J. Microbiol. Biotechnol. (2012), **22**(2), 207–214
http://dx.doi.org/10.4014/jmb.1106.06031
First published online January 4, 2012

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Laccase- and Peroxidase-Free Tyrosinase Production by Isolated Microbial Strain

Sambasiva Rao, K. R. S.[1,2]*, N. K. Tripathy[2], Y. Mahalaxmi[3], and R. S. Prakasham[3]

[1]*Department of Biotechnology, Acharya Nagarjuna University, Nagarjunanagar – 522 510, Guntur, A.P., India*
[2]*Department of Zoology, Berhampur University, Berhampur – 760 007, Orissa, India*
[3]*Bioengineering and Environmental Center, Indian Institute of Chemical Technology, Hyderabad – 500 007, India*

Received: June 15, 2011 / Revised: October 29, 2011 / Accepted: October 30, 2011

Laccase- and peroxidase-free tyrosinase has commercial importance in the production of L-3, 4-dihydroxyphenylalanine (L-DOPA), which is mainly used in the treatment of Parkinson's disease. In the present study, isolation of an actinomycetes microbial strain capable of producing only tyrosinase is reported. Among all soil isolates, three individual colonies revealed black color around the colony in the presence of tyrosine. Further screening for laccase and peroxidase activities using syringaldazine denoted that one of the isolates, designated as RSP-T1, is laccase and peroxidase negative and produces only tyrosinase. The microbe was authenticated as *Streptomyces antibioticus* based on 16S ribotyping. Effective growth of this isolate was noticed with the use of medium (pH 5.5) containing casein acid hydrolysate (10.0 g/l), K_2HPO_4 (5.0 g/l), $MgSO_4$ (0.25 g/l), L-tyrosine (1.0 g/l), and agar (15 g/l). The scanning electron micrograph depicted that the microbe is highly branched and filamentous in nature. The enzyme production was positively regulated in the presence of copper sulfate. The impact of different fermentation parameters on tyrosinase production depicted that the maximized enzyme titer values were observed when this isolate was grown at 6.5 pH and at 30°C temperature under agitated conditions (220 rpm). Among all the studied physiological parameters, agitation played a significant role on tyrosinase production. Upon optimization of the parameters, the yield of tyrosinase was improved more than 100% compared with the initial yield.

Keywords: Actinomycetes, enzyme, fermentation, isolation, tyrosinase

Tyrosinase oxidizes tyrosine to melanin *via* L-3,4-dihydroxyphenylalanine (L-DOPA) and dopaquinone. Dopaquinone is a neurotransmitter and its deficiency in human results in the degenenerative disorder Parkinson's disease that affects the central nervous system [27]. However, dopaquinone cannot be administered as such because it cannot cross the blood brain barrier (BBB), whereas L-DOPA, the precursor of dopaquinone, can cross the BBB. Tyrosinase belongs to a class of polyphenol oxidases [19] that is characterized by copper-containing bifunctional biocatalysts that catalyze the hydroxylation of monophenols, and subsequent oxidation by the same enzyme leading to the production of L-DOPA [10, 13, 14, 18, 21, 30]. L-DOPA is a commercially and pharmaceutically important compound, but its production by chemical synthesis is not an economically viable process; therefore, the scientific community is involved in the development of a cost-effective biotechnological process. Hence, the isolation and purification of this enzyme has drawn the attention of the scientific world, as it catalyzes the synthesis of the naturally occurring amino acid L-3,4-dihydroxyphenylalanine (L-DOPA) by the oxidation of tyrosine.

Tyrosinase is essential for all living beings to carry out various functions, including melanin biosynthesis in bacterial and human species as defense against the harmful effects of UV light [23]. In plants, it is required for biosynthesis of phenol polymers such as lignin, flavinoids, and tannins. Tyrosinases have been isolated and purified from various sources such as animals, plants, insects, and microorganisms [1, 5, 6, 8, 15].

The commercial production of tyrosinase is mostly reported from mushroom *Agaricus bisporus.* Extensive research has been carried out on the production and immobilization of tyrosinase from *Agaricus bisporus.* Recently, Dalfard *et al.* [2] isolated and characterized a melanogenic soil bacterium capable of producing tyrosinase as well as laccase enzymes, and reported a tyrosinase activity of 2.38 U/ml. Almost all reported tyrosinase-producing microbial strains also produce other polyphenol oxidases such as peroxidase and laccase concomitantly. The presence of laccase and peroxidase along with tyrosinase is considered to be contamination

*Corresponding author
Phone: +91-863-2346355; Fax: +91-863-2293378;
E-mail: krssrao@yahoo.com, prakasam@iict.res.in

and as a serious problem at the commercial level [12] as all these enzymes use tyrosine as substrate material and produce different products leading to a decrease of yield values as well as increased cost for the downstream process. In view of the above, the present study assumes importance where the authors report the production of only tyrosinase enzyme by an isolated actinomycete microorganism. The added advantage with the selected microbial species is that it produces only tyrosinase and does not exhibit peroxidase or laccase activities. In addition, our data suggest that the speed of agitation plays a significant role in tyrosinase production.

Materials and Methods

Screening for Isolation of Tyrosinase-Producing Microorganism

Soil samples were collected from different places of the Indian Institute of Chemical Technology (IICT), Hyderabad premises. The collected soil samples were screened according to the method of Dalfard *et al*. [2] for isolation of microbes having the potential to produce tyrosinase. To isolate the tyrosinase-producing microbial strain, 1 g of an air-dried soil sample was introduced into a 250 ml Erlenmeyer flask containing 100 ml of sterile distilled water and diluted serially up to the concentration of 10^{-9}. About 0.1 ml of each dilution was distributed on sterilized (autoclaved 15 min at 121°C) screening medium containing casein broth hydrosylate 10 g/l, K_2HPO_4 0.5 g/l, $MgSO_4$ 0.25 g/l, L-tyrosine 1.0 g/l, and agar 1.5 g/l, adjusted to pH 5.5. Plates were incubated at 37°C for 24 to 72 h. The appearance of black or brown color around the margin of colonies indicated possession of tyrosinase activity. Further confirmation studies for tyrosinase enzyme production by these microrganisms were carried out by inoculating a loopful of each organism in liquid media (above medium without agar) and the tyrosinase activity in cell-free broths was determined.

16S rRNA Gene Analysis

Amplification of the 16S rDNA was performed in 30 cycles as described by Weisburg *et al*. [29] using the conditions of 95°C (30 s), 52°C (40 s), and 68°C (1.30 min) plus one additional cycle with a final 7 min chain elongation. The amplified product was submitted to DNA sequencing. Then the sequence obtained was subjected to nucleotide blast analysis to know the sequence similarity with existing sequences and aligned with related sequences using the Clustal W alignment program in MEGA 5 according to Tamura *et al*. [27]. Subsequently, the phylogenetic tree was constructed by the neighbourjoining method using the MEGA 5 program [27]. Bootstrap analysis of the neighbourjoining data, using 1,000 resamplings, was carried out to evaluate the validity and reliability of the tree topology. The 16S rDNA tree was rooted using the sequence of *Streptomyces olivochromogenes* NBRC 13067 (AB184289) as an out group.

Maintenance of Isolated Cultures and Production Medium

The melanogenic strain was maintained in preculture medium containing nutrient broth 8.0 g/l, extract of meat 10.0 g/l, peptone 10.0 g/l, tryptone 10.0 g/l, agar 20.0 g/l, and NaCl 0.5 g/l at 37°C. The microbes were subcultured at regular intervals on slants and stored at 4°C. The production medium consisted of yeast extract 1.5 g/l, tryptone 1.5 g/l, and NaCl 5.0 g/l.

Scanning Electron Microscopy (SEM) Analysis

SEM was used to investigate the morphology of the isolated strain. The sample for SEM was prepared by transferring the microbial strain to a clean eppendorff containing approximately 1.5 ml of 3.5% glutaraldehyde solution. Then, the culture was incubated for 4 h at room temperature followed by a wash with phosphate buffer (100 mM, pH 7.2). The culture was then dehydrated with alcohol gradient from 10% to 100%. The dehydrated samples were air dried and fixed on the stubs using double adhesive tape. A thin layer of gold was coated over the sample using an HUS-5GB Hitachi vacuum evaporator for 90 s. These samples were then observed under a scanning electron microscope (Hitachi S-3000N, Japan) at the various magnifications at acceleration voltage of 10.0 kV.

Polyphenol Oxidase Activities Measurement and Melanin Biosynthesis Evaluation

Polyphenol oxidase activities such as laccase and peroxidase activities were carried out according to Harkin and Obst [7]. For evaluation of peroxidase and laccase production by isolated strains, syringaldazine solution (1.0 g/l) was added to fresh culture, grown on agar surface in the presence and absence of hydrogen peroxide, respectively. The appearance of purple color was considered as positive test. To confirm the tyrosinase-based pigmentation and not an artifact, the effect of manganese was studied using Whatmann filter papers dipped in 5 M $MnCl_2$, as manganese is known to interact with microbial metabolic products and produce black color only in acidic pH environment. The manganese dipped papers were placed at one end of the screening medium plates (without tyrosine) inoculated with melanogenic strains and incubated for one week and observed for pigmentation. To confirm that the appearance of black color is not an artifact due to other microbial metabolites, microbes were grown on media of different compositions, tabulated in Table 1.

Tyrosinase Assay

The amount of enzyme produced was estimated using tyrosine as substrate, which is specific for tyrosinase. The intensity of yellow color produced with maximum absorption in the region of 475 nm wavelength determines the amount of tyrosinase present in the sample. One unit was defined as the amount of enzyme that catalyzes the appearance of 1.0 μmol of product per minute at 37°C.

Spore Suspension and Development of Inoculum

The strain RSP-T1 was grown on nutrient agar slants for more than two days to obtain the spores. To these slants, 5.0 ml of sterile 1.0 g/l (w/v) Tween was added. The spores were then scraped to release from the agar plates and collected in the above solution under sterile

Table 1. Compositions of media used for evaluation of the tyrosinase production potential of isolated microbial strain, *Streptomyces antibioticus* RSP-T1.

Medium	NA	SP	TY	$CuSO_4$
Medium 1	-	+	-	-
Medium 2	-	+	+	-
Medium 3	-	+	+	+

NA, Nutrient agar; SP, sucrose peptone; TY, tryptone.

environment. The spore solution was preserved in a refrigerator for further use. A 25 ml sample of preculture medium was inoculated with 1.0 ml of spore solution and incubated in a shaker for 24 h at 200 rpm. The inoculum thus developed was used for further studies.

Optimization of Conditions for the Growth of the Isolated Melanogenic Strain

To select the optimum pH, temperature, and speed of agitation, tyrosinase enzyme production was investigated at different pH environments (3.5, 4.5, 5.5, 6.5, 7.5, and 8.5), at temperatures 25 to 40°C with an interval of 5°C and speed of agitation from 100 to 220 rpm, respectively, in separate flasks. The samples were collected every 24 h for two days to carry out tyrosinase assay.

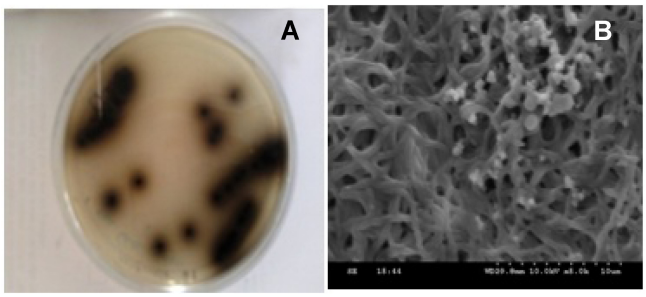

Fig. 1. (**A**) The petri plate showing the isolate with potential to produce extracellular tyrosinase, and (**B**) a scanning electron micrograph of isolate *Streptomyces antibioticus* RSP-T1.

RESULTS

Screening for Isolation of Tyrosinase-Producing Microorganism

Various soil samples collected from different places in the Indian Institute of Chemical Technology, Hyderabad premises were used for isolation of tyrosinase-producing microbial strains using tyrosinase-containing agar medium plates. Among developed colonies, three individual colonies revealed black color around their margin from the back side of the agar plate after two days of incubation at 37°C in an incubator, indicating these three different strains have the potential of producing tyrosinase enzyme. Similar observations have been noticed by Dalford *et al*. [2] while screening for tyrosinase-producing microbial strains. In the present study, further analysis of the color intensity revealed variation with microbial strain to strain, revealing that these three strains may be different in their biochemical and genetic behaviors, especially with respect to tyrosinase enzyme production. Furthermore, careful observation of the colony development of these isolated strains indicated that color formation around the colony changed with respect to incubation time. Initially, the blackish brown color appeared within 24 h of incubation, which with increase in incubation time to 48 h turned to charcoal black color. Based on the diameter of the color zone and intensity of pigmentation (black color), only one strain among the isolated three strains was selected for further evaluation of enzyme production pattern under submerged fermentation conditions. The pure culture of the isolate was obtained from a single colony and subjected to secondary screening with the production medium described in the Materials and Methods. The strain was designated as RSP-T1 (Fig. 1A).

Morphological Characteristics of Strain RSP-T1

Morphologically, individual colonies are circular in shape with a dry surface and rough edges and deep seated into the agar. The scanning electron micrograph of the microorganism depicted that the microbe is filamentous and highly branched (Fig. 1B). The isolated microbial species colony growth analysis indicated it to be pale white in color initially for the first 24 h of incubation, which turned to black owing to development of pigmentation after 48 h of incubation at 37°C. White spores were also produced within 48 h of incubation and remained unchanged with further incubation, even beyond 72 h.

Identification of Strain RSP-T1 by 16S rRNA Gene Analysis

To identify the isolated actinomycetes strain RSP-T1, 16S rRNA gene sequence analysis was performed. Blast analysis revealed that the sequence of the tyrosinase-producing actinomycetes isolate RSP-T1 showed 100% identity to that of two actinomycetes strains, *Streptomyces antibioticus* CFCC 3075 (FJ792547) and *Streptomyces* sp. SS (AY507122), indicating that this isolate belongs to *Streptomyces antibioticus*; hence, it was referred to as *Streptomyces antibioticus* RSP-T1 and the obtained sequence deposited in the EMBL database under Accession No. FR853171. Fig. 2 shows the phylogenetic position of *S. antibioticus* RSP-T1 with reference to related public database sequences, with *Streptomyces olivochromogenes* NBRC 13067 (AB184289) as the out group.

Evaluation of *S. antibioticus* RSP-T1 for Tyrosinase Specificity

Most of the microbial strains producing tyrosinase enzyme are also producers of laccase and peroxidase enzymes concomitantly [9, 20, 22]. Hence, evaluation of any new microbial strain is one of the essential requirements for production of laccase- and peroxidase- free tyrosinase. In general, identification of laccase- and peroxidase-free tyrosinase activity in the presence of tyrosine is difficult, as this amino acid is a substrate for peroxidase and tyrosinase. The fundamental difference between peroxidase and tyrosinase is that peroxidase requires hydrogen peroxide as an additional substrate, whereas tyrosinase does not require any such additional substrate, although both of these enzymes produce black pigmentation upon use of tyrosine as substrate. Therefore, laccase- and peroxidase-specific tests were

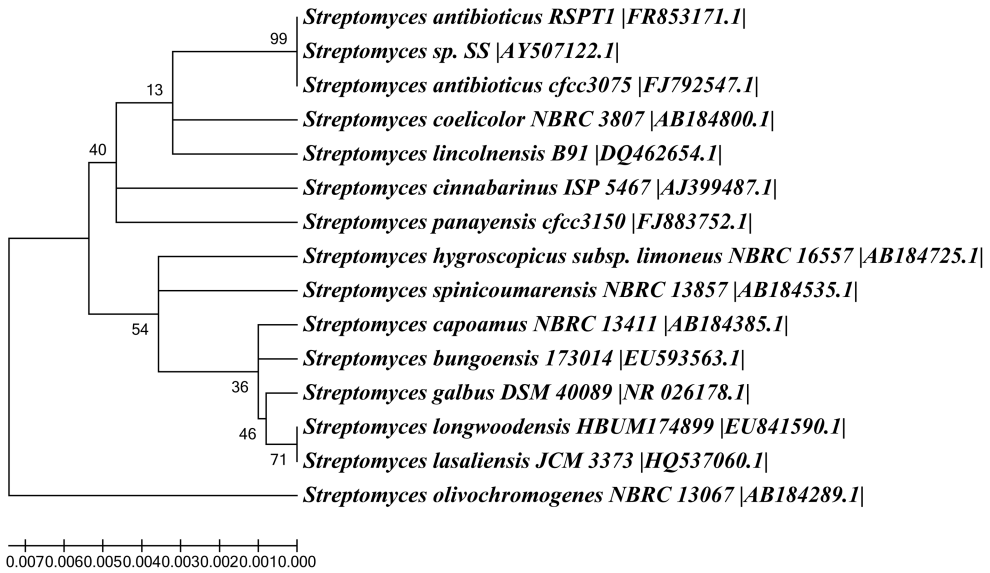

Fig. 2. Phylogenetic analysis of isolate *Streptomyces antibioticus* RSP-T1, based on 16S rRNA sequencing.

considered in the present study. Syringaldazine is a substrate for laccase and peroxidase but not for tyrosinase. Laccase and/or peroxidase catalyze the syringaldazine and produce a purple color product in the presence of oxygen or hydrogen peroxide, respectively. Hence, the absence of purple color production is considered as evidence for laccase and peroxidase activities. In the present study, addition of syringaldazine to the freshly grown culture did not develop purple color either in the presence or absence of hydrogen peroxide. This observation thus suggested that the isolated microbe does not produce either peroxidase and/or laccase during its growth. Moreover, this strain showed the charcoal blackish color during its growth on tyrosine-supplemented agar plates, indicating that this isolated microbial strain produces laccase- and peroxidase-free tyrosinase enzyme.

In order to confirm further that the produced pigmentation by isolated microbial strain, RSP-T1 is only because of tyrosinase enzyme and not an artifact as noticed in certain microbial strains by [2, 9], a manganese-specific color development experiment was conducted. This was performed by growing the isolated microbial strain RSP-T1 on tyrosine-negative and -positive agar plates and by placing the sterile manganese chloride (5 M) dipped Whatmann filter paper in the vicinity of the growing colony on agar plates (as described in Materials and Methods). The pigmentation was noticed only around the colonies, which were grown on tyrosine-containing agar plates after one week of incubation at 37°C. This observation further suggested that the produced pigmentation is exclusively due to the production of tyrosine catalysis product by tyrosinase enzyme. Therefore, the observed pigmentation was attributed to the activity of the tyrosinase enzyme and not manganese oxidation.

During the biocatalysis process, tyrosine is first converted to L-Dopa and then to melanin, which is an indicator for estimation of tyrosinase enzyme production by microbial strains. Hence, melanin formation was investigated by growing the isolated microbe in media of different compositions (Table 1). The results of these investigations are depicted in Fig. 3. Fig. 3A, 3B, and 3C depict the growth of microbe on sucrose peptone plates without external added tyrosine (Fig 3A), with tyrosine (Fig 3B),

Fig. 3. Petri plates showing (**A**) *Streptomyces antibioticus* RSP-T1 growth on sucrose peptone medium, (**B**) tyrosinase production in the presence of tyrosine alone, and (**C**) tyrosinase production in the presence of copper sulfate along with tyrosine.

and with copper sulfate along with tyrosine (Fig 3C), respectively. The sucrose peptone plate supported the microbial growth along with a little brown pigmentation (Fig. 3A), which may be attributed to the tyrosine present in the medium, as the medium consists of peptone, a protein digest. However, with external tyrosine-supplemented environment (Fig. 3B), characteristic charcoal black pigmentation (melanin formation) was observed, the intensity of which increased in the presence of copper sulfate (Fig. 3C). This increased black color intensity with copper sulfate may be attributed to the fact that copper is a cofactor for tyrosinase, and supplementation of copper sulfate might have enhanced the enzyme production in this microbial strain.

Optimization of Fermentation Process Parameters

In view of the isolated microbial strain potential in production of laccase- and peroxidase- free tyrosinase, optimization studies were carried out to maximize the tyrosinase enzyme by the microbe using conventional, one variable at a time, methodology. Initially, the pattern of tyrosinase enzyme production was studied against fermentation time. The data revealed that enzyme production is associated with growth of organism, and tyrosinase titer values increased up to 48 h of incubation time (data not shown). Further increase in fermentation time resulted in a decrease in enzyme titers. Preliminary investigations were made to understand various requirements such as incubation temperature (25 to 40°C), medium pH (3.5 to 8.5), and speed of agitation (100 to 200 rpm) using this isolated tyrosinase-producing strain, RSP-T1, by analyzing enzyme values at 48 h of incubation.

The role of incubation temperature on tyrosinase production could be evidenced from Fig. 4, where the enzyme production varied with incubation temperature. Optimized tyrosinase enzyme production (3.29 U/ml) was observed at 30°C and any variation resulted in a decrease of enzyme yield. A change in 5°C variation from 30 to 25°C resulted in >20% decrease in enzyme productivity, whereas the shift of incubation temperature from 30 to 35°C altered the enzyme production from 3.29 to 2.99 U/ml, indicating a decrease of 10% (Fig. 4). However, further increase of temperature from 35 to 40°C caused a further 10% reduction in enzyme production. These data suggested that higher temperature has more detrimental influence on tyrosinase enzyme production by this isolated microbial strain compared with lower incubation temperature.

Hence, to select the suitable pH for optimum production of tyrosinase by selected strain RSP-T1, studies were carried out in different pH environments in the range of 3.5 to 8.5 with an interval of 1.0 unit by incubating at 30°C. It was evident from the depicted data in Fig. 4 that the selected range of pH from 4.5 to 8.5 supports tyrosinase production, indicating the isolated microbe potential in

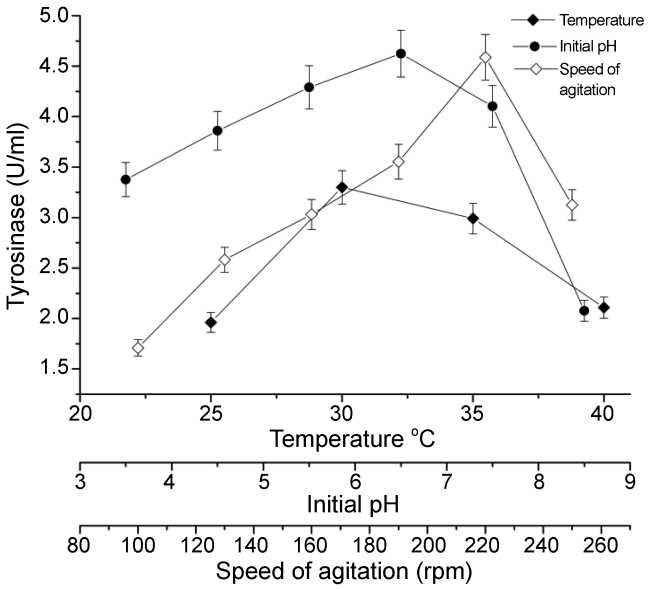

Fig. 4. Effects of pH, temperature, and speed of agitation on production of tyrosinase by isolate *Streptomyces antibioticus* RSP-T1.

growth and subsequent metabolism in a wide range of pH environment. However, the optimized tyrosinase production was noticed at 6.5 pH, and minimum production of 2.07 U/ml was obtained at pH 8.5. The data also suggested that the tyrosinase production values increased with increase in pH from 4.5 to 6.5 and then decreased on increasing the pH to 7.5 and 8.5. This could be evidenced from the fact that the maximum production of 4.62 U/ml was observed at pH 6.5. The production values also suggested that acidic and neutral pH's are relatively favorable for tyrosinase production compared with alkaline conditions. This is confirmed based on the fact that variation of 2.0 pH units towards the acidic side (6.5 to 4.5) caused 18% reduction, whereas increase of pH from 6.5 to 8.5 resulted in 65% decrease with respect to tyrosinase production at 6.5 pH environment. Thus, whereas the acidic as well as neutral pH conditions support tyrosinase production, alkaline environments are highly unsuitable for tyrosinase production by RSP-T1. To select the optimum speed of agitation that supports maximum titers of tyrosinase, fermentation experiments were conducted at different speeds of agitation in the range 100 to 220 rpm with an interval of 30 rpm investigated. These results presented in Fig. 4 suggested that the production of tyrosinase increased with increase in the rpm from 100 to 220. The maximum and minimum titers of tyrosinase at 220 and 100 rpm were noticed to be 4.815 and 1.745 U/ml, respectively. Thus, 2-fold increase in agitation resulted in 4-fold improvement in tyrosinase production values. These studies suggested that high agitation and aeration play significant roles in the production of tyrosinase by the isolated strain RSP-T1.

DISCUSSION

Melanin biosynthesis (charcoal blackish pigmentation) is a two-step biocatalytic process mediated by tyrosinase enzyme using tyrosine as the substrate material. Biochemically, tyrosinase enzyme possesses both monophenolase activity (hydroxylation of monophenols to *o*-diphenols) and diphenolase activity (oxidation of *o*-diphenols to *o*-quinones). In the first reaction, the tyrosine is converted to L-Dopa (3,4-dihydroxyphenyl alanine), which upon subsequent catalysis step results in production of melanin. Since L-Dopa is pivotal in the treatment of Parkinson's disease, the present isolate with high tyrosinase-producing character compared with literature-reported strains assumes importance at industrial and pharmaceutical sectors [28].

Cell wall composition analysis of the isolated microbial strain RSP-T1 depicted the presence of two actinomycetes-specific amino acids (*viz*., diaminopimilic acid and glycine), indicating that this isolated tyrosinase-producing microorganism belongs to the *Streptomyces* group (data not shown). This is further supported by the morphological characteristic, that is, the presence of substrate and aerial mycelium followed by white spores formation upon increase in incubation time on starch-agar plates (Fig. 3A). These data are in accordance with literature-reported tyrosinase-producing actinomycetes strains such as *Streptomyces griseus* [4] and *Streptomyces* sp. [1].

The 16S rRNA sequence of the present isolate RSP-T1 revealed 100% similarity with the EMBL sequence of *Streptomyces antibioticus* CFCC 3075 (FJ792547), suggesting both these strains may be similar in genetic and morphological features. However, the present isolate is a mesophile in nature and isolated from soil samples collected at the Indian Institute of Chemical Technology, Hyderabad campus, whereas the *Streptomyces antibioticus* CFCC 3075 (FJ792547) is reported from China. The authors did not reveal whether this strain produces any tyrosinase activity. However, another strain, *Streptomyces antibioticus* IMRU 3720 (culture from Institute of Microbiology, Rutgers University, USA), was reported to have the ability to produce tyrosinase enzyme [11]. The authors, with this microbial strain too, did not report whether it produces only tyrosinase or laccase/peroxidase-associated tyrosinase. These 16S rRNA studies further confirmed that the isolated strain belongs to *Streptomyces antibioticus*, hence this isolate is designated as *Streptomyces antibioticus* RSP-T1.

The novelty of this isolated *S. antibioticus* RSP-T1 is its production of tyrosinase-specific enzyme without associated laccase and peroxidase activities, unlike most of the reported tyrosinase enzyme-producing microbial strains (known to produce laccases and peroxidase concomitantly) [9, 20], which has been elucidated based on biochemical and genomic studies [22]. The isolated strain potential to produce only tyrosinase and not other polyphenolic enzymes (laccases and peroxidases) was confirmed based on the syringaldazine test. Syringaldazine, being chemically polyaromatic in nature, can be oxidized to a purple color compound by laccase and peroxidases in the presence of either atmospheric oxygen (specific to laccases) or hydrogen peroxide (specific to peroxidase), but not by tyrosinase. The addition of syringaldazine to fresh isolated *S. antibioticus* RSP-T1 culture did not develop purple color in either the presence or absence of hydrogen peroxide (data not shown). This observation thus suggested that the isolated microbe does not produce either peroxidase and/or laccase. Moreover, this strain showed the charcoal blackish color during its growth on tyrosine-supplemented agar plates, indicating the produced enzyme is tyrosinase (Fig. 3). These observations are categorically expletive that the isolated microbial strain has potential to produce tyrosinase enzyme exclusively but not other polyphenol oxidases, unlike other reported microbial strains like *Bacillus* sp., *Pseudomonas putida* F6 [20], mushroom tyrosinase [3], *Bacillus subtilis* [24], *Pseudomonas putida* [25], and *Streptomyces griseus* [4].

Hullo *et al*. [9] and Dalfard *et al*. [2] reported that charcoal blackish pigmentation during the growth of microbial strain may also be associated with the variations in hydrogen ion concentration in the presence of manganese. Under alkaline environment, manganese is converted to manganese dioxide in the presence of oxygen and under acidic conditions. The black color appearance by the growth of isolated *S. antibioticus* RSP-T1 is independent of interaction of any microbial metabolites with the traces of manganese present in the fermentation medium. This could be evidenced from the fact that (i) the isolated strain produced black color pigmentation only in presence of tyrosine, (ii) the microbe growth could be seen effectively at pH 6.5, and (iii) supplementation of external manganese to the growing cultures did not show black color.

The yield improvement of any enzyme production, in general, by any microbial system depends on the physiological, nutritional, and biochemical natures of the microbe employed, and these factors vary from organism to organism [17, 26]. This is one of the essential requirements for evaluating the isolate's commercial importance, which can be judged by characterization and optimization of each factor of growth, the nutritional requirement [16]. The notable environmental and fermentation factors that influence metabolism-mediated production yields include pH, temperature, aeration, carbon and nitrogen sources, incubation time, initial inoculum size, *etc*. and whose interactive behaviors influence the economic production of the desired metabolite [17]. Similar trends have been observed by other researchers in microbial metabolites production. An optimum temperature of 30°C has been observed for effective tyrosinase enzyme production by isolated *S. antibioticus* RSP-T1 (Fig. 4), whereas 37°C was the optimum for *Bacillus* sp. [24], indicating the tyrosinase production pattern is influenced

by the metabolic as well as genetic nature of the microbial strain employed. The optimum pH is a necessary requirement for effective growth as well as improved metabolite production by any microorganism, as the hydrogen ion concentration influences the transport of nutrients and products in addition to regulation of the metabolic process by altering the ionic configuration of reactants as well as enzymes. A similar trend of tyrosinase production by bacterial strain *Bacillus* sp. was reported by Dalford *et al.* [2]; however, the reported optimum production was at pH 6.0, whereas the isolated strain's optimum growth and enzyme production were observed at pH 6.5. Tyrosinase-based catalysis is an aerobic process and any variation in oxygen concentration in the fermentation environment highly influences the metabolism of the tyrosinase-producing microbial strain. However, it is not clear that oxygen requirement is also essential for tyrosinase enzyme production. Dalford *et al*. [2] also reported the requirement of a high agitated environment for optimized production of tyrosinase, suggesting oxygen is one of the essential parameter. Although enzyme production is improved (Fig. 4) with agitation (known to improve the oxygen transfer), this improvement may also be attributed to maintaining the equilibrated mass transfer of nutrients and products during fermentation and to improve the dissolved oxygen concentration in the medium, which is evidenced with the present strain too (Fig. 4).

Overall, the study indicated isolation of a laccase- and peroxidase-free tyrosinase-producing microbial strain from soil samples. The isolated microbial strain growth along with tyrosinase yields are influenced by physiological pH, incubation temperature, and oxygenic environment. This strain has been identified as *Streptomyces antibioticus* based on 16S rRNA analysis. Qualitative investigation of enzyme production in the presence of copper sulfate revealed improved tyrosinase yield. Among the studied physiological parameters, variation of aeration levels altered 4-fold enzyme production in this isolated microbial strain.

Acknowledgment

One of the authors, Y. Mahalaxmi, is greatly indebted to the Council of Scientific and Industrial Research, New Delhi for financial support in the form of a senior research fellowship.

REFERENCES

1. Claus, H. and H. Decker. 2006. Bacterial tyrosinases. *Syst. Appl. Microbiol.* **29:** 3–14.
2. Dalfard, A. B., K. Khajeh, M. R. Soudi, H. N. Manesh, B. Ranjbar, and R. H. Sajedi. 2006. Isolation and biochemical characterization of laccase and tyrosinase activities in a novel melanogenic soil bacterium. *Enzyme Microbial Technol.* **39:** 1409–1416.
3. Dawley, R. M. and W. H. Flurkey. 1993. Differentiation of tyrosinase and laccase using 4-hexyl-resorcinol. *Phytochemistry.* **33:** 281–284.
4. Endo, K., Y. Hayashi, T. Hibi, K. Hosono, T. Beppu, and K. Ueda. 2003. Enzymological characterization of EpoA, a laccase-like phenol oxidase produced by *Streptomyces griseus*. *J. Biochem.* **33:** 671–677.
5. Espin, J. C., M. F. Trujano, J. Tudela, and F. Garcia-Canovas. 1997. Study of stereospecificity in pear and strawberry polyphenol oxidases. *J. Agric. Food Chem.* **45:** 1091–1096.
6. Halaouli, S., M. Asther, K. Krus, L. Guo, M. Hamdi, and J. C. Sigoillot. 2005. Characterization of a new tyrosinase from *Pycnoporus* species with high potential for food technological applications. *J. Appl. Microbiol*. **98:** 332–343.
7. Harkin, M. J. and R. J. Obst. 1973. Syringaldazine, an effective reagent for detecting laccase and peroxidase in fungi. *Experientia*. **29:** 381–387.
8. Hearing, V. J. 1987. Mammalian monophenol monooxigenase (tyrosinase): Purification, properties, and reactions catalyzed. *Methods Enzymol*. **142:** 154–163.
9. Hullo, M. F., I. Moszer, A. Danchin, and M. I. Verstraete. 2001. CotA of *Bacillus subtilis* is a copper-dependent laccase. *J. Bacteriol*. **183:** 5426–5430.
10. Inagaki, H., A. Koga, Y. Bessho, and H. Hori. 1998. The tyrosinase gene from medakafish: Transgenic expression rescues albino mutation. *Pigment Cell Res*. **1:** 1283–1290.
11. Katz, E., C. J. Thompsons, and D. A. Hopwood. 1983. Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans. J. Gen. Microbiol.* **129:** 2703–2714.
12. Kumar, M. and W. H. Flurkey. 1991. Activity, isoenzymes and purity of mushroom tyrosinase in commercial preparations. *Phytochemistry* **30:** 3899–3902.
13. Kupper, U., D. M. Nidermann, G. Travaglini, and K. Lerch. Isolation and characterization of the tyrosinase gene from *Neurospora crassa*. *J. Biol. Chem*. **264:** 17250–17258.
14. Kwon, B. S., A. K. Haq, S. H. Pomerantz, and R. Halaban. 1087. Isolation and sequence of cDNA for human tyrosinase that maps at the mouse-c-albino locus. *Proc. Natl. Acad. Sci. USA* **84:** 7473–7477.
15. Lerch, K. and L. Ettlinger. 1972. Purification and characterization of a tyrosinase from *Streptomyces glaucescens*. *Eur. J. Biochem.* **31:** 427–437.
16. Mahalaxmi, Y., C. S. Rao, G. Suvarnalaxmi, T. Sathish, P. Sudhakar, and R. S. Prakasham. 2008. Rifamycin B production pattern in *Nocardia* RSP-3 strain and influence of barbital on antibiotic production. *Curr. Trends Biotechnol. Pharm*. **2:** 173–181.
17. Mahalaxmi, Y., T. Sathish, C. S. Rao, and R. S. Prakasham. 2010. Corn husk as a novel substrate for the production of rifamycin B by isolated *Amycolatopsis* sp. RSP 3 under SSF. *Process Biochem.* **45:** 47–53.
18. Mason, H. S. 1965. Oxidases. *Annu. Rev. Biochem*. **34:** 595–634.
19. Mayer, A. M. 2006. Polyphenol oxidases in plants and fungi: Going places? A review. *Phytochemistry* **67:** 2318–2331.
20. McMahona, A. M., E. M. Doyle, S. Brooks, and K. E. O. Connor. 2007. Biochemical characterisation of the coexisting

tyrosinase and laccase in the soil bacterium *Pseudomonas putida* F6. *Enzyme. Microbial Technol*. **40:** 1435–1441.
21. Porta, G. and R. H. Thomson. 1976. Melanin pigmentation in mammals. *Endeavour* **35:** 32–38.
22. Romero, H. D., F. Solano, and S. A. Amat. Polyphenol oxidase activity expression in *Ralstonia solanacearum*. *Appl. Environ. Microbiol*. **71:** 6808–6815.
23. Ruan, L., Y. Huang, G. Zhang, D. Yu, and S. Ping. 2002. Expression of the *mel* gene from *Pseudomonas maltophilia* in *Bacillus thuringiensis*. *Lett. Appl. Microbiol*. **34:** 244–248.
24. Sanchez, A. A. and F. Solano. 1997. A pluripotent polyphenol oxidase from the melanogenic marine *Alteromonas* sp. shares catalytic capabilities of tyrosinases and laccases. *Biochem. Biophys. Res. Commun*. **240:** 787–792.
25. Solano, F., P. Lucas-Elio, E. Fernandez, and A. Sanchez-Amat. 2000. *Marinomonas mediterranea* MMB-1 transposon mutagenesis: Isolation of a multipotent polyphenol oxidase mutant. *J. Bacteriol.* **182:** 3754–3760.
26. Suvarna Laxmi, G., T. Sathish, C. S. Rao, P. Brahmaiah, M. Hymavathi, and R. S. Prakasham. 2008. Palm fiber as novel substrate for enhanced xylanase production by isolated *Aspergillus* sp. RSP-6. *Curr. Trends Biotechnol. Pharm.* **2:** 447–455.
27. Tamura, K., J. Dudley, M. Nei, and Kumar, S. 2007. Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molec. Biol. Evol*. 24**:** 1596–1599.
28. Tuncagil, S., S. K. Kayahan, G. Bayramoglu, M. Y. Arica, and L. Toppare L. 2009. L-Dopa synthesis using tyrosinase immobilized on magnetic beads. *J. Mol. Cat. B Enz.* **58:** 187–193.
29. Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **173:** 697–703.
30. Wichers, H. J., K. Recourt, M. Hendriks, C. E. M. Ebbelaar, G. Biancone, F. A. Hoeberichts, *et al*. 2003. Cloning, expression and characterisation of two tyrosinase cDNAs from *Agaricus bisporus*. *Appl. Microbiol. Biotechnol.* **61:** 336–341.