components needed for the synthesis of protein, nucleic acids, cell wall materials and also the reserved food materials.

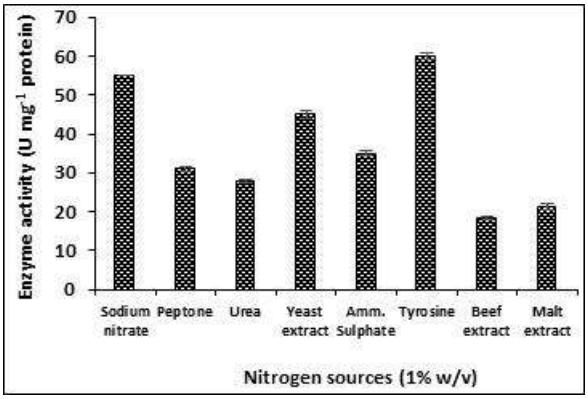

Figure 5: Effect of nitrogen sources on the production of tyrosinase from *A. nidulans*.

This experiment aimed to study the effect of inoculum size on the production of tyrosinase. The tested inoculum sizes (25, 50, 75, 100, 125, 175 and 200 µl). The results in Figure 6 reveal that there was continuous increase in the enzyme production with increasing the inoculum size from 25 µl to 125 µl which seems likely to be the optimal size where the enzyme activity was 21.3 units mg^{-1} protein. In the present work, the optimal inoculum size for tyrosinase from *A. nidulans* was 125 µl. Increasing or decreasing the inoculum size above or below 125 µl resulted in lowering the productivity of tyrosinase which may be due to the presence of an inadequate level of inoculum in balance with the aeration, nutrition and other factors controlling the production. It is well known that the initial biomass controls the kinetics of growth and several biological metabolic functions, leading to the overall biomass and extracellular production (Wakayama et al. 2005).

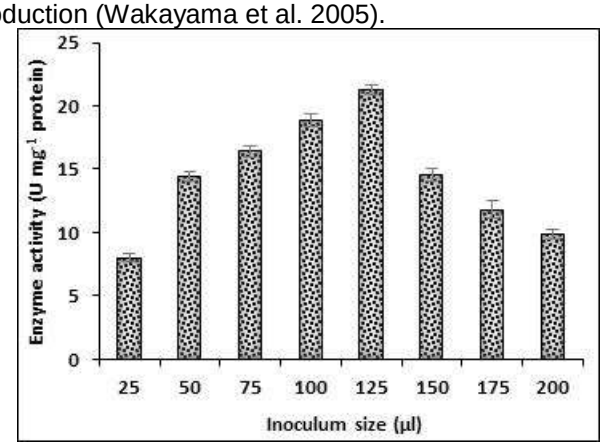

Figure 6: Effect of inoculum size on the production of tyrosinase from *A. nidulans.*

The effect of agitation speed was tested regarding to its effect on tyrosinase production. The agitation speeds were 25, 50, 75, 100, 125, 175 and 200 rpm. The results in Figure 7 indicate continuous increase in the enzyme production with increasing the speed of agitation from 25 rpm to 100 rpm where the activity was 19.9 units mg^{-1} protein. Any further increase in the agitation speed resulted in reduction of the enzyme production. Agitation speed has been shown to affect production of many enzymes by microorganisms. In the present study the results demonstrated that the highest production of tyrosinase was recorded at 100 rpm. This probably due to aeration of the culture medium was increased and dissolved oxygen in the media was sufficient. Nutrient uptake by the fungus increased tyrosinase production. Increasing the agitation of speed over 100 rpm reduced the production of tyrosinase. T he adequate aeration and nutrient might cause the fungus to grow well but shear forces and cell damage finally had negative effect on enzyme production (Whitaker *et al.* 1984; Ibrahim et al. 2015).

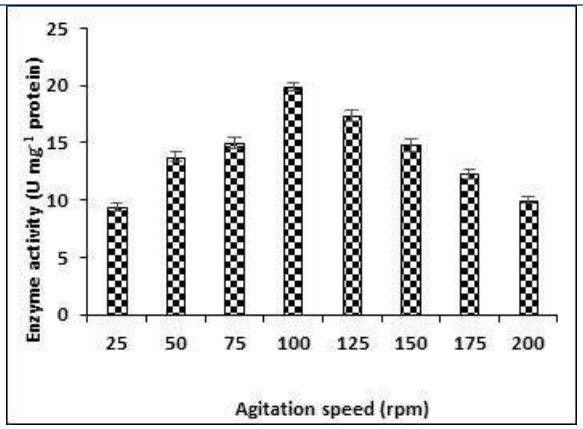

Figure 7: Effect of agitation speed on the production of tyrosinase from *A. nidulans.*

CONCLUSION

The production of extracellular tyrosinase from *A. nidulans* was optimized and the highest productivity was obtained on the 6th day of fungal growth at pH 7.0 and temperature of 40°C with inoculum size of 125 µl.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

AUTHOR CONTRIBUTIONS

H.M.E.-S. and M.E.I.; methodology, H.M.E.-S. and M.W.A.; software, H.M.E.-S., B.Y., and M.W.A; investigation, H.M.E.-S., M.E.I. and B.Y.; resources, H.M.E.-S., M.E.I. and B.Y.; writing—original draft preparation, H.M.E.-S. and M.W.A.; writing—review and editing, H.M.E.-S. and B.Y.; visualization, H.M.E.-S., M.E.I.; supervision, H.M.E. All authors have read and agreed to the published version of the manuscript.

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