International Journal for Multidisciplinary Research (IJFMR)
E-ISSN: 2582-2160 ● Website: www.ijfmr.com ● Email: editor@ijfmr.com

Isolation of tyrosinase producing bacteria from Rhizosphere soil samples

Barate D. L.[1], Sonar N. R.[2]

[1]Assistant Professor, P. G. Department of Microbiology, Shri Shivaji College of Arts, Commerce and Science, Akola – 444001 (M.S.) India.
[2]Student, P. G. Department of Microbiology, Shri Shivaji College of Arts, Commerce and Science, Akola – 444001 (M.S.) India.

Abstract

The present study is based on the isolation and identification of the bacteria producing tyrosinase enzyme which play an important role in production of L-DOPA amino acid which is used to treat Parkinson's disease. In the study, 42 rhizosphere soil samples were collected from Akola and Amravati district, out of which 15 isolates were found to be tyrosinase enzyme producers when screened on tyrosine agar medium. Two isolates NT and ST showed most prominent tyrosinase production which latter checked for the effect of parameters on tyrosinase activity. Results revealed that tyrosinase activity was optimum at temperature range 37°C - 42°C, pH 6 for NT isolate and pH 9 for ST isolate and time course 72 hours. Further effect of nitrogen sources showed prominent increase in tyrosinase activity after addition of 1% sodium and potassium nitrate while addition of carbon sources did not show any remarkable increase in tyrosinase activity.

Keywords: Tyrosinase, L-DOPA, L-Tyrosine, Rhizosphere

1. Introduction

Tyrosinase is a multifunctional membrane bound copper containing glycoprotein which is located in the membrane of melanosome. Tyrosinase is also known as polyphenol oxidase, DOPA oxidase, phenolase and catechol oxidase (Pradhan and Sarkar, 2017). The enzyme tyrosinase can take part in biosynthesis of melanin in which monophenolase catalyses substrate tyrosine into 3,4-dihydroxyphenylalanine or L-DOPA. L- DOPA is an amino acid a favored medication for treatment of Parkinson's disease (Franciscon *et al.,* 2012).

In humans, tyrosinase produces melanin as a defence against the harmful effects of UV light, X-ray, gamma rays. (Kumar *et al.,* 2011; Gare *et al.,* 2016). In bacteria, tyrosinase is the key enzyme in initiating the melanin biosynthesis pathway and an important protective and survival role (Valipour and Arikan, 2015). While in fungi it is of crucial importance in survival and virulence, reproductive organ differentiation, spore formation and tissue protection after injury (Bell and Wheeler, 1986).

The monophenol hydroxylase and diphenol oxidase activities of tyrosinase are used in environmental technology for the detoxification of phenol containing waste water and contaminated soils as a construction of a biosensor for the detection of phenolic compounds (Dos Santos *et al.,* 2013). Tyrosinase also plays an important role in wound healing and the primary immune response of plant life, sponges and many invertebrates (Danial *et al.,* 2018; Decker and Tuczek, 2000). Melanocytes produces

two types of melanin "Eumelanin (brown-black)" and "Pheomelanin (red-yellow)" formed by the conjugation of cysteine.

The excessive production of dopaquinones results in neuronal damage and cell death. This suggests that tyrosinase might play a significant role in neuromelanin formation in the human brain and responsible for the neurodegeneration associated with Parkinson's disease and Huntington's diseases (Pillaiyar *et al.,* 2017). Tyrosinase has been extracted, isolated and purified from various sources such as animals, plants, insects, microorganisms (Saratale *et al.,* 2011; Xu *et al.,* 2012). Thus, the present study was aimed to isolate and identify tyrosinase producers from the rhizosphere soil samples for their possible effective utilization in melanin production.

2. Material and Methods

➢ Collection of rhizosphere soil samples

A total of 42 soil samples from rhizosphere of plants were collected from Amravati and Akola region. 10gm of soil samples were collected in sterile zip lock polythene bags. The samples were carried to the laboratory for further use.

➢ Isolation and screening of bacteria from soil samples.

The 1gm rhizosphere soil samples were serially diluted and inoculated on nutrient agar plates at 37°C for 24 hrs. The pure colonies obtained were streaked on nutrient agar slant and maintained at 4°C in refrigerator for further use.

➢ Primary screening of tyrosinase producing bacteria

The soil isolates were then subjected to primary screening for tyrosinase production. For this the isolates were inoculated on sterilized Tyrosine Agar Medium and incubated at 37°C for 2-3 days. The brown to black colored colonies developed by the isolates were selected as tyrosinase producers and subjected for secondary screening.

➢ Secondary screening of tyrosinase producing bacteria.

In the secondary screening all the positive isolates were inoculated on sterile Tyrosine Agar Medium by Spot (1mm diameter) inoculation method and incubated for 2-3 days at 37°C temperature. After the incubation the brown or black color spots were observed. The positive isolates were also inoculated in sterile Tyrosine Broth with few drops of chloroform as per the method assigned by Gare and Kulkarni (2015) and tubes were incubated at 37°C for 2-3 days. On the basis of brown or black color observed the isolates were assigned as Weak (+), Moderate (++), Strong (+++), and Very strong (++++).

➢ Production of Tyrosinase

Tyrosinase production was carried out in 250ml flask containing 200ml of basal medium (L-Tyrosine broth). The broth was sterilized in autoclave at 121°C for 15 minutes. The sterile broth was inoculated with potent isolates and incubated on rotary shaker at 37°C. After incubation, the broth was centrifuged at 10,000 rpm for 15 minutes and the cell free supernatant was separated and used as a crude enzyme. Also, protein content was estimated using Lowry a method (Lowry *et al.,* 1951).

➢ **Determination of Tyrosinase Activity**

Tyrosinase activity of two prominent isolates was determined by using L-Tyrosine as substrate. 5 ml of culture was centrifuged at 10000 rpm for 10 min. The reaction mixture was consisted of 0.7 ml distilled water, 0.1 ml supernatant of crude enzyme, 0.1 ml L-Tyrosine, 0.1 ml of HCL. The reaction mixture without crude enzyme extract was used as blank and activity was determined by using spectrophotometer at 480nm wavelength (Pradhan and Sarkar, 2017),

$$\text{IEU/ml} = \frac{(\text{OD of sample} - \text{OD of blank}) \times \text{volume of assay mixture}}{\text{volume of enzyme}}$$

➢ **Effect of various parameters on tyrosinase production**

For the optimization of tyrosinase production the effect of various parameters such as Temperature, pH, Nitrogen sources and Carbon sources were studied using one factor at a time method. And the activity was checked by using spectrophotometer at 480nm wavelength (Pradhan and Sarkar, 2017).

▪ **Effect of Temperature on tyrosinase production**

Concerning the effect of tyrosinase production by potent isolates at various temperature. The basal medium (broth) was prepared in tubes. The tubes were then sterilized and inoculated with two potent isolates. The inoculated tubes were incubated at various temperature such as 15°C, 30°C, 37°C, 42°C for 2-3 days to determine the optimum temperature of tyrosinase production by isolates.

▪ **Effect of pH on tyrosinase production**

To check the effect of cultivation pH on growth and tyrosinase production, the broth was prepared in 250 ml flask and then separated in tubes and different pH were adjusted by using HCL (2N) and NaOH (2N). The sterilized tubes containing broth were then inoculated and incubated at 37°C for 2-3 days.

▪ **Effect of Nitrogen Sources on tyrosinase production**

The effect of Nitrogen sources on tyrosinase production were detected by using the 1% nitrogen sources such as Ammonium chloride, Ammonium sulphate, Sodium nitrate and Potassium nitrate. The broth was prepared in flask and later separated in tubes with corresponding nitrogen source. The tubes were autoclaved separately. Sterilized tubes were inoculated with the two potent isolates and incubated at 37°C for 2-3 days.

▪ **Effect of Carbon Sources on tyrosinase production**

To check the activity of tyrosinase production by potent isolates various carbon sources were used. The broth tubes were supplemented with 1% carbon sources such as Glucose, Lactose, Maltose, Sucrose. The carbon sources were prepared in separate tubes and sterilized in autoclave. After the sterilization, the tubes were inoculated with the isolates which are prominent in tyrosinase production and incubated at 37°C for 2-3 days.

3. Results and Discussion

Rhizosphere is an area of plant which provides favorable environment and nutrition for the growth of bacteria. In this view the 42 rhizosphere soil samples were collected from various places of Akola and Amravati. A total of 50 bacterial isolates were obtained by serial dilution which were further checked for the tyrosinase activity on Tyrosine Agar Medium. Out of 50 bacterial isolates 15 (30.0%) isolates showed tyrosinase activity by showing brown to black colored colony forming in the primary screening. In the secondary screening, the positive isolates were further inoculated on Tyrosine Agar Medium by spot inoculation and checked for the zone of brown or black color. The efficacy of isolates producing tyrosinase was decided by the zone as weak producer, good producer, and excellent producer (Fig. 1). Out of 15 isolates 2 isolates ST and NT were found to be prominent tyrosinase producers. These isolates were selected for the further studies. The two isolates ST and NT were identified by Morphological, Cultural and Biochemical characteristics (Table 1). It was found that both the isolates were belongs to the *Bacillus spp*.

The isolates were further used for tyrosinase production by inoculating in the production media and crude enzyme obtained was further used for the study. The protein estimation from the crude enzyme was determined by Folin Lowry method. It was found that Protein content of crude enzyme from isolate ST was 292 µg/ml and for NT isolate it was 316 µg/ml. The enzyme assay for tyrosinase activity was done and it was found that crude enzyme from isolate ST showed 3.4 U/ml, while NT showed 4.0 U/ml activity.

The effect of various parameters like Temperature, pH, Nitrogen sources and Carbon sources on tyrosinase activity was also checked.

The effect of temperature on tyrosinase activity showed that isolate ST showed 0.9 U/ml. 3.8 U/ml, 5.1 U/ml and 3.9 U/ml activity at 15°C, 30°C, 37°C and 42°C respectively, while isolate NT showed 0.7 U/ml, 6.9 U/ml, 9.1 U/ml and 10.1 U/ml activity at 15°C, 30°C, 37°C and 42°C temperature respectively (Fig. 2).

The effect of pH on the tyrosinase activity showed that isolate ST showed 4.4 U/ml, 3.4 U/ml, 4.5 U/ml, 4.1 U/ml and 5.3 U/ml activity at 5,6,7,8 and 9 pH respectively, while isolate NT showed 6.4 U/ml, 14.6 U/ml, 4.7 U/ml, 3.3 U/ml and 4.5 U/ml activity at 5-9 pH respectively (Fig. 3).

The effect of nitrogen sources on the tyrosinase activity showed that isolate ST showed 6.1 U/ml, 10.4 U/ml, 3.5 U/ml and 3.0 U/ml activity for Potassium nitrate, Sodium nitrate, Ammonium sulphate and Ammonium chloride respectively, while isolate NT showed 19.8 U/ml, 19.5 U/ml, 7.7 U/ml and 6.7 U/ml activity for Potassium nitrate, Sodium nitrate, Ammonium sulphate and Ammonium chloride respectively (Fig. 4).

The effect of carbon sources on the tyrosinase activity showed that isolate ST showed 2.5 U/ml, 6.7 U/ml, 6.1 U/ml and 4.9 U/ml activity for Glucose, Sucrose, Lactose and Maltose respectively, while isolate NT showed 6.9 U/ml, 5.3 U/ml, 7.3 U/ml and 4.2 U/ml activity for Glucose, Sucrose, Lactose and Maltose respectively (Fig. 5)

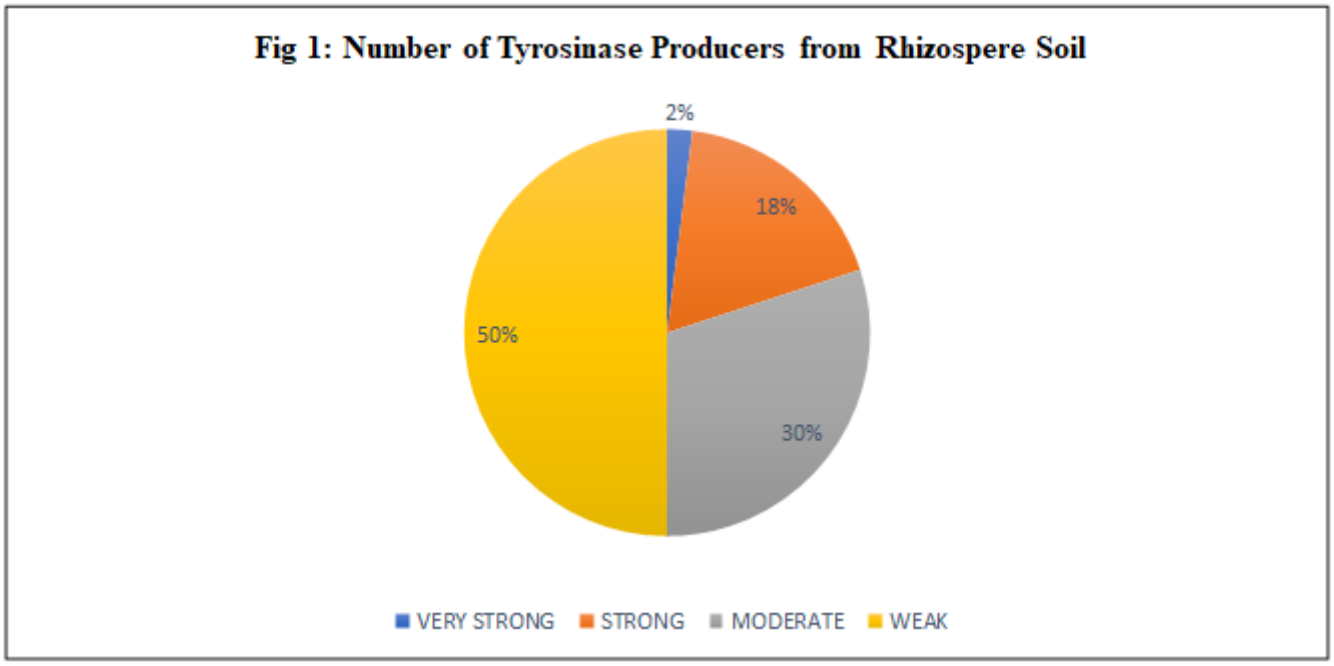
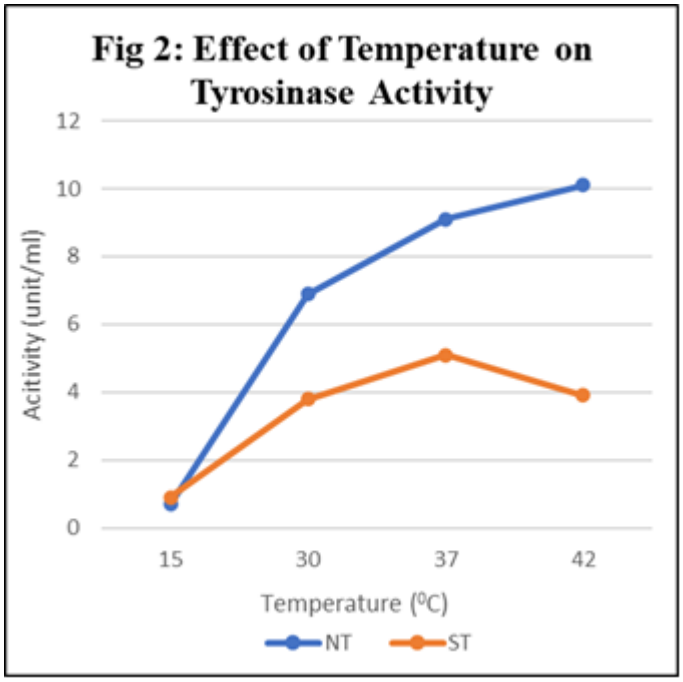
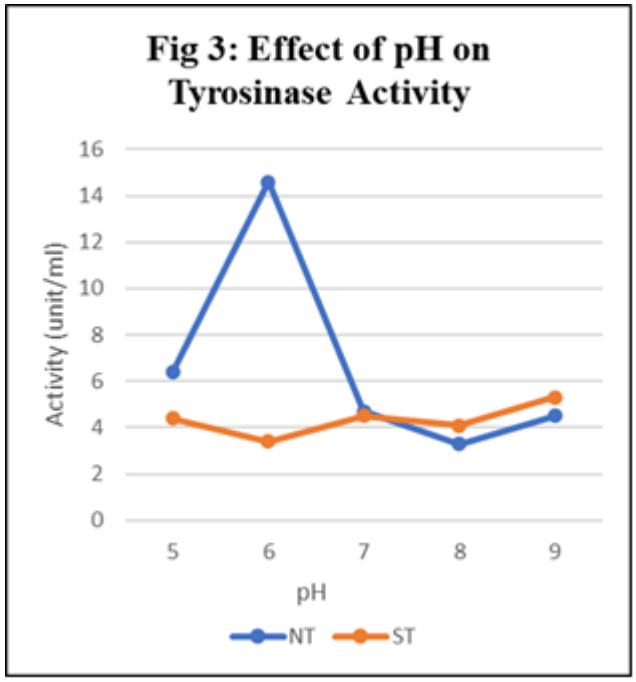


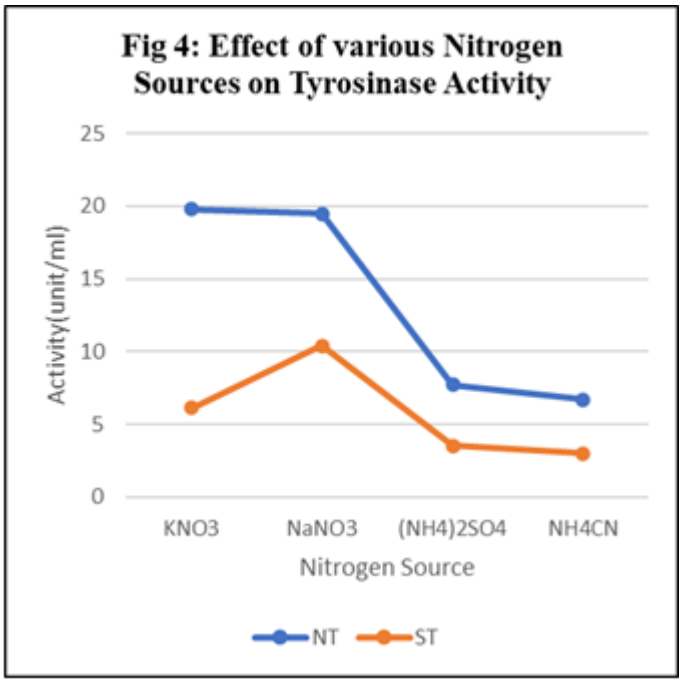
Table 1: Morphological, Cultural and Biochemical identification of prominent isolates.

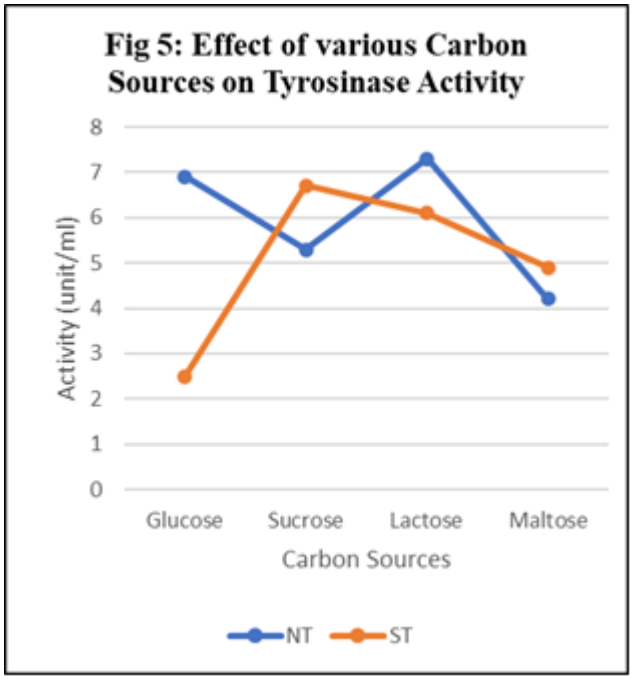
Characteristics	**ST**	**NT**
Gram character	Gm +ve rod	Gm +ve rod
Shape	Circular	Circular
Size	0.5-0.8 µm	0.4-0.7 µm
Color	White	Cream
Opacity	Opaque	Opaque
Margin	Undulate	Undulate
Motility	Motile	Motile
Elevation	Raised	Raised
Surface	Smooth	Smooth
Fermentation of Sugar		
Glucose	+ve	+ve
Sucrose	+ve	+ve
Lactose	-ve	-ve
Maltose	-ve	-ve
IMViC Test		
Indole	-ve	-ve

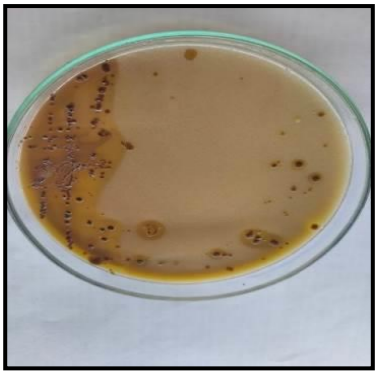
Methyl Red	-ve	-ve
Voges Proskauer	+ve	+ve
Citrate	-ve	+ve
Enzyme Study		
Catalase	+ve	+ve
Oxidase	+ve	-ve
Amylase	-ve	+ve
Urease	+ve	+ve
Gelatinase	-ve	+ve
Probable Isolate	*Bacillus spp.*	*Bacillus spp.*





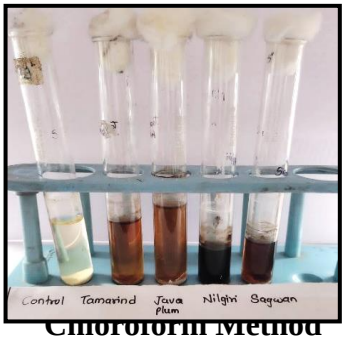






Tyrosine Agar

Spot Inoculation



Chloroform Method

Primary screening

Secondary screening

Discussion

In the present study, 42 rhizosphere soil samples were collected from various places of Akola and Amravati from which 15 isolates found to be positive for tyrosinase production. Out of 15 isolates 2 were prominent in tyrosinase production. After the identification both the isolates were found to be of *Bacillus spp.* (Table 1). Several other studies like, Valipour and Arikan (2016), isolated tyrosinase producing bacteria from soil samples collected from a Tomato field in city of Adana in Turkey and identified the bacteria as *Bacillus megaterium* strain M36. Similar study was carried out by Elsayed and Danial (2018), for the isolation of tyrosinase producing bacteria from the soil samples collected from Potato planted field, Mansoura, Egypt and found *Bacillus subtilis* NA2 strain.

For the optimization of tyrosinase activity, the effect of various parameters were checked in which temperature for the NT isolate was 42°C for maximum tyrosinase activity (10.1 U/ml), while ST isolate gives maximum tyrosinase activity at 37°C temperature (5.1 U/ml) (Fig: 2). Similarly, Valipour and Arikan (2016), found the maximum tyrosinase production at 36°C and also the optimum tyrosinase

activity ranging between 30°C-40°C. Pradhan and Sarkar (2017), also found the increase in tyrosinase production at 40°C.

When the effect of pH checked for the tyrosinase activity then maximum tyrosinase production was observed at pH-6.0 for NT isolate (14.6 U/ml) and for ST isolate it was found to be pH-9.0 (5.3 U/ml) (Fig: 3). Valipour and Arikan (2015), reported the same observation for tyrosinase production at pH-9. Likewise, Valipour and Arikan (2016) found the maximum tyrosinase activity at pH range of 6.0 – 7.0.

After detecting the effect of nitrogen sources, the maximum tyrosinase activity was found by addition of 1% Potassium nitrate (KNO3) by NT isolate (19.8 U/ml), whereas for ST isolate the maximum tyrosinase activity was showed by Sodium nitrate (NaNO3) (10.4 U/ml) (Fig: 4). Similarly, Pradhan and Sarkar (2017), reported sodium nitrate as a nitrogen source which increase the yield of tyrosinase production. Ingle and Khobragade (2013), also reported the highest tyrosinase enzyme production by the addition of potassium nitrate as a nitrogen source.

After detecting the tyrosinase activity by the addition of 1% carbon source such as lactose it was found that there was an increase in tyrosinase production by NT isolate (7.3 U/ml), while for ST isolate sucrose was the carbon source (6.7 U/ml) increases tyrosinase production (Fig: 5). The results are opposite to Valipour and Arikan (2015) reported that Glucose and Maltose increases the tyrosinase yield. While the results are in accordance with Elsayed and Danial (2018), reporting that, sucrose was the most suitable carbon source for tyrosinase activity.

4. Conclusion

In the present study it was found that two prominent isolates showing tyrosinase production were belongs to *Bacillus spp*. Optimization studies conclude that isolate ST showed highest activity at 37°C temperature, pH – 9 and after 72 hrs. of incubation. While, isolate NT showed highest activity at 42°C temperature, pH – 6 and 72 hrs. of incubation. Also, addition of 1% Carbon and Nitrogen sources showed enhanced production of tyrosinase. It was observed that further detail studies on tyrosinase enzyme from the isolates would have potential in melanin production and its application in future.

5. References

1. Bell AA and Wheeler MH (1986). Biosynthesis functions of fungal melanin. *Annu Rev Phytopathol*; 24: 411-451.
2. Danial NE,and Al-Birshi WM (2018). Optimization of medium composition for increased production of tyrosinase enzyme in recombinant *Bacillus megaterium. J. pharmaceutical Biol Chem Sci* 2018; 9(1): 480-486.
3. Decker H, Tuczek F: Tyrosinase (catechol oxidase activity of hemocyanins: structure basis & molecular mechanism, *Trends Biochemical Science* 2000; 25: 392-397.
4. Dos S., V. P., Silvaa, L. M., Salgadoa, A. M., & Pereirab, K. S. (2013). Application of Agaricus bisporus extract for benzoate sodium detection based on tyrosinase inhibition for biosensor development. *Chemical Engineering* 32(2).
5. Elsayed, E. A., & Danial, E. N. (2018). Isolation, identification and medium optimization for tyrosinase production by a newly isolated Bacillus subtilis NA2 strain. *Journal of Applied Pharmaceutical Science*, 8(9), 093-101.

6. Fransciscon E., Grossman, M.J., Paschoal, J.A.R., Reyes F.G.R., and Durrant, L.R. (2012). Decolorization & Biodegradation of reactive sulfonated azo dyes by a newly isolated *Brevibacterium Sp*. strain VN-I5. *Springerplus*, 1(1), 1-10.
7. Gare, S. S. and Kulkarni, S. W. (2015). Isolation and characterization of tyrosinase producing Streptomyces luteogriseus. *World J. Pharmaceutical Research* 4(4), 1385-1395.
8. Gare Sandip Subhash, D.D. Karad and S.W. kulkarni (2016). Screening of tyrosinase producing soil *actinomycetes* from shirala region of Maharashtra, India, *International Journal of current microbiology and applied sciences*, volume 5(3): 345-353.
9. Ingle SS and Khobragade CN (2013). Production and purification of the tyrosinase enzyme from soil bacteria. *Helix*; 6: 436-440.
10. Kumar CG, Mongolla P, Pombala S, Kamle A, Joseph J (2011). Physiochemical characterization and antioxidant activity of melanin from a novel strain of Aspergillus bridgeri ICTF-201. *Lett Applied Microbiology*, 53 (3): 350-358.
11. Lowry O.H.; Rosebrough N.J. Farr A.L. Randell R.J. (1951). J Biological Chemical; 193: 265-275.
12. Pillaiyar T, Manickam M and Namasivayam v (2017). Skin Whitening agents: medicinal chemistry perspective of tyrosinase inbitiors, *J Enzyme inhibitor Medical Chemistry*; 32(1): 403-425.
13. Pradhan, P., and Sarkar, P. (2017). Production, optimization and application of tyrosinase from Bacillus sp. CGR6 in dye degradation. *World J. Pharmaceutical Medical Research*. 3, 212-218.
14. Rani, N., Joy, B., & Abraham, T. E. (2007). Cell suspension cultures of Portulaca grandiflora. As potent catalysts for biotransformation of L-tyrosine into L-DOPA, an anti-Parkinson's drug. *Pharmaceutical Biology*, 45(1), 48-53.
15. Saratale, R. G., Saratale, G. D., Chang, J. S., & Govindwar, S. P. (2011). Bacterial decolorization and degradation of azo dyes: a review. *Journal of the Taiwan institute of Chemical Engineers*, 42(1), 138-157.
16. Valipour, E. and Arikan, B. (2015). Optimization of tyrosinase enzyme production from native *Bacillus spp*. MV29 isolate. *J Appl. Biol. Sci.*, 9(2), 77-82.
17. Valipour, E. and Arikan, B. (2016). Increased production of tyrosinase from Bacillus megaterium strain M36 by the response surface method. *Archives of biological sciences*, 68(3), 659-668.
18. Xu DY, Chen JY, Yang Z (2012). Use of cross linked tyrosinase aggregates as catalyst for synthesis of L-DOPA, *J. Biochemical engineering*; 63: 88-94.