Front. Biol. 2014, 9(4): 306–316
DOI 10.1007/s11515-014-1324-0

RESEARCH ARTICLE

Isolation and characterization of tyrosinase produced by marine actinobacteria and its application in the removal of phenol from aqueous environment

Suki ROY[1], Ishita DAS[1], Minki MUNJAL[1], Loganathan KARTHIK[1,2], Gaurav KUMAR[1,2], Sathish KUMAR[1], Kokati Venkata Bhaskara RAO (✉)[1]

[1] *Molecular and Microbiology Research Laboratory, School of Bio Sciences and Technology, VIT University, Vellore-632014, Tamil Nadu, India*
[2] *Department of Biotechnology, Shri JJT University, Jhunjhunu, Rajasthan, India*

Abstract **The present study was focused on screening and characterization of tyrosinase enzyme produced by marine actinobacteria and its application in phenolic compounds removal from aqueous solution. 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. Based on the result of secondary screening LK-4 was selected for further analysis, such as tyrosinase assay, protein content and specific activity of the enzyme. The tyrosinase enzyme was produced in a SS medium and was partially purified by ammonium sulfate precipitation, dialysis and SDS PAGE. The isolate (LK-4) was identified as *Streptomyces espinosus* using 16S rRNA gene sequencing and named as "*Streptomyces espinosus* strain LK4 (KF806735)". The tyrosinase enzyme was immobilized in sodium alginate which was applied to remove phenolic compounds from water. The enzyme efficiently removed the phenolic compounds from aqueous solution within few hours which indicated that tyrosinase enzyme produced by *Streptomyces espinosus* strain LK-4 can be potently used for the removal of phenol and phenolic compounds from wastewater in industries.**

Keywords actinobacteria, phenolic compounds, immobilization, tyrosinase

Introduction

Phenol and phenolic compounds are the common pollutants abundant in industrial wastewater of several industries viz., metal, steel, coal conversion, petroleum refining, resin, plastic, agrochemicals, pharmaceuticals, dye industries etc. (Grady, 1990; Ha et al., 2000). Phenolic compounds are toxic in nature and these compounds are able to cause environmental hazards as well as health hazards which include cardiac arrhythmias, renal diseases, skin cancer and even death (Rice and Cohen, 1996; Adeyemi et al., 2009). Physical, chemical and biological methods have been applied to eliminate such toxic contaminants from industrial wastewater. Pulse high-voltage discharge system, quick sorption on the activated sludge (Zhao et al., 2008) are some of the good physical methods to remove organic contaminants (Shi et al., 2009). Whereas chemical methods includes Photo-Fenton reaction for the removal of natural or synthetic aromatic compounds (Gernjak et al., 2003) and solvent-impregnated resin system for the removal of phenols and thiophenols from Cuypers et al. (2010). Phytoremediation and microbiological removal process are also in use to remove phenol from wastewater by biological degradation. There are processes where plant and some plant associating bacteria (symbiotic and asymbiotic) directly involve in the biodegradation of phenols from contaminated wastewater or industrial leachate (Saiyood et al., 2010). However these methods are having several disadvantages, as the phenol or some other contaminants are not particularly bio-refectory and suffer from various limitations including high cost, incomplete removal, formation of toxic byproducts, low efficiency, high energy

Received March 13, 2014; accepted June 16, 2014
Correspondence: Kokati Venkata Bhaskara RAO
E-mail: kokatibhaskar@yahoo.co.in

requirements and are effective only in a low concentration range (Chung et al., 2003). Enzymatic treatment is better than those physical and chemical ways to remove phenols from industrial wastewater. Treatment of aqueous phenols using oxidoreductive enzymes is an efficient and cost effective method and peroxidases can be better utilized for the removal of phenolic contaminants (Klibanov et al., 1980). Peroxidase is a copper containing enzyme and it is present in several microorganism, plants and animals. Peroxidase mainly exists in two groups namely, laccases and tyrosinases which catalyze the oxidative transformation of a large number of phenolic and non-phenolic aromatic compounds viz., phenol, o- and p-cresol, catechin, gallic acid, phenylenediamines (o-, m-, p-phenylenediamines) and benzenediols (catechol, resorcinol, and hydro quinone) to their corresponding quinones (Escribano et al., 1997; Dura'n et al., 2002; Mayer and Staples, 2002; Saboury et al., 2006). Tyrosinase (monophenol monooxygenase and o-diphenol oxidoreductase or catechol oxidase) is widely distributed throughout the phylogenetic scale from bacteria to mammals and even present in different parts of the same organism and it contributes to various metabolic activities like biosynthesis of melanin in bacteria and animal, promotes plant immunity etc. (Peralta-Zamora et al., 2003). It is reported that tyrosinase involve in two different enzymatic reaction; the orthohydroxylation of monophenols to o-diphenols (monophenolase activity) and the oxidation of o-diphenols to o-quinones (diphenolase activity). The active quinones polymerizes spontaneously to the macromolecular melanin (Seo et al., 2003). Tyrosinases are belong to a larger group of proteins named type-3 copper proteins, which include the catechol oxidases that exhibit only catecholase activity and the oxygen-carrying hemocyanins from mollusks and arthropods (Decker and Tuczek, 2000; Halaouli et al., 2006). Although much of the work on tyrosinase and their applications has been done with mushroom tyrosinases, however there are several limitations in the cultivation of mushrooms (low quality spawn, competing wood rotting fungi, molds and variables weather pattern). Hence, bacteria can be considered as a good source of tyrosinase enzyme and bacterial tyrosinases are more advantageous than mushroom tyrosinases.

Actinomycetes are Gram positive bacteria having high G + C ($>55\%$) content in their DNA. 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 compounds like antibiotics, pigments and many extracellular enzymes as glucose isomerase, amylase, cellulases and proteases. *Streptomyces* tyrosinases are the most thoroughly characterized enzymes of bacterial origin (Della-Cioppa et al., 1998a, 1998b; Matoba et al., 2006; Popa and Bahrim, 2011). Current study was focused on the isolation and characterization of tyrosinases from the marine actinobacteria and their application in the removal of phenolic compound from aqueous solutions.

Materials and methods

Medium and chemicals

All the media and chemicals used in the current study were purchased from HiMedia Chemicals, Mumbai, India and SRL Pvt. Ltd., Mumbai, India.

Isolation of marine actinobacteria

The marine water sample was collected from Marina beach (13.05°N, 80.28°E), Chennai, Tamil Nadu, India. A 10-fold serial dilution of the sample was prepared up to 10^{-6}. 100 µL aliquots of each dilution was inoculated into starch casein agar medium (pH 7.2) prepared with 50% seawater to sustain the growth of actinobacteria. To avoid the growth of fungal and bacterial contaminant, potassium dichromate (50 µg/mL) and nalidixic acid (15 µg/mL) were supplemented to the medium. The plates were incubated at room temperature (28°C) and monitored periodically over 3 months for actinomycetes growth. The pure isolates of the actinobacteria were transferred to ISP2 (Isolation streptomyces project medium No.2) slants and preserved at 4±2°C (Kathiresan et al., 2005; Karthik et al., 2010).

Primary screening method

Primary screening of tyrosinase enzymes producing marine actinobacteria was carried on skim milk agar plates. All the isolates were streaked into skim milk agar plates (pH 6.5–7.2); containing peptone 1%, sodium chloride 0.5%, yeast extract 0.3%, agar 2% and skim 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 interrupted as follows '–' no zone of clearness and '+' shows zone of clearness (Raval et al., 2012).

Secondary screening method

Tyrosinase enzyme producing marine actinobacteria were further screened by following different methods like tyrosine agar plate and tyrosine broth.

Tyrosine agar

The isolates were streaked on tyrosine agar (pH 7) containing peptone 0.5%, beef extract 0.3%, agar 2% and L-tyrosine 0.5% and all the plates were incubated at 30°C for 2–3 days. The occurrence of brown pigmented colonies that gradually changed its color to black (melanin formation) was indication of tyrosinase positive organism (Raval et al., 2012).

Tyrosine broth

The isolates were inoculated into 50 mL of 0.1% tyrosine broth with few drops of chloroform in 100 mL Erlenmeyer flasks and incubated at 30°C for 2–3 days. The deep red color shows the positive results (Raval et al., 2012).

Production media for tyrosinase

One loop full culture of the isolate LK-4 was inoculated in 100 mL of SS media (soluble starch 25 g/L, glucose 10 g/L, yeast extract 2 g/L, calcium carbonate 3 g/L, trace salt solution ($FeSO_4$, $CuSO_4$, $ZnSO_4$ and $MnCl_2$) 1mL enriched with 0.1% tyrosine and 10 mg/mL of copper sulfate and incubated at room temperature for seven days. After seven days of incubation the deep brown color showed the production of the tyrosinase enzyme.

Characterization of tyrosinase produced by isolates

The tyrosinase produced by the isolates was characterized by using tyrosinase assay and the protein content was estimated by following Bradford's method.

Tyrosinase assay

To 0.1 mL culture supernatant of positive isolates, 1.0 mL of 0.5 M phosphate buffer (pH 6.5), 1.0 mL of 0.001 M L-tyrosine and 0.9 mL of reagent grade water were added into a test tube. The reaction mixture was oxygenated by bubbling through a capillary tube for 4–5 min to reach temperature equilibration and absorbance was recorded at 280 nm by using UV-Vis spectrophotometer (Raval et al., 2012).

The calculation of enzyme activity was done by using the following formula:

$$\text{Units of enzyme/mL} = \frac{\Delta A280\ \text{nm/min Test} - \Delta A280\text{nm/min Blank(df)}}{(0.001)(0.1)}$$

Bradford's method for protein content

The protein content was measured by Bradford's method, using Bovine Serum Albumin (BSA) as a standard protein. 1 mL of sample was mixed with 5 mL of Bradford reagent (Coomassie brilliant blue G-250) and incubated for 5 min. After that, absorbance was measured at 595 nm. Protein content was expressed as milligrams of protein per milliliter of sample (Raval et al., 2012). Following tyrosinase assay and Bradford's method, specific activity of tyrosinase was calculated using the following formula:

$$\text{Specific activity} = \frac{\text{Units of enzyme mL/min}}{\text{mg of protein/mL}}$$

Purification of tyrosinase enzyme

The enriched culture was re-inoculated in tyrosine broth and after incubation the culture was centrifuged and the supernatant was used for further experiments. Tyrosinase was further purified from the supernatant by the following methods like ammonium sulfate precipitation, dialysis and SDS-PAGE.

Ammonium sulfate precipitation

Ammonium sulfate powder was added to the supernatant slowly with continuous stirring in an ice bath, until 60% saturation is achieved. The mixture was kept at 4°C overnight followed by centrifugation at 6000–10000 r/min in a cooling centrifuge. The supernatant was discarded and the precipitate was dissolved in 0.2 M phosphate buffer (pH7.0) with continuous mixing using magnetic stirrer. The suspended precipitate was then checked for enzymatic activity as well as total protein content (Dolashki and Gushterova, 2009).

Dialysis

The dialysis membrane was cut and pre-treated in boiling water for 60 min and stored in 0.2 M phosphate buffer (pH 7.0). The membrane was packed up with the dissolved precipitate and sealed by clamps on both ends. The dialysis bag was suspended overnight at 4°C in a glass beaker containing 0.2 m phosphate buffer (pH 7.0) with continuous mixing using a magnetic stirrer. The dialysis sample was checked for tyrosinase activity as well as total protein content (Bradford, 1976).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was done with 12% polyacrylamide gel using Tris-glycine buffer (pH 8.3). The crude extract of enzyme from LK-4 was loaded on to a denaturing polyacrylamide gel and compared with protein marker. Coomassie blue staining was performed in order to visualize the protein bands (Haghbeen et al., 2004).

High performance liquid chromatography (HPLC)

The culture free supernatant was analyzed by high performance liquid chromatography using C18 column (250 mm × 4.6 mm × 5 mm i.d.). The HPLC gradient program was: starting at 8% solvent B and holding for 1 min, then ramping to 75% solvent B in 20 min, holding at 75% solvent B for 10 min, backing to 8% solvent B in 1 min and holding at 8% solvent B for 5 min. Solvent A was 98:2 (v/v) water: acetonitrile with 0.1% acetic acid, and solvent B is 10:90 (v/v) water: acetonitrile with 0.1% acetic acid. The HPLC analysis was performed at a flow rate of 1.0 mL/min, with

detection wavelength at 244 nm. The injection volume used was 10 μL (Wang et al., 2007).

Stability studies

To evaluate the temperature effect, ten milliliters of the culture free supernatant were exposed at 20°C, 40°C, 60°C and 80°C for seven days and cooled to room temperature. To examine the pH effect, the pH of the cell free broth was adjusted to pH values of 2.0, 4.0, 6.0, 7.0 and 8.0. The effect of tyrosine concentration was detected by adding tyrosine into the tyrosine broth to achieve the concentration of (0.02%, 0.04%, 0.06%, 0.08% and 1.0% w/v). Tyrosinase enzyme activity was measured by tyrosinase assay as well as Bardford's method.

Molecular characterization and identification of potential isolate

The potent isolate that demonstrated expressive enzyme activity was molecularly characterized with 16S rRNA gene sequencing. The actinobacterial DNA was isolated by bacterial DNA Mini spin kit (2 μL) (Amion, India) and PCR amplification was done by using an initial denaturation step at 94°C for 5 min followed by 35 cycle of 1 min at 94°C, 30 s at 55°C and 1.5 min at 72°C and final extension at 72°C for 5 min cooled at 4°C, the *Taq* polymerase were used as 10× AMTaq Pol. Buffer: 5 μL AMTaq, polymerase enzyme: 3 U and PCR grade 8 water to make the volume up to 50 μL (Amnion, India) as per user manual (Saiki et al., 1985). The forward and reverse primers used are 5′-CWG RCC TAN CAC ATG SAA GTC-3′(100 ng) and 5′-GRC GGW GTG TAC NAG GC-3′(100 ng) respectively. The resemblance or homogeneity of the 16S rRNA partial gene sequence was analyzed with the analogous sequences already present in the records bank— National Center for Biotechnology Information (NCBI) via BLAST search. The applied DNA sequences were allied and phylogenetic tree was created with the function of neighbor joining technique, the used on line software is known as ClustalW (Saitou and Nei, 1987).The topologist of resulted tree were calculated through bootstrap analyses of the neighbor-joining technique based on 1000 re-samplings. The phylogenetic analysis was conceded by using the TREEVIEW software (version 1.3.3).

Immobilization of tyrosinase in sodium alginate beads

The partially purified enzyme solution was mixed with sodium alginate solution in 1:1 ratio. The mixture was added dropwise into calcium chloride (0.2 M) solution with shaking at 4°C. The beads were allowed to wash with distilled water followed by phosphate buffer of pH 7 (Anwar et al., 2009).

Immobilization efficiency

Initial activity of free enzyme = 1672.94 U/(mg·min)

Volume of enzyme solution = 10 mL

Weight of beads formed after immobilization of enzyme solution = 8.44 g

Enzyme entrapped in 0.5 g beads = 1.18 mL

Based on initial activity of the free enzyme, the activity of the enzyme entrapped must be = 1974.07 U/(mg·min)

Activity of immobilized enzyme obtained in 0.5g beads = 947.55 U/(mg·min)

Therefore, total enzyme activities after entrapment = 48% (Anwar et al., 2009)

Phenol removal from aqueous solution by using immobilized tyrosinase enzyme

The immobilized beads of 20 ml of enzymes were prepared and exposed to various concentration of phenol in water. The phenol concentration varied from 1mM to 5mM. The concentration of phenol remained was estimated by Folin-Ciocalteu reagent after each half an hour (Maurya and Singh, 2010). The phenol concentration was determined for each set of concentration till 4 h.

Statistical analysis

All experiments were carried out in triplicates. Data are presented as mean ± standard deviation (SD). To prove significant differences, results were evaluated statistically, at 95% confidence level ($p < 0.05$), using the Analysis of Variance (ANOVA) available in Graphpad Prism v.6.00 (La, Jolla, CA, USA).

Results

A total of 20 marine actinobacteria were isolated from marine water sample. Among these 20 isolates, 2 isolates LK-4 and LK-20 showed positive proteolytic activity in skimmed milk agar were selected for further studies. In the tyrosine agar plate, both the isolates LK-4 and LK-20 showed brown colored pigmentation. The occurrence of a brown pigmentation around the colonies gave a positive indication for the tyrosinase production. The color of the inoculated tyrosine broth changed from light pink to brown and ultimately to deep red with further incubation. The color intensity of tyrosine broth produced by LK-4 was much higher than that of LK-20 as shown in Fig. 1. In the characterization assay, the tyrosinase enzyme activity was found to be 6 U/(mL·min) for the isolate LK-4, whereas the enzyme activity of LK-20 showed 2 U/(mL·min) as shown in Table 1. The overall specific activity of LK-4 was found to be four times higher than that of LK-20. Based on the characterization result, LK-4 was selected for production of tyrosinase enzyme in SS media. After incubation period the crude extract was further purified by ammonium sulfate precipitation and dialysis. The detailed results of the purification of tyrosinase from LK-4 were summarized in Table 2. The specific activity of the crude

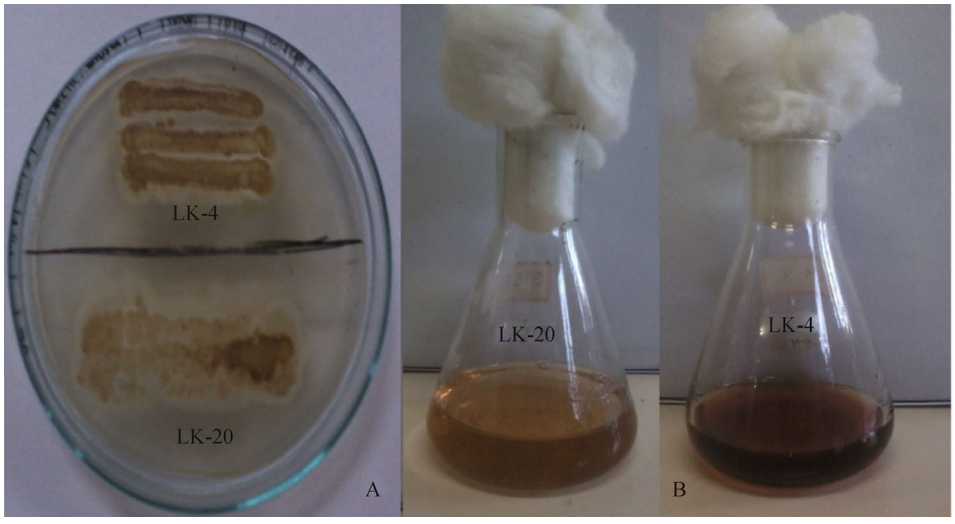

Figure 1 Secondary screening of tyrosinase production by LK-4 and LK-20. (A) Tyrosine agar plate, (B) tyrosine broth.

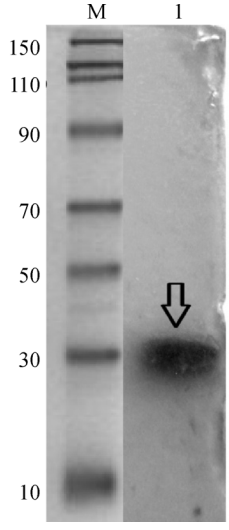

Figure 2 SDS-PAGE of tyrosinase produced by LK-4.

enzyme was found to be 986.52 which ultimately increased to 2556.89 after purification of the enzyme. The total yield of the tyrosinase enzyme came to around 50.69% with a purification fold of 2.592. The partially purified enzyme was allowed to run on a polyacrylamide gel under denaturing conditions with a mid-range protein marker. The SDS-PAGE analysis of the partially purified enzyme revealed the presence of single protein band that corresponds to approximately 30 kDa as shown in Fig. 2. The HPLC chromatogram of the partial purified enzyme with phosphate buffer as mobile phase showed a single peak at a retention time of 7.298 min which corresponds to the tyrosinase enzymes as shown in Fig. 3. The optimum conditions for the production of tyrosinase enzyme were examined at different pH, temperature and concentration of the substrate. With regards to the production of the tyrosinase enzyme at different pH, the optimum pH was found to be at 8 which showed highest specific activity of the

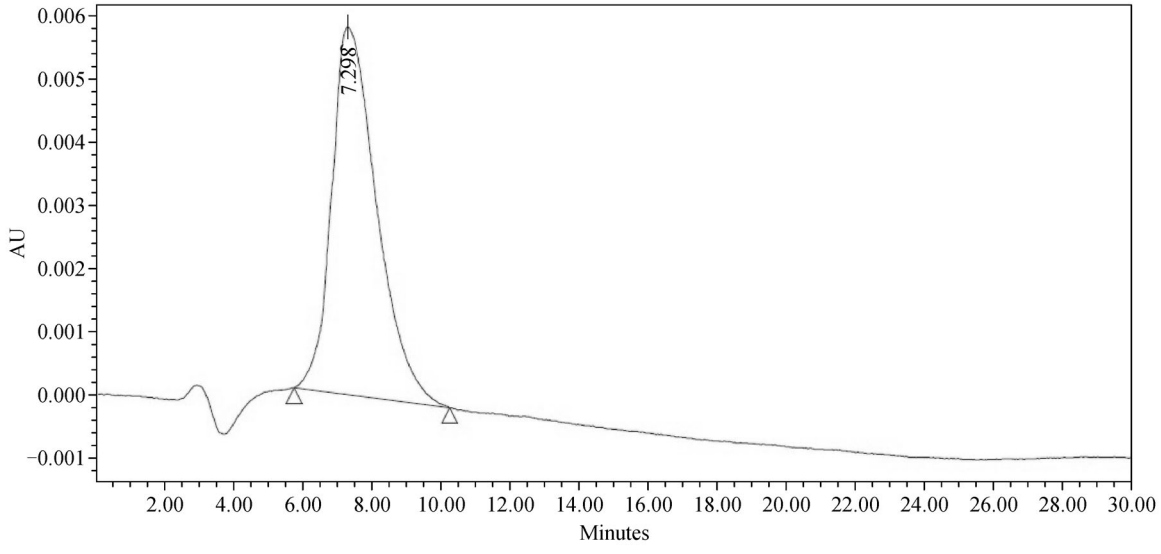

Figure 3 HPLC profile of partially purified tyrosinase enzyme from LK-4.

tyrosinase enzyme as shown in Fig. 4. A temperature of 40°C showed maximum specific activity of the tyrosinase enzyme which was followed by a temperature of 60°C as shown in Fig. 5. The optimum concentration of tyrosine for the production of tyrosinase was found to be 0.02 g/mL which showed highest tyrosinase activity in the media as shown in Fig. 6. The potent isolate was sequenced and the taxonomic position of *Streptomyces espinosus* strain LK4 (KF806735) was determined based on 16S rRNA partial gene sequencing. A phylogenetic tree was constructed on the basis of the neighbor- joining method using Treeview software as shown in Fig. 7. The tyrosinase enzyme was immobilized in Sodium alginate and the entrapment efficiency was calculated. The entrapment efficiency of the sodium alginate beads came around 48%. The immobilized beads of tyrosinase enzyme efficiently removed the phenolic compounds from the water. The phenol removal by the tyrosinase was checked at various concentrations for 4 h. The result of phenol removal by tyrosinase enzyme was depicted in Fig. 8. There were significant reductions in the levels of phenols from water with increased in time of exposure to the enzyme. About 60% phenol removal was achieved in case of 1mM of phenol which was followed by 30%, 22%, 15% and 26% respectively for 2 mM, 3 mM, 4 mM and 5 mM of phenol. The tyrosinase enzyme was able to remove phenol over a wide range of initial phenol concentrations without any precipitate formation.

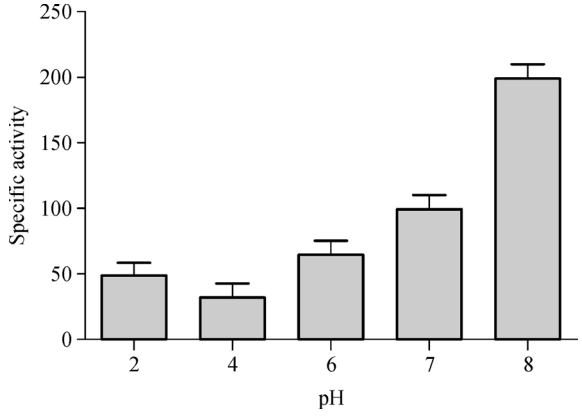

Figure 4 Optimum pH for tyrosinase production by LK-4. Data is given as mean±SD ($n = 3$).

Discussion

Marine actinobacteria comprise an unlimited pool of novel chemistry which is widely used in the field of bioremediation, pharmaceutical and cosmetic products. With this regard, we paid more attention for isolation of marine actinobacteria. In this present study, 20 isolates were isolated from marine water sample which is designated as LK-1 to LK-20. Likewise, Shubhrasekhar et al. (2013) also isolated 20 actinobacterial strains from marine sample.

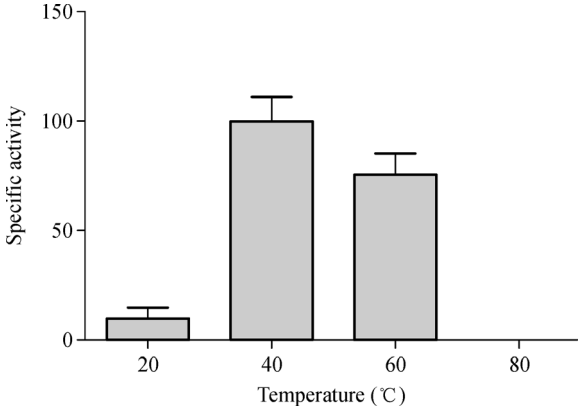

Figure 5 Optimum temperature for tyrosinase production by LK-4. Data is given as mean ± SD ($n = 3$).

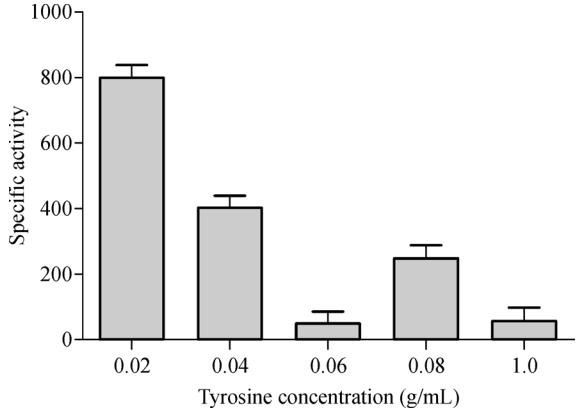

Figure 6 Optimum concentration of tyrosine for tyrosinase production by LK-4. Data is given as mean ± SD ($n = 3$).

Screening test of tyrosinase production

In the present study, the isolates were primarily screened for tyrosinase production by using skimmed milk agar. Among the isolates, only two isolates namely, LK-4 and LK-20 showed the clear zone on skimmed milk agar. The formation of the zone on the agar plates reflected the production of proteolytic enzyme by the isolates. The hydrolysis zone produced by the microorganism on the skimmed milk agar plate directly was correlated to the concentration of protease produced by it (Vermelho et al., 1996). Dajanta et al. (2009) used this method to screen the protease producing bacteria. Jones et al. (2007) described that the skimmed-milk agar method to find proteases was not significantly strong to provide a perfect screen. Some other non protease activity might also give the same clear zone on the skimmed milk agar plate and these would lead to a false positive result. Hence, tyrosinase production was further checked on tyrosine agar and tyrosine broth. The isolates produced brown coloration in both tyrosine agar plate and tyrosine broth which reveals the ability of the isolates to produce tyrosinase. This result showed similarity with the observation by Dalfard et al.

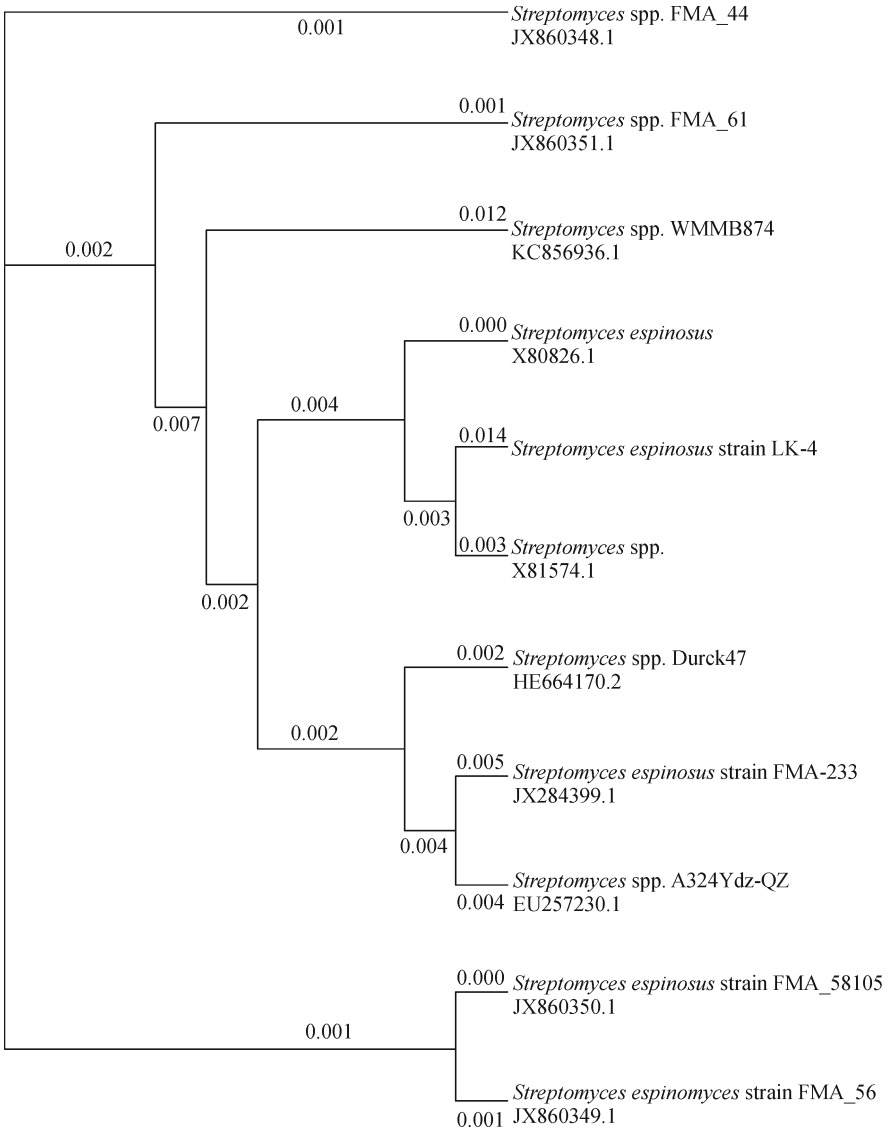

Figure 7 Phylogenetic tree of *Streptomyces espinosus* strain LK-4 (KF806735).

(2006). The formation of brown color was mainly due to the melanin production by oxidative polymerization of phenolic compound by tyrosinase.

Characterization of the tyrosinase enzyme produced by isolates

The activity of the tyrosinase enzyme can be analyzed by the disappearance of tyrosine through tyrosinase assay. The tyrosinase activity of LK-4 and LK-20 was estimated by using tyrosine as substrate as similar to the work of Dolashki and Gushterova (2009). One of the widely used substrate of tyrosinase enzyme is tyrosine. Tyrosinase is a bi-functional enzyme that catalyzes both the orthohydroxylation of monophenols (tyrosine) followed oxidation of diphenols (L-DOPA) to orthoquinones (dopachrome). Dopachrome can then be transformed to the brown/black melanin pigments (Fairhead and Thöny-Meyer, 2012). The protein content was estimated by one of the preferred method for quantifying protein. According to Kruger (1994), the Bradford's method is a simple, fast and more sensitive method than Lowry method and subject to less intervention by nonprotein components and general reagents; hence, the method is used to estimate the protein in the medium. The total protein content of LK-20 came higher than LK-4. A higher specific activity represents a higher activity of tyrosinase enzyme on the substrate.

Production and purification of tyrosinase enzyme

The enzyme purification was started with 600 mL of culture filtrate which was purified to obtain 26 mL of purified enzyme corresponding to a yield of 31.88%. The yield of tyrosinase enzyme was found to be higher than enzyme production by

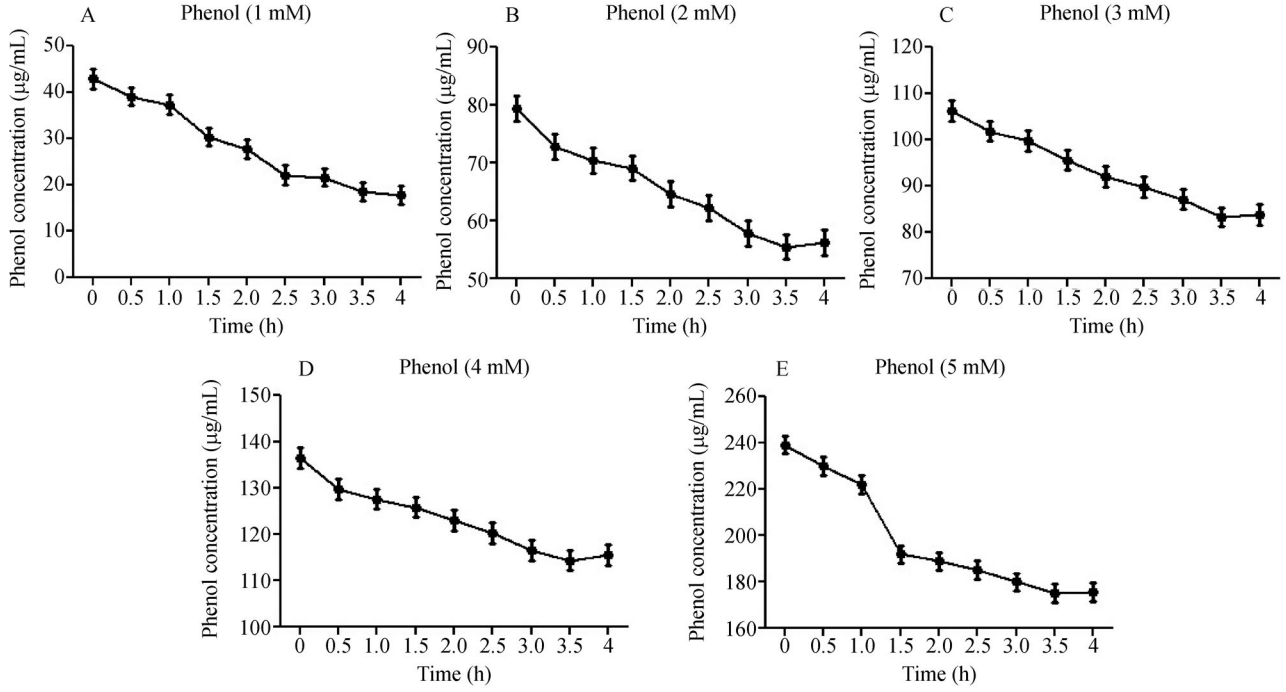

Figure 8 Removal of different concentrations of phenol from aqueous solution by immobilized tyrosinase enzyme produced by *Streptomyces espinosus* strain LK-4.(A) Phenol 1mM. (B) Phenol 2 mM. (C) Phenol 3 mM. (D) Phenol 4 mM. (E) Phenol 5 mM.

Table 1 Secondary screening of tyrosinase produced by two isolate LK-4 and LK-20

Isolate name	Total activity (U/(mL·min))	Protein content (mg/mL)	Specific activity (U/mg)
LK-4	6±1.5	1.3±0.05	120±2.3
LK-20	2±1.4	1.1±0.08	25±1.9

Table 2 Characterization of tyrosinase produced by isolate LK-4

Purification stage	Total volume (mL)	Protein content (mg/mL)	Total activity (U/(mL·min))	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	600	0.51	505	986.52	1	100
Precipitation	156	0.23	389	1672.94	1.696	77.03
Dialysis	90	0.10	256	2556.89	2.592	50.69

Streptomyces albus as reported by Dolashki and Gushterova (2009). In comparison to the yield of first bacterial tyrosinase from *Streptomyces nigrifaciens* (Nambudiri and Bhat, 1972) of 17% and extracellular tyrosinase from *Streptomyces michiganensis* with a yield of 1.7% yield (Philipp et al., 1991), the yield of tyrosinase enzyme from LK-4 showed a mush promising result. There was increase in the specific activity of the enzyme with gradual purification steps. The total protein content decreased to 7.8% of the crude extracted protein whereas the specific enzyme activity rose to more than three times of the initial activity. In comparison to the report of Haghbeen et al. (2004), the specific activity of crude tyrosinase enzyme from LK-4 came higher than mushroom tyrosinase. The molecular mass was determined by SDS-PAGE, where the protein mass was found to be 30 kDa. This result was in reasonable agreement with the reported molecular mass of other tyrosinases from *Streptomyces* species. The HPLC analysis of the partially purified tyrosinase enzyme with phosphate buffer as mobile phase revealed a single intense peak which confirmed the purity of the enzyme. The result obtained in this study is in agreement with the work of Dolashki and Gushterova (2009).

Stability analysis of the tyrosinase enzyme

The production of tyrosinase by microorganism is regulated by factors such as temperature, pH of the medium, the presence of enzyme inducers, genetics of the microorganism, the composition of the medium (Katz and Betancourt, 1988). Maintaining enzyme activity at different physical parameters

is one of the important features for potent biotechnological application of tyrosinase. Therefore, the optimum conditions for the enzyme production were investigated at different pH, temperature and tyrosine concentration. The enzyme activity as a function of the pH was found to be optimum at 8.0 which was in agreement with the result of optimum pH of enzyme activity of *Streptomyces albus* (Dolashki and Gushterova, 2009). In contrast to the result of Xu et al. (2011), the tyrosinase enzyme was found to be stable even at a high pH. The current demand of industrial enzyme is preferable to show high activity in various temperatures. In the present study the impact of temperature on the tyrosinase production was determined by establishing the growth at temperature range from 20°C to 80°C. The maximum tyrosinase activity was found to be at 40°C. The enzyme activity showed a decrease in its activity with the increase in temperature. At high temperature, the necessity of energy for the cell was high which was contributed to thermal denaturation of other enzymes of the metabolic pathway and therefore the production of tyrosinase becomes less. The tyrosinase enzyme from LK-4 was found to be more stable than enzyme from *Penicillium jensenii* which showed maximum stability at a temperature of 30°C (Raval et al., 2012). Higher concentration of substrate inhibits the production of enzyme tyrosinase which may be due to formation of high amount of intermediary products. The optimum tyrosine concentration for the tyrosinase production was found out to be at 0.02 g/mL which was less than the optimum tyrosine concentration required by *Penicillium jensenii* for the enzyme production (Raval et al., 2012).

Molecular characterization and Identification of potential isolate

Streptomyces espinosus strain LK4 was found to be closely related to *Streptomyces espinosus* with 99% similarity. This was the first report on the production of tyrosinase by marine *Streptomyces espinosus*.

Removal of phenol from aqueous solution

The immobilized enzyme provides improved resistance to alteration in conditions such as temperature or pH. In such a condition, enzymes remain held in position throughout the reaction which leads to easy separation from product, reusability and continuous operation. It is an efficient technique that is being used in industry for enzyme catalyzed reactions. In regards to enzymatic water treatment, immobilized tyrosinase showed better efficiency in terms of reusability, stability and longer viability (Bevilaqua et al., 2002; Molina et al., 2003; Kameda et al., 2006). The immobilization of tyrosinase improved the thermal stability and the gel-entrapped tyrosinase was almost entirely preserved from proteolysis maintaining more than 80% of its activity (Crecchio et al., 1995). The use of cheaper supports for the preparation of immobilized enzyme for such applications is always considered necessary. Hence, sodium alginate was used in order to immobilize enzyme to remove phenol from aqueous solution. Tyrosinase has ability to oxidize a wide range of polyphenolic and phenolics components to their related nontoxic quinones and hence this enzyme has been utilized to remove hazardous and toxic phenolic contaminants from effluent and wastewater (Robb, 1995). In the present study, the partially purified enzyme was immobilized in sodium alginate in order to remove the phenol from the water. The immobilization efficiency came around 48% which showed a better than the work of Anwar et al. (2009), where the efficiency of the immobilized protease enzyme was found to be 45%. The immobilised enzyme was exposed to various concentration of phenol ranging from 1mM to 5mM for a time period of 4 h which resulted in the reduction in the concentration of phenol with increase in exposure time. The reduction of phenolic components was function of exposure time. There was a gradual decrease in the percentage of phenol removal from 1 mM of phenol to 5 mM of phenol. The phenol removal was around 60% in case of 1 mM of phenol whereas the removal percentage greatly reduced to 15% at 4 mM of phenol. There was a slight increase in phenol removal percentage to 26% in case of 5 mM of phenol. The result of phenol removal showed slight similarity with the work of Shesterenko et al. (2012).

Conclusions

The marine actinobacteria *Streptomyces espinosus* strain LK-4 isolated from marine water sample was found to be the proficient producer of tyrosinase as well as potent remover of phenol from aqueous solution. The enzyme was found to be stable even at high temperature, pH and it is also having high enzymatic activity and greater stability than mushroom tyrosinase enzyme. Immobilized tyrosinase enzyme from *Streptomyces espinosus* strain LK-4 can act as a promising technique for phenol removal from wastewater along with maintaining high stability of the enzyme. Hence, it can be concluded that the tyrosinase enzyme from *Streptomyces espinosus* strain LK-4 can be potently use in industries in order to remove phenol from wastewater.

Acknowledgements

We are very thankful to the management of VIT University for providing laboratory and facilities to carry out our project work. We are also thankful to School of Advanced Science (SAS) and Chemistry Division for providing HPLC facility.

Compliance with ethics guidelines

The authors declare no competing financial interests. Suki Roy, Ishita Das, Minki Munjal, Loganathan Karthik, Gaurav Kumar,

Sathish Kumar, Kokati Venkata Bhaskara Rao declare that they have no conflict of interest.

References

Adeyemi O, Oginni O, Osubor C C, Adeyemi O, Oloyede O B, Oladiji A T, Adebayo E A (2009). Effect of water contaminated with phthalate, benzene and cyclohexane on *Clarias gariepinus*' cellular system. Food Chem Toxicol, 47(8): 1941–1944

Anwar A, Qader S A, Raiz A, Iqbal S, Azhar A (2009). Calcium alginate: a support material for immobilization of proteases from newly isolated strain of *Bacillus subtilis* KIBGE-HAS. World Appl Sci J, 7: 1281–1286

Bevilaqua J V, Freire M C, Anna S (2002). Phenol removal through combined biological and enzymatic treatments. Braz J Chem Eng, 19 (2): 151–158

Bradford M M (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem, 72(1–2): 248–254

Chung T P, Tseng H Y, Juang R S (2003). Mass transfer effect and intermediate detection for phenol degradation in immobilized *Pseudomonas putida* systems. Process Biochem, 38(10): 1497–1507

Crecchio C, Ruggiero P, Pizzigallo M D R (1995). Polyphenoloxidases immobilized in organic gels: Properties and applications in the detoxification of aromatic compounds. Biotechnol Bioeng, 48(6): 585–591

Cuypers R, Sudhölter E J, Zuilhof H (2010). Hydrogen bonding in phosphine oxide/phosphate-phenol complexes. ChemPhysChem, 11 (10): 2230–2240

Dajanta K, Wongkham S, Thirach P, Baophoeng P, Apichartsrangkoon A, Santithum P, Chukeatirote E (2009). Comparative study of proteolytic activity of protease-producing bacteria isolated from *Thua nao*. Maejo Int J Sci Technol, 3: 269–276

Dalfard B, Khajeh K, Soudi M R, Manesh H N, Ranjbar B, Sajedi R H (2006). Isolation and biochemical characterization of laccase and tyrosinase activities in a novel melanogenic soil bacterium. Enzyme Microb Technol, 39(7): 1409–1416

Decker H, Tuczek F (2000). Tyrosinase/catecholoxidase activity of hemocyanins: structural basis and molecular mechanism. Trends Biochem Sci, 25(8): 392–397

Della-Cioppa G, Garger S J, Holtz R B, McCulloch M J, Sverlow G G (1998a). Method for making stable extracellular tyrosinase and synthesis of polyphenolic polymers therefrom. US Patent 5801047

Della-Cioppa G, Garger S J, Sverlow G G, Turpen T H, Grill L K, Chedekal M R (1998b). Melanin production by *Streptomyces*. US Patent 5814495

Dolashki A, Gushterova A (2009). Identification and characterization of tyrosinase from *Streptomyces albus* by mass spectrometry. Biotechnol & Biotechnol, 23: 946–950

Dura'n N, Rosa M A, D'Annibale A, Gianfreda L (2002). Applications of laccases and tyrosinases (phenoloxidases) immobilized on different supports: a review. Enzyme Microb Technol, 31(7): 907–931

Escribano J, Cabanes J, Chazarra S, Garcı'a-Carmona F (1997). Characterization of monophenolase activity of table beet polyphenol oxidase. Determination of kinetic parameters on the tyramine/dopamine pair. J Agric Food Chem, 45(11): 4209–4214

Fairhead M, Thöny-Meyer L (2012). Bacterial tyrosinases: old enzymes with new relevance to biotechnology. New Biotechnol, 29(2): 183–191

Gernjak W, Krutzler T, Glaser A, Malato S, Caceres J, Bauer R, Fernández-Alba A R (2003). Photo-Fenton treatment of water containing natural phenolic pollutants. Chemosphere, 50(1): 71–78

Grady C P L Jr (1990). Biodegradation of toxic organics: status and potential. J Environ Eng, 116(5): 805–828

Ha S R, Vinitnantharat S, Ozaki H (2000). Bioregeneration by mixed microorganisms of granular activated carbon loaded with a mixture of phenols. Biotechnol Lett, 22(13): 1093–1096

Haghbeen K, Jazii F R, Karkhane A A, Borojerdi S S (2004). Purification of tyrosinase from edible mushroom. Iranian J Biotechnol, 2: 189–194

Halaouli S, Asther M, Sigoillot J C, Hamdi M, Lomascolo A (2006). Fungal tyrosinases: new prospects in molecular characteristics, bioengineering and biotechnological applications. J Appl Microbiol, 100(2): 219–232

Jones B V, Sun F, Marchesi J R (2007). Using skimmed milk agar to functionally screen a gut metagenomic library for proteases may lead to false positives. Lett Appl Microbiol, 45(4): 418–420

Kameda E, Langone M A, Coelho M A (2006). Tyrosinase extract from Agaricus bisporus mushroom and its in natura tissue for specific phenol removal. Environ Technol, 27(11): 1209–1215

Karthik L, Kumar G, Bhaskara Rao K V (2010). Diversity of marine actinomycetes from Nicobar marine sediments and its antifungal activity. Int J Pharm Pharm Sci, 2: 199–203

Kathiresan K, Balagurunathan R, Manilamani M, Selvan (2005). Fungicidal effect of marine actinomycetes against phytopathogenic fungi. Indian J Biotechnol, 4: 271–276

Katz E, Betancourt A (1988). Induction of tyrosinase by L-methionine in Streptomyces antibioticus. Can J Microbiol, 34(12): 1297–1303

Klibanov A M, Alberti B N, Morris E D, Felshin L M (1980). Enzymatic removal of toxic phenols and anilines from waste waters. J Appl Biochem, 2: 414–421

Kruger N J (1994). The Bradford method for protein quantitation. Methods Mol Biol, 32: 9–15

Matoba Y, Kumagai T, Yamamoto A, Yoshitsu H, Sugiyama M (2006). Crystallographic evidence that the dinuclear copper center of tyrosinase is flexible during catalysis. J Biol Chem, 281(13): 8981–8990

Maurya S, Singh D (2010). Quantitative Analysis of Total Phenolic Content in Adhatoda vasica Nees Extracts. Int J Pharm Tech Res, 2: 2403–2406

Mayer A M, Staples R C (2002). Laccase: new functions for an old enzyme. Phytochemistry, 60(6): 551–565

Molina L P, Hiner A N P, Tudela J, Garcı'a-Ca'novas F, Rodrı'guez-Lo'pez J N (2003). Enzymatic removal of phenolsfrom aqueous solution by artichoke (*Cynara scolymus L.*)extracts. Enzyme Microb Technol, 33(5): 738–742

Nambudiri A M D, Bhat J V (1972). Conversion of *p*-cumarate into caffeate by *Streptomyces nigrifaciens*. Biochem J, 130: 425–433

Peralta-Zamora P, Pereira C M, Tiburtius E R L, Moraes S G, Rosa M A, Minussi R C, Dura'n N (2003). Decolorization of reactive dyes by immobilized laccase. Appl Catal B, 42(2): 131–144

Philipp S, Held T, Kutzner H J (1991). Purifi cation and characterization

of the tyrosinase of *Streptomyces michiganensis* DSM 40015. J Basic Microbiol, 31(4): 293–300

Popa C, Bahrim G (2011). Streptomyces tyrosinase: production and practical applications. Innov Rom Food Biotechnol, 8: 1–7

Raval K M, Vaswani P S, Majumder D R (2012). Biotransformation of a single amino acid L tyrosine into a bioactive molecule L-DOPA. Int J Sci Res, 2: 2250–3153

Rice R H, Cohen D E (1996). The basic science of poisons. McGraw-Hill, New York

Robb D A (1995). Exploiting tyrosinase activity in aqueous and nonaqueous media. Acs Sym Ser, 600: 159–165

Saboury A A, Zolghadri S, Haghbeen K, Moosavi-Movahedi A A (2006). The inhibitory effect of benzenethiol on the cresolase and catecholase activities of mushroom tyrosinase. J Enzyme Inhib Med Chem, 21(6): 711–717

Saiki R K, Scharf S, Faloona F, Mullis K B, Horn G T, Erlich H A, Arnheim N (1985). Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science, 230(4732): 1350–1354

Saitou N, Nei M (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol, 4(4): 406–425

Saiyood S, Vangnai A S, Thiravetyan P, Inthorn D (2010). Bisphenol A removal by the Dracaena plant and the role of plant-associating bacteria. J Hazard Mater, 178(1–3): 777–785

Seo S Y, Sharma V K, Sharma N (2003). Mushroom tyrosinase: recent prospects. J Agric Food Chem, 51(10): 2837–2853

Shesterenko Y A, Sevastyanov O V, Romanoyskaya L L (2012). Removal of phenols from aqueous solutions using Tyrosinase immobilized on polymer carriers and inorganic coagulants. J Water Chem Technol, 34(2): 107–111

Shi J, Bian W, Yin X (2009). Organic contaminants removal by the technique of pulsed high-voltage discharge in water. J Hazard Mater, 171(1–3): 924–931

Shubhrasekhar C, Supriya M, Karthik L, Gaurav K, Bhaskara Rao K V (2013). Isolation, characterization and application of biosurfactant produced by marine actinobacteria isolated from Saltpan soil from coastal area of Andhra Pradesh, India. Res J Biotechnol, 8: 18–25

Vermelho A B, Meirelles M N L, Lopes A, Petinate S D G, Chaia A A, Branquinha M H (1996). Detection of extracellular proteases from microorganisms on agar plates. Mem Inst Oswaldo Cruz, 91(6): 755–760

Wang Q, Fang X, Bai B, Liang X, Shuler P J, Goddard W A 3rd, Tang Y (2007). Engineering bacteria for production of rhamnolipid as an agent for enhanced oil recovery. Biotechnol Bioeng, 98(4): 842–853

Xu D Y, Yang Y, Yang Z (2011). Activity and stability of cross-linked tyrosinase aggregates in aqueous and nonaqueous media. J Biotechnol, 152(1–2): 30–36

Zhao J, Li Y, Zhang C, Zeng Q, Zhou Q (2008). Sorption and degradation of bisphenol A by aerobic activated sludge. J Hazard Mater, 155(1–2): 305–311