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Antioxidant and haemolytic activity of tyrosinase producing marine actinobacteria from salterns

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ABSTRACT

The present work was concerned with the isolation and screening of tyrosinase enzyme producing marine actinobacteria from marine salterns soil. It was also focussed on testing the antioxidant and haemolytic activity of the isolated marine actinobacteria. A total of 17 actinobacterial isolates were isolated from salterns soil samples from Kothapattanam, Ongole, Andhra Pradesh. They were screened for tyrosinase production using skim milk agar medium. In the primary screening, among the 17 isolates, isolate NSRB showed a maximum zone of hydrolysis. 8 potent isolates were studied for secondary screening using tyrosinase agar. Based on the result of secondary screening, NSRB was selected for further analysis, such as tyrosinase assay and protein content. The isolate NSRB showed moderately higher antioxidant activity in various screening tests viz., DPPH scavenging assay (88.20%), metal chelating assay (75.37%) and reducing power assay (0.59%). The isolate was found to be non haemolytic towards human RBC. Further the isolate NSRB was partially characterized by conventional methods, using Nonomura key. It showed similar characteristics to Streptomyces aureofasciculus.

Keywords: Marine actinobacteria, Tyrosinase enzyme, Antioxidant activity, Haemolytic activity

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INTRODUCTION

Tyrosinase is a polyphenol oxidase found in various species of bacteria and yeast and is associated with melanin production [1]. The capability of microorganism to produce melanin compounds has been well known. Tyrosinase acts as a key enzyme for initiating the biosynthetic pathway to produce melanin in bacteria and animals. It has been identified in many of the bacterial genomes sequenced to date. Tyrosinase enzymes are also reported to be in actinobacteria, fungi, plants and animals [2]. The microbial melanin contributes to defence mechanism in different ways: it defends DNA from the damages of UV radiation and reactive oxygen species [3] it is able to bind toxic heavy metals [4] and to interact with DNA, possibly slowing down the metabolism [5]. Tyrosinases are thus important for the survival of organisms. It is reported that tyrosinases are involved in two different enzymatic reactions; 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 [6]. Tyrosinases 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 [7]. Although much of the work on tyrosinase and their applications have been done with mushroom tyrosinases, 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 better source of tyrosinase enzyme and bacterial tyrosinases are more advantageous than mushroom tyrosinases.

Anti-oxidants have the ability to scavenge and inhibit free radicals. Free radicals are able to oxidize nucleic acids, proteins, lipids and therefore can initiate degenerative diseases. Biological systems have highly reactive free radicals and oxygen species. Antioxidants have wide applications as dietary supplements and can prevent diseases like

cancer, coronary heart disease and even altitude sickness [8-10]. Antioxidants also have numerous industrial uses, such as food preservatives, in cosmetics and to prevent the degradation of rubber and gasoline [11]. Marine environment covers almost 70% of the earth surface [12]. Organisms present in these environments are extremely rich sources of bioactive compounds [13,14]. Actinobacteria are gram positive, filamentous bacteria which are supreme secondary metabolite producers [15-17]. Among the members of actinomycetes genus, Streptomyces sp is a potential producer of Tyrosinase enzyme. CU^{2+} ions are the major constituents of the enzyme[18].

Actinobacteria from terrestrial origin produce hundreds of potential metabolites which are widely used at present. Some differences could be expected among organisms existing in marine and terrestrial environments due to variation in the physical, chemical and biological factors [19]. It is apparent that the marine environment is a potent source for finding new actinobacteria and new biologically active substances [20]. Hence, the present work was undertaken to isolate potent actinobacteria from salt pan soil sample to elucidate their tyrosinase enzyme activity, antioxidant and haemolytic activity.

MATERIALS AND METHODS

Isolation of Actinobacteria

The soil samples were collected from a salt pan in Kothapattanam, Ongole, Andhra Pradesh (15° 30’ 0” N, 80° 3’ 0” E) India during December 2013. The isolation of actinobcateria was performed by using selective media such as Actinomycetes Isolation Agar (AIA), Kuster’s agar and Starch Caesin Agar. All the media were supplemented with a final concentration of 100 µg/l cyclohexamide and 15 µg/l nalidixic acid, to inhibit fungal and bacterial growth respectively [21]. A 10 fold serial dilution was made and plated in triplicates on the different agar plates. The plates were incubated at room temperature (28°C) and monitored periodically over 3 months for actinobacterial growth [22]. Morphologically distinct colonies were separated and purified.

Primary screening method

Primary screening of tyrosinase enzymes producing marine actinobacteria was carried on skim milk agar plates. All the isolates were streaked onto skim milk agar plates (pH 7±7.2). and the plates were incubated at 30ºC for 2-3 days. After incubation, the plates were observed for the zone of clearance around the colony [23].

Secondary screening method

Tyrosinase enzyme producing marine actinobacteria was screened by inoculating the isolates on tyrosine agar plate and in tyrosine broth.

Test on 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 appearance of brown pigmented colonies that gradually changed its color to black (melanin formation) was the indication of tyrosinase positive organisms [23].

Test in 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 were incubated at 30°C for 2-3 days. A deep red color shows the positive results [23].

Production media for tyrosinase

One loop full culture of the isolate NSRB 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 $MnCl_2$- 1 ml/l) enriched with 0.1% tyrosine and 10 mg/ml of copper sulphate 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 the enzyme tyrosinase

The tyrosinase enzyme produced by the isolate was characterized by performing tyrosinase assay and the protein content was estimated by Bradford’s method.

Tyrosinase assay

To 0.1 ml supernatant of the isolate NSRB, 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 minutes to reach temperature equilibration and absorbance was recorded at 280 nm by using UV-Vis spectrophotometer [23].

The enzyme activity was calculated by using the following formula:

$$\text{Units of enzyme/ml} = \frac{\Delta A280\ nm/\ min\ Test - \Delta A280nm/\ min\ Blank\ (df)}{(0.001)\ (0.1)}$$

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 5ml of Bradford reagent (Coomassie brilliant blue G-250) and incubated for 5 minutes. After that, absorbance was measured at 595 nm. Protein content was expressed as milligrams of protein per millilitre of sample [23].

Following tyrosinase assay and Bradford's method, specific activity of tyrosinase was calculated using the following formula-

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

Anti-oxidant assays

The potent isolate NSRB was assessed by performing anti-oxidant assays.

DPPH assay

The isolate NSRB was centrifuged at 5000 rpm for 10 minutes. Supernatant was collected for anti-oxidant assay. In test tubes, 1ml of supernatant from each strain was taken and 2 ml DPPH reagent (2,2-diphenyl-1-picrylhydrazyl) was added in dark. DPPH is a free radical. Test tubes were incubated for 20 minutes at room temperature in dark condition. The degree of reduction of the reagent was measured as absorbance in UV-Visible spectrophotometer at 517 nm [24].

Percentage of scavenging activity was calculated as:

% scavenging rate= (Control-Absorbance of test/Control)*10

Reducing power Assay

Reducing power was measured using the protocol of Daljit Singh and Priyanka [25] given as follows. 1.0 ml of the sample was dissolved in 1.0ml of phosphate buffer and was mixed well with 1.0 ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$] and then this mixture was incubated at 50^{o}C for 20 minutes. Subsequently 1.0 ml of trichloroacetic acid, and 1.0 ml milliQ water were added. Finally the solution was mixed with 0.1 ml of 0.1% ferric chloride. Mixture was incubated at 50^{o}C for 10 minutes. Absorbance was measured at 700 nm [26].

Metal chelating assay

The chelating activity was measured using the protocol of Zhao et al. [27]. In this method 0.5 ml of each extract was mixed with 1.6 ml milliQ water. Subsequently, 0.05 ml of $FeCl_2$ was added in each test tube. This reaction was initiated by the addition of 0.1 ml of 5Mm ferrozine. The solution was mixed well and allowed to stand for 10 minutes at 40°C. After incubation period, absorbance was measured using a spectrophotometer at 562 nm. Distilled water was used as control. Instead of $FeCl_2$ distilled water was used as blank.

Ferrous ion-chelating ability was calculated as follows:

%scavenging activity

(Ferrous ion chelating ability)= $[1- (A_1-A_2)/A_0] * 100$

Where, A_0 is absorbance of control, A_1 is reaction mixture; A_2 is absorbance without $FeCl_2$.

Haemolytic activity

The haemolytic activity of tyrosinase producing actinobacteria was measured using the protocol of Gaurav et al. [28]. In this assay 5 ml of blood containing heparin was centrifuged at 15000 rpm for 30 minutes. Supernatant containing plasma was discarded and pellet containing red blood cells was washed with 0.75% saline by centrifugation at 1500 rpm for 5 minutes. The cells were suspended in normal saline. 0.5 ml of cell suspension was then mixed with sample. The mixture was incubated for 30 minutes at 37°C and then centrifuged at 1500 rpm for 10 minutes. The free haemoglobin in supernatant was measured using UV-Vis spectrophotometer at 540 nm. Distilled water and phosphate buffer saline was used as minimal and maximal haemolytic controls.

The activity was calculated as follows-

%Haemolytic activity = $(A_t - A_n) / (A_c - A_n) * 100$

Where A_t is absorbance of sample, A_n is absorbance of control and A_c absorbance of control

Taxonomic Identification
The efficacious actinobacteria isolate was characterized by morphological and biochemical method and the results were compared with Nonomura key 1974, Shirling and Gottlieb 1966 and with Bergey's manual of Determinative Bacteriology [29].

Morphological characteristics
Actinobacteria isolate was inoculated on seven different International Streptomyces Project mediums (ISP 1 to ISP 7) and incubated for 7 days at room temperature. The colonies were observed under a high power magnifying lens and colony morphology was noted with respect to aerial mycelium color, nature of colony and reverse side color.

Assimilation of Carbon Source
The ability of the isolate species in utilizing various carbon sources is analysed. viz., arabinose, xylose, inositol, mannitol, fructose, rhamnose, sucrose and raffinose as sources of energy were studied based on the method recommended by International Streptomyces Project (ISP). These carbon sources were sterilized by ether sterilization [30].

Generic investigation
The genus of potent actinobacteria was identified using cell wall composition (amino acid and whole cell sugars) analysis [30].

Statistical Analysis
All tests were conducted in triplicate. Data are reported as means ± standard deviation (SD). Results were analysed statistically by using Microsoft Excel 2007 (Roselle, IL, USA).

RESULTS AND DISCUSSION

Marine sources are rich potential environment of novel microorganisms that are capable of producing a wide variety of biologically active compounds including antibiotics and other secondary metabolites [31]. Hence, the present study involved in the isolation of actinobacteria from coastal salterns and assessing them in the tyrosinase enzyme production and other beneficial activities.

Primary screening of tyrosinase enzyme production
A total 17 different types of actinobacterial isolates were isolated from collected salterns salt sample based on colony morphology and microscopic appearance. The actinomycetes isolation agar recovered more actinobacteria colonies, when compared to other media. All these isolated actinobacteria were primarily screened for tyrosinase enzyme production. Among the 17 actinobacterial isolates only one isolate showed positive result for tyrosinase enzyme production and the potential isolate was named as NSRB. In the primary screening study, on skim milk agar NSRB isolate showed the zone formation and indicates the secretion of proteolytic enzyme. The zone formation of actinobacteria on the skim milk agar plates was directly proportional to the production of protease enzyme [32, 33]. Skim milk agar method is not an accurate procedure for the screening of tyrosinase enzyme production [34]. Hence, the screening tyrosinase enzyme production was further carried out in tyrosine agar. On tyrosine agar plate NSRB isolate showed brown colored pigmentation. The production of brown color pigment indicates the tyrosinase enzyme production [35]. The production of brown color was due to the production of melanin by oxidative polymerization of phenolic compound by tyrosinase.

Secondary Screening and estimation of tyrosinase enzyme
On the basis of the results obtained on skim milk and tyrosine agar plates, the potent isolate was inoculated in tyrosine broth. After incubation the tyrosine broth color was changed from pink to brown. The production of brown color indicates the secretion of tyrosinase enzyme and the enzyme activity of the potent actinobacteria isolate NSRB, showed tyrosinase enzyme was found to be $5Uml^{-1}$ min^{-1}. During the tyrosinase estimation assay, the production of tyrosinase enzyme was detected by the disappearance of tyrosine [36]. Tyrosine is as a basic substrate for the production of tyrosinase enzyme. The tyrosinase enzyme acts as a bi-functional for the reaction of orthohydroxylation of monophenols (tyrosine) followed oxidation of diphenols (L-DOPA) to orthoquinones (dopachrome). Dopachrome can then be transformed to the brown/ black melanin pigments [37]. The protein content

was estimated using Bradford's method and the Bradford's method is a simpler, faster, more sensitive method than Lowry's method and subjects to less intervention by non-protein components and general reagents; hence, the method is used to estimate the protein in the medium [38].

DPPH Assay

2, 2-diphenyl-1-picrylhydrazyl (DPPH) is a biological free radical. The magnitude of anti-oxidation ability of sample can be expressed by their capacity to scavenge DPPH radical. Based on this property, antioxidants present in the sample will turn the free radical from violet to yellow color. This change of color indicates a positive test. The isolate NSRB has high free radical scavenging activity and the results are depicted in Fig.1.

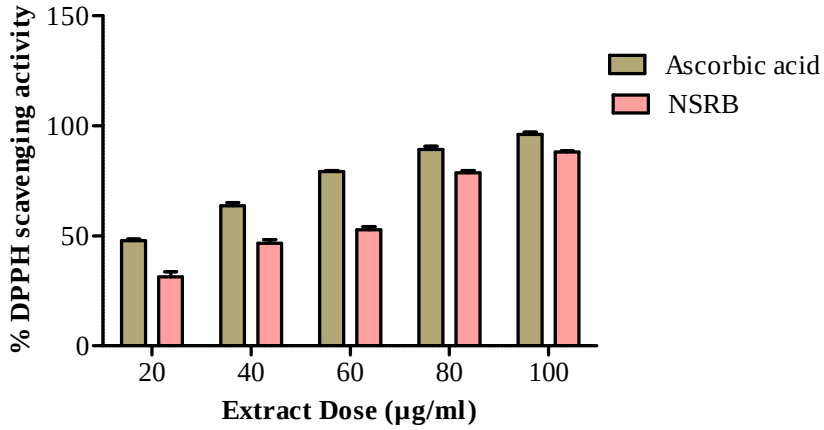

FIG1. DPPH ASSAY

Reducing Power Assay

The electron donating capability of an antioxidant is measured by the reducing power assay. When reducers are present they convert Fe^{3+} (ferricyanide) complex to the ferrous form, which is a significant indicator of antioxidant activity. Green color indicates positive test. Increase in the absorbance of the reaction mixtures indicates an increase in reducing power. Potent isolate NSRB showed maximum reducing power (Fig. 2).

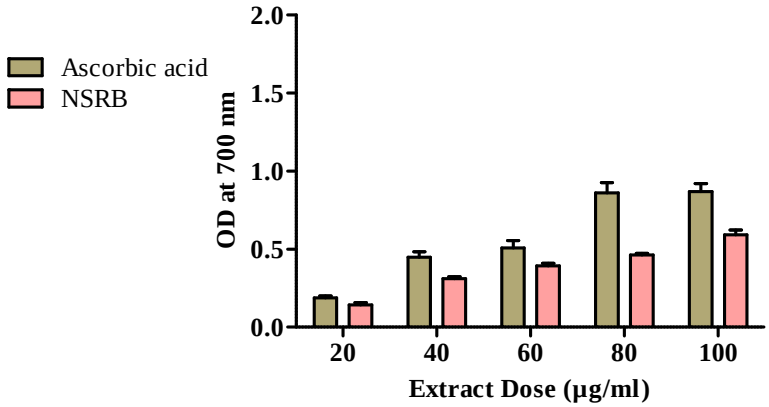

FIG 2. REDUCING POWER ASSAY

Metal Chelating Assay

Metal chelating property of the transition metals prevents catalysis of hydrogen peroxide decomposition. If chelating agents are present, the complex formation is inhibited and there is reduction in the red color. Chelating activity of sample is determined by measurement of color. The transition metal ion Fe^{2+} has the ability to move electron, by this it can allow propagation and formation of radical reaction. The potent actinobacterial isolate NSRB showed high metal chelating activity (Fig. 3).

Haemolytic assay

In vitro haemolytic assay using spectroscopic methods provides an effective and easy method for the quantitative measurement of haemolysis. This method helps to evaluate the effect of different concentration on biomolecules of the human erythrocytes. No haemolytic activity was observed, indicating that the tyrosinase producing isolates are not toxic to the human red blood cells.

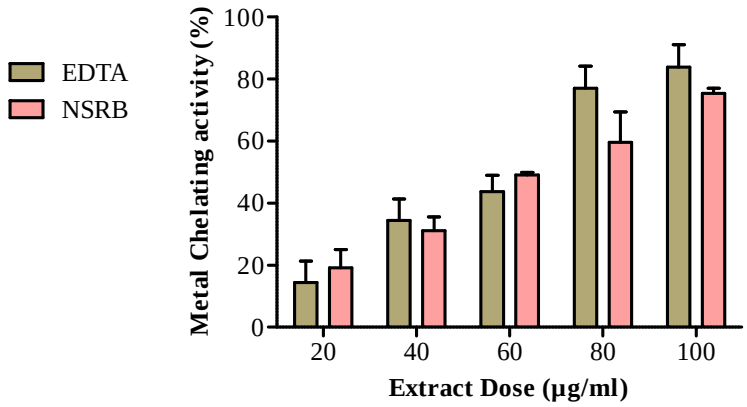

FIG3. METAL CHELATING ASSAY

Characterization of the potential strain

The strain NSRB possesses LL-Diaminopimelic acid and it contains glycine in its cell wall. Presence of L,L-Diaminopimelic acid along with glycine indicate the cell wall chemotype-I, which is the characteristic of the genera *Streptomyces*, *Streptoverticillium*, *Chainia*, *Actinopycnidium*, *Actinosporangium*, *Elyptrosporangium* and *Microellobosporia*. The morphological characters of the strain NSRB are similar to the genus *Streptomyces*. The morphological and biochemical characteristics of tyrosinase producing isolate NSRB was tested in the present study, the results are compared with those of *Streptomyces* species given in the key of Nonomura and also with the species described in the Bergey's Manual of Determinative Bacteriology. The strain NSRB showed similar characters to the reference strain *Streptomyces aureofasciculus*. Hence the NSRB has been tentatively identified as *S. aureofasciculus* (Table.1and 2).

Table1. Morphological and biochemical analysis

Characteristics	NSRB	*Streptomyces aureofasciculus*
Morphological characteristics		
Colour of aerial mycelium	White	White
Melanoid pigment	Nil	Nil
Reverse side pigment	Nil	Nil
Soluble pigment	Nil	Nil
Spore chain morphology	Spirales	Spirales
Spore surface morphology	Smooth	Smooth
Utilisation of sole carbon sources		
Arabinose	-	-
xylose	+	+
Inositol	+	+
Sucrose	-	-
Fructose	+	+
Raffinose	-	-

Table2. Cell wall analysis

Strain no	LL-DAP	Meso-DAP	Glycine	Whole cell sugars	Wall type
S. aureofasciculus	+	-	+	-	I
NSRB	+	-	+	-	I

CONCLUSION

The marine actinobacteria *S. aureofasciculus* isolated from marine saltern soil sample was found to be the proficient producer of tyrosinase enzyme. The tyrosinase enzyme from *S. aureofasciculus* strain can act as a promising source for industries. The isolates also showed anti-oxidant properties like scavenging of free radical, reducing power and metal chelating capability. The potent actinobacteria are non -toxic to human erythrocytes. Future studies will be focussed to increase the enzyme activity of *S.aureofasciculus*.

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