WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.045
Volume 4, Issue 4, 1385-1395. Research Article ISSN 2277– 7105

ISOLATION AND CHARACTERIZATION OF TYROSINASE PRODUCING *STREPTOMYCES LUTEOGRISEUS*.

Gare Sandip Subhash[1] and Kulkarni S.W.[2*]

[1]Department of Microbiology, Vishwasrao Naik Art's, Commerce and Baba Naik Science Mahavidyalaya, Shirala 415 408, Dist-Sangli M.S, India.

*[2]Research Department of Microbiology, Shriman Bhausaheb Zadbuke Mahavidyalaya, Barshi 413 401, Dist-Solapur M.S, India.

Article Received on
29 Jan 2015,

Revised on 24 Feb 2015,
Accepted on 22 March 2015

***Correspondence for Author**
Kulkarni S.W.
Research Department of Microbiology, Shriman Bhausaheb Zadbuke Mahavidyalaya, Barshi 413 401, Dist-Solapur M.S, India.

ABSTRACT

The majority of actinomycetes are free living, saprophytic bacteria found widely distributed in soil, water and colonizing plants. Several species of *Streptomyces* genus produces bioactive molecules like antibiotics, pigments and many extracellular enzymes as glucose isomerase, amylase, cellulases and proteases. The present study was focused on isolation and characterization of *Streptomyces* producing tyrosinase enzyme. A total four soil samples were collected from the Shirala region Dist-Sangli. The total 70 actinomycetes isolates were obtained from soil by performing serial dilution technique and using Glycerol asparagine agar supplemented with Cycloheximide (100µg/ml). The primary screening of 70 isolates for tyrosinase production was carried out on skimmed milk agar. 19 isolates showed zone of hydrolysis and these were taken for secondary screening by using tyrosine agar medium and peptone yeast extract iron agar. The blackish brown diffusible pigment was reported on tyrosine agar and peptone yeast extract iron agar. The qualitative test for tyrosinase activity were carried out by inoculating in tyrosine broth supplemented with traces of chloroform and incubating at 30^0 C for 48 hrs. Red color was reported in tyrosine broth indicated positive tyrosinase activity. Among 19 isolates, 5 were reported as potential tyrosinase producers on the basis of intensity of red color in tyrosine broth. Out of them isolate C7 was identified on the basis of morphological, cultural, biochemical and 16S rRNA gene sequencing as *Streptomyces luteogriseus.*

KEYWORDS: Actinomycetes, *Streptomyces*, Tyrosinase, Screening.

INTRODUCTION

The actinomycetes are Gram positive bacteria having high G+C (>55%) content in their DNA. Actinomycetes were originally considered to be an intermediate group between bacteria and fungi but now are recognized as prokaryotic organisms. The majority of actinomycetes are free living, saprophytic bacteria found widely distributed in soil, water and colonizing plants. Several species of *Streptomyces* genus produces bioactive molecules like antibiotics, pigments and many extracellular enzymes as glucose isomerase, amylase, cellulases and proteases. Their capacity to produce tyrosinase was studied in a lesser extent. In addition, this group of actinomycetes is also able, when are cultivated on organic media, to synthesize and excrete dark pigments, melanin or melanoid, which are considered as useful criteria in taxonomic studies.[1, 2] *Streptomyces* tyrosinases are the most thoroughly characterized enzymes of bacterial origin.[3, 4] The first bacterial tyrosinases have been purified from cell extracts of *Streptomyces nigrifaciens*[5] and *Streptomyces glaucescens*.[6]

Tyrosinase is a copper-containing enzyme that has been found widely distributed in microorganisms, plants and animals.[7, 8, 9] Tyrosinase catalyses the *o*-hydroxylation of monophenols into their corresponding *o*-diphenols (monophenolase or tyrosine hydroxylase (TH) activity), and the oxidation of *o* diphenols to *o*-quinones (diphenolase or dopa oxidase activity), using molecular oxygen, which then polymerise to form brown or black pigments.[10] The synthesis of *o*-diphenols (catechols) is a potentially valuable catalytic ability and thus tyrosinase has attracted a lot of attention with respect to biotechnological applications.

Conventional methods of phenolic wastewater degradation are much coast and too much time consuming. Tyrosinase can be an efficient and feasible alternative for the oxidation of many types of phenolic compounds such as chlorophenols, methylphenols, diphenols, and naphthols.[11] L-DOPA is useful drug in the treatment of Parkinson's myocardial diseases following neurogenic injury.[12] Most of the L-DOPA sold commercially is synthesized from vanillin and hydantoin by a chemical process that involves eight reaction steps. Chemical synthesis of L-DOPA is a time consuming process, which involves several chemicals that are extremely costly and requires catalysts that are not eco-friendly.[12, 13] Conversion of L-tyrosine to L-DOPA is a one-step oxidation reaction catalysed by the enzyme tyrosinase.[14] The worlds market for L-DOPA is about 250 tons per year.[15] Melanin biosynthesis pathway is regulated by an enzyme tyrosinase. Tyrosinase catalyses the first two rate limiting steps.

Melanin might be effective against most of the skin disorders.[16] Synthetic melanins have applications as protectives against radiation (UV, X-ray, and gamma-ray), cation exchanger, and carrier for drugs, antioxidants; antiviral agents and immunogens. In spite of this microbial originated tyrosinase is significant. An enzyme tyrosinase has remarkable properties and many applications in different sectors as mentioned above. Different aspects of an enzyme tyrosinase have been so far studied to some extent throughout the world by scientists. Geographically shirala region is situated at heavy rain fall. No one had reported *Streptomyces* from this region which can produce tyrosinase. This study focuses on *Streptomyces* which have ability to produce an enzyme tyrosinase.

MATERIALS AND METHODS

Materials

1. Soil samples- Four soil samples were collected from the villages around Shirala Dist. -Sangli,
   M.S. India and used in this study for isolation of actinomycetes.
2. Glycerol asparagine broth, Glycerol asparagine agar and Cycloheximide.
3. Skimmed milk agar, Tyrosine agar, Peptone yeast extract iron agar, Tyrosine broth and Chloroform.

Methods

1. Isolation of Actinomycetes

The soil samples were collected from the villages around Shirala Dist.-Sangli, M.S. India and enrichment of soil samples were carried out in Glycerol asparagine broth supplemented with Cycloheximide (100µg/ml). A 10-fold serial dilution of the sample was prepared up to 10^{-6} and 0.1ml aliquots of each dilution was inoculated into Glycerol asparagine agar (L-asparagine- 0.1g, K_2HPO_4-0.1g, glycerol- 1%, trace salt solution- 0.1ml, agar- 2.5g, distilled water-100ml pH-7.4). To avoid the growth of fungal contaminant medium were supplemented with Cycloheximide (100µg/ml). Plates were incubated at room temperature (28°C) and monitored periodically over 5 to 7 days. Pure isolates were transferred on same medium as slants and preserved at 4±2°C for further study.

2. Identification of *Streptomyces*

Morphological characteristics were studied with cover slip culture technique. Cultural characteristics were recorded on Glycerol asparagine agar medium. Biochemical characters were recorded on the basis of sugar utilization potential, enzymatic activities and growth

under inhibitory substances. On the basis of spore mass color, the substrate mycelium color, the shape of the spore chain, morphological and cultural characters of actinomycetes suspected to be *Streptomyces* were sorted. Biochemical characterisations of *Streptomyces* producing tyrosinase were carried out.

3. Primary screening

The primary screening of tyrosinase enzyme producing *Streptomyces* were carried on Skimmed milk plates (pH 6.5–7.2); containing peptone-1%, sodium chloride-0.5%, yeast extract- 0.3%, agar-2% and skimmed milk-10%. All the plates were incubated at 30°C for 2–3 days. After incubation, the plates were observed for the zone of clearness around the colony. The results were interpreted as follows '–' no zone of clearness and '+' shows zone of clearness.[14]

4. Secondary screening methods

Tyrosinase enzyme producing Soil *Streptomyces* were further screened by following different methods like tyrosine agar plate, peptone yeast extract iron agar and tyrosine broth.

Tyrosine agar

The isolates were streaked on tyrosine agar (pH 7) containing Asparagine-0.1%, L-tyrosine-0.5%, K_2HPO_4-0.05%, $MgSO_4.7H_2O$-0.05%, NaCl-0.05%, $FeSO_4.7H_2O$-0.000001%, $CuCl_2.2H_2O$-0.0000027% , $CoCl_2.6H_2O$-0.000004%, Sodium molybdate.$2H_2O$-0.0000025%, Zinc chloride-0.000002%, Boric chloride-0.000285%, Manganese chloride $4H_2O$-0.00018%, Sodium tartarate-0.000177% and agar-2%. All the plates were incubated at 30°C for 48 hrs the occurrence of brown pigmented colonies that gradually changed its color to black (melanin formation) was indication of tyrosinase positive organism.[14]

Peptone yeast extract iron agar

The isolates were streaked on Peptone yeast extract iron agar (pH 6.7) containing Peptic digest of animal tissue-1.5%, Protease peptone-0.5%, Yeast extract-0.01%, Ferric ammonium citrate-0.005%, K_2HPO_4-0.1%, sodium thiosulphate-0.008% and Agar-2%. Plates were incubated at 30^0C for 48hrs to observe brown pigmented colonies that gradually changed its color to black were indication of tyrosinase positive organism.

Tyrosine broth

The isolates were inoculated into 50 mL of 0.1% tyrosine broth with few drops of Chloroform in 100mL Erlenmeyer flask and incubated at 30^0C for 48hrs. The deep red color shows the positive results.[14]

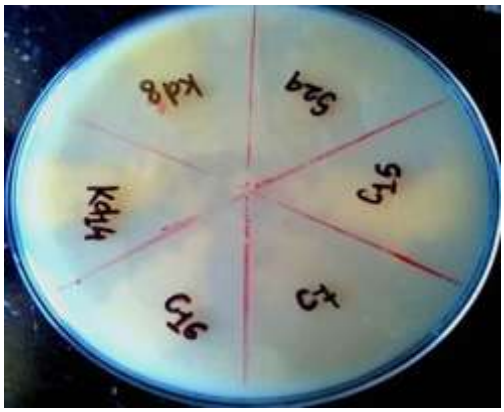
Molecular Identification of *Streptomyces*

One of the *Streptomyces* having potential to produce maximum tyrosinase was identified by using 16s rRNA Sequencing. Genomic DNA was isolated using the Insta Gene ™ Matrix Genomic DNA isolation kit. Using below 16S rRNA Universal primers gene fragment was amplified using MJ Research Peltier Thermal Cycler. Name of the primer used for forward sequencing was 27F with sequence details AGAGTTTGATCMTGGCTCAG having number of Base 20. Name of the primer used for reverse sequencing was 1492R with sequence details TACGGYTACCTTGTTACGACTT having number of bases 22. 16S rRNA gene fragment was amplified using universal primers such as above mentioned. Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labelled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The phylogeny analysis of sequence with the closely related sequence of BLAST results was performed followed by multiple sequence alignment.

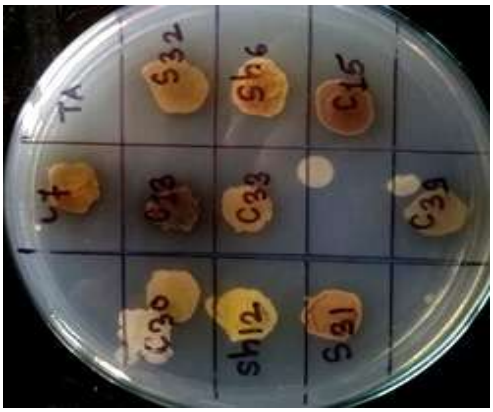
RESULTS AND DISCUSSION

Isolation of tyrosinase producing *Streptomyces*

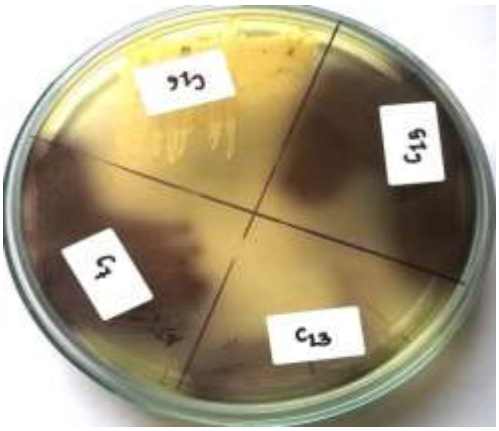
A total 70 actinomycetes were screened from soil samples. Out of 70 isolates 57 were belonging to genus *Streptomyces* on the basis of morphological and cultural characteristics. Among 57 isolates, 19 isolates showed positive proteolytic activity in skimmed milk agar (Fig. 1) were selected for further studies. On tyrosine agar (Fig. 2) and peptone yeast extract iron agar medium (Fig. 3) 19 isolates showed brown colored pigmentation. Pigmentation around the colonies gave positive indication for the tyrosinase production. All isolates were inoculated in tyrosine broth supplemented with few drops of chloroform for confirmation test of tyrosinase. The color of the inoculated tyrosine broth changed from light pink to brown and ultimately to deep red with further incubation (Fig. 4). The color intensity of isolates C7, C15, Kd8, Kd14 and S29 were much higher than rest of isolates. C7 were selected for further study.



"Fig.1 Primary screening of isolate on Skimmed milk agar"



"Fig.2 Secondary screening of isolates on tyrosine agar"

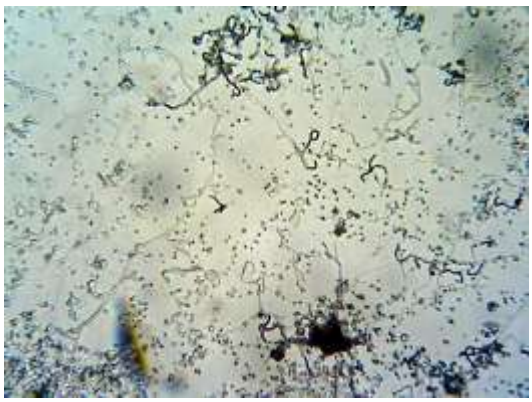


"Fig.3 Secondary screening of isolates on PYIA"

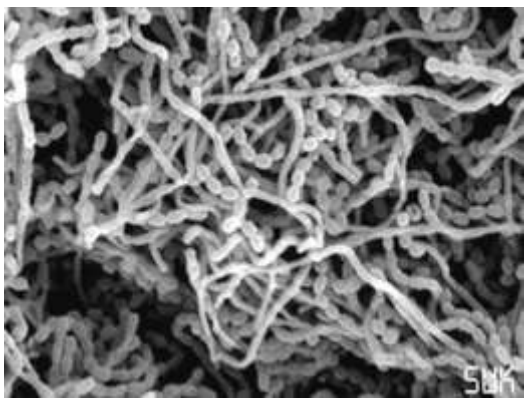
"Fig.4 Deep red color of isolate (C7) in tyrosine broth"

Identification of *Streptomyces*

Isolate C7 was identified as *Streptomyces luteogriseus* on the basis of morphological, cultural, biochemical and 16S rRNA identification. Morphological characters were studied by using cover slip culture technique and scanning electron microscopy (Aerial and substrate mycelia with spore chain arrangement showed in Fig. 5 and 6). Biochemical characters of tyrosinase producing isolate C7 were reported (Table.1). 16S rRNA and Phylogeny analysis of isolate C7 were carried out (Fig.7).

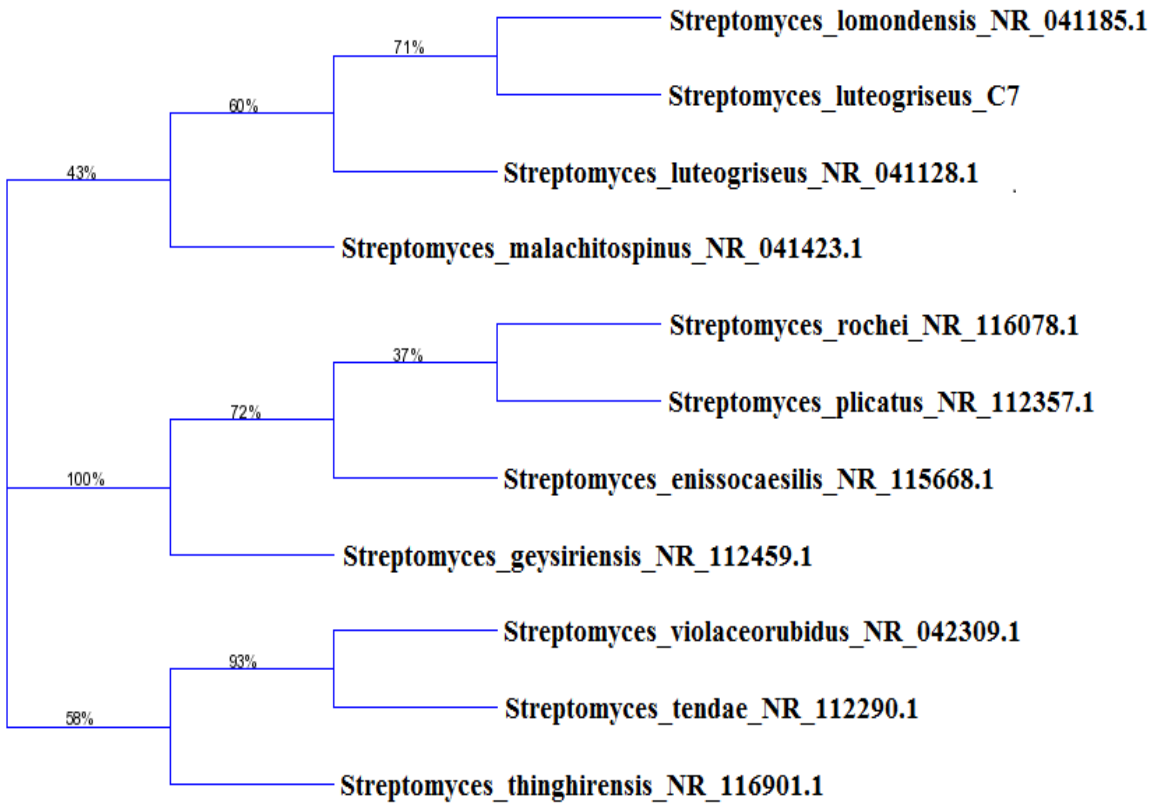


"Fig.5 Spore chain morphology under Light Microscope (C7)"



"Fig.6 Scanning Electron Microscope image of isolate (C7)"

The products from *Streptomyces* have immense importance in different sectors. No one had reported *Streptomyces* from Shirala region which can produce tyrosinase. *Streptomyces* tyrosinases are the most thoroughly characterized enzymes of bacterial origin. [3, 4] The first bacterial tyrosinases have been purified from cell extracts of *Streptomyces nigrifaciens* [5] and *Streptomyces glaucescens.*



"Fig.7 Phylogenetic tree of *Streptomyces luteogriseus* (C7)''

This study focuses on *Streptomyces* which have ability to produce an enzyme tyrosinase. Tyrosinase catalyses the *o*-hydroxylation of monophenols into their corresponding *o*-diphenols (monophenolase or tyrosine hydroxylase (TH) activity), and the oxidation of *o* diphenols to *o*-quinones (diphenolase or dopa oxidase activity), using molecular oxygen, which then polymerise to form brown or black pigments.[10]

Patil and Rathod[13] studied on Inductive effect of L-methionine in transformation of L-tyrosine to L-Dopa and Tyrosinase production by *Streptomyces sp.*VRS9. Among the 52 isolates, 9 have produced melanoid and/or diffused pigments when cultivated on PYIA and tyrosinase agar, respectively, confirming that they are tyrosinase producer.

Raval and Mujumder[14] studied on Biotransformation of a single amino-acid L-tyrosine into a bioactive molecule L-DOPA. From the soil sample 10 isolates were obtained that gave a zone of hydrolysis on (casein) milk agar. Out of these only 8 isolates showed positive result on Tyrosine agar plate. The occurrence of a distinct brown spot which gradually changed its color to black (melanin formation) was indicative of the fact that the above isolates were tyrosinase positive.

Table1. Characterization of isolate C7

Sr.No.	Isolate	Characteristic		Result
1	C7	**Morphological characters**	Spore chain morphology (spirals)	+
			Pigmentation of substrate mycelium (colony reverse) Yellowish brown.	+
			Diffusible pigments(Melanoid pigments)	+
		Pigmentation characters	Pigmentation on PYIA (Blackish brown)	+
			Pigmentation on Tyrosine agar (Blackish brown)	+
			Tyrosine broth (Red color)	+
		Carbon utilization	Glucose	+
			Sucrose	+
			Mannitol	+
			Xylose	+
			Arabinose	+
			Lactose	-
			Trehalose	+
			Fructose	+
		Nitrogen utilization	L-phenylalanine	+
			L-Cysteine	-
			L-Histidine	+
			DL-Valine	+
		Enzyme activity	Catalase	+
			Oxidase	+
			Lecithinase	+
			Lipolysis	+
			Proteolysis	+
			Nitrate reduction	+
			H_2S production	-
		Degradation activity	Gelatin	+
			Starch	+
			L-Tyrosine	+
			Urea	+
		Growth temperatures	4^0C	-
			10^0C	-

			37^0C		+
			50^0C		-
		Growth in presence of inhibitory compounds	Crystal violet (0.0001%)		-
			Phenol (0.1%)		+
			Sodium azide	0.001%	+
				0.002%	-
			Sodium chloride	4%	+
				7%	-
				10%	-
				13%	-

**** Where + = positive - = negative***

Gare and Kulkarni[18] studied tyrosinase producing actinomycetes from soil of Shirala region. A total 60 actinomycetes were isolated from different soil samples. Out of sixty isolates 14 isolates belonging to Kokrud region were screened for tyrosinase activity by using tyrosine agar. Two isolates were showing change in color from colorless to pink to brown and finally turned into black at 30^0c for 24 to 48 hrs.

Roy and Rao[19] studied isolation and characterization of tyrosinase produced by marine actinobacteria and its application in the removal of phenol from aqueous environment. A total of 20 strains were isolated from marine sediment sample and screened for tyrosinase production by using skimmed milk agar medium. Among 20 isolates, two isolates LK-4 and LK-20 showed zone of hydrolysis and these were taken for secondary screening by using tyrosine agar medium.

CONCLUSION

The actinomycete *Streptomyces luteogriseus* isolated from soil sample were found to be the potential producer of tyrosinase.

ACKNOWLEDGEMENT

Researchers are thankful to the Principal of Shriman Bhausaheb Zadbuke Mahavidyalaya Barshi Dist. Solapur for providing laboratory and library facilities to carry out this study.

REFERENCES

1. Zonova G.M. Melanoid pigments of Actinomycetes. *Mikrobiologiya,* 1965; (34): 278-283. www.isca.in/rics/Archives/vol1/15/ISCA RICS 04 2011 90.pdf.
2. Arai T. and Mikami Y. Choromogenecity of *Streptomyces. Appl. Microbiol,* 1972; (23):402-406. www.bioaliment.ugal.ro/revista/8/paper%2081.pdf.

3. Della-Cioppa G., Garger S.J., Sverlow G.G., Turpen T.H., Grill L.K., and Chedekal M.R. (1998b). Melanin production by *Streptomyces*. US Patent 581445. DOI: 10.1016/0141 - 0229 (91)90030-E.
4. Matoba Y., Kumagai T., Yamamoto A., Yoshitsu H., Sugiyama M. Crystallo-graphic evidence that the dinuclear copper center of tyrosinase is flexible during catalysis. *J. Biol. Chem ;* 2006 *;* (281): 89818990.DOI:10.1074/jbc.M509785200.
5. Nambudiri A.M.D. and Bhat J.V. Conversion of p-cumarate into caffeate *Streptomyces nigrifaciens*. *Biochem. J ;* 1972*; (130):425-433*.DOI: 10.1002/jobm.3620310412.
6. Lerch K. and Ettlinger L. Purification and characterization of tyrosinase from *Streptomyces glaucescens. European Journal of Biochemistry,* 1972; (31):427-437. DOI: 10. 1111/j.1432-1033.1972.tb02549.x.
7. Claus H. and Decker H. Bacterial tyrosinases. *Syst Appl Microbiol;* 2006; *29(1):3-14*. DOI.org/10.1016/j.syapm.2005.07.012.
8. Garcia-Borron J.C. and Solano F. Molecular anatomy of tyrosinase and its related Proteins: beyond the histidine- bound metal catalytic center.*Pigm Cell Res,* 2002; *15(3):162- 173.* DOI:10.1034/j.1600-0749.2002.02012.x.
9. Van Gelder C.W.G., Flurkey W.H.and Wichers H.J. Sequence and structural features Of Plant and fungal tyrosinases. *Phytochemistry;* 1997; 45(7):1309-23 www. aseanbiotechnology.info/Abstract/21028575.pdf.
10. Martinez M.V., Whitaker J.R. The biochemistry and control of enzymatic Browning. *Trends Food Sci Techno;* 1995; *(6):195-200.*www.okyanusbilgiambari.com.
11. Atlow S. T., Bonadonna-Aparo L. and Klibanov A. M. (1984).Dephenolization of industrial waste water catalysed by oxidase. *Biotechnology and Bioengineering; (26):599.*https://tspace.library.utoronto.ca/bitstream/1807/.../1/ NQ49835.pdf.
12. Raju B.G. S., Rao G. H., Ayyanna C. (1993).Bioconversion of L-tyrosine to L-DOPA

a. using *Aspergillus oryzae* CBS publishers Vishakhapatnam India: 106-110.

13. Patil S., Rathod V. and Ranganath E. Inductive effect of L-methionine in transformation of L-tyrosine to L-DOPA and Tyrosinase production by *Streptomyces* Sp.VRS9.Indian *Journal of Biotechnology,* 2012*;* (11): 320-325**.** eprints.icrisat.ac.in/view /subjects/002.html.
14. Raval K.M., Vasawani P.S. and Mujumder D.R. Biotransformation of a single amino-acid L-tyrosine into a bioactive molecule L-DOPA. *International Journal of scientific and. research Publication, Vol;* 2012; *(2):2250-3153*. www.ijsrp.org/print- journal/ijsrp.

15. Para G.M. and Baratti J.C. Effect of culture conditions on the production of Tyrosinase phenol-lyase by *Erwinia herbicola, Appl Environ Microbiol;* 1984; (48): 1256- 1258. DOI:10.1007/BF01089004.
16. Koyanagi T., Katayama T., Suzuki H., Nakazawa H. and Yokosuka K *et al*. Effective production of 3, 4-dihydroxyphenyl L-alanine (L-DOPA) with *Erwinia herbicola* Cells carrying a mutant transcriptional regulator Tyr*, J Biotechnol;* 2005*; (115):303- 306.*DOI: org/10.1016/j.jbiotec.2004.08.016.
17. Wang N., and Hebert D.N., Tyrosinase maturation through the mammalian Secretory pathway: Bringing colour to life. *Pigment Cell Res*; 2006; (19): 3-18 DOI: 10.1111 /j.1600-0749.2005.00288. X.
18. Gare S.S. and Kulkarni S.W. Tyrosinase producing Actinomycetes from soil of Shirala region. *J. Microb. World*, 2013; 15(2): 5-8.
19. Roy S., Das I., Munjal M., Karthik L., Kumar G., Kumar S. and Rao K.V.B.(2014). Isolation and characterization of tyrosinase produced by marine actinobacteria and its application in the removal of phenol from aqueous environment. *Front. Biol.* FIB-10324-br.3d. DOI: 10.1007/s11515-014-1324-0.