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Der Pharmacia Lettre, 2016, 8 (3):166-172
(http://scholarsresearchlibrary.com/archive.html)

ISSN 0975-5071
USA CODEN: DPLEB4

Assessment of anti-typhoid and antioxidant activity of marine actinobacteria isolated from Chennai marine sediments

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ABSTRACT

The present study was carried out to screen the actinobacteria isolated from marine sediments collected from Chennai, Tamilnadu, India for their antibacterial and antioxidant activites. A total of 6 isolates of marine Actinobacteria were isolated and were designated as VSKB 1 to VSKB 6. Out of the six isolates only one isolates VSKB-3 showed activity against salmonella typhi with a zone of inhibition of 18mm. In solvent extraction butanol was found to extract more active compounds as it showed a zone of 22mm against salmonella typhi. The potential isolated VSKB-3 showed moderately higher antioxidant activity in various screening tests viz., DPPH scavenging assay (88.32%), metal chelating assay (80.7%) and reducing power assay (0.80%). Father the isolate VSKB-3 was partially characterized by conventional methods, using Nonomura key. It showed similar characteristics to Streptomyces bluensi.

Keywords: *Salmonella typhi*, Marine actinobacteria, Antioxidant activity, Typhoid fever

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INTRODUCTION

Typhoid fever is a systemic disease caused by bacteria *Salmonella typhi* which is acquired by ingestion of contaminated water and food [1]. This fever received various names, such as gastric fever, abdominal typhus, infantile remittant fever, slow fever, nervous fever, pythogenic fever etc. Salmonella is named after the scientist who discovered them, Salmon. This organism is a Gram-negative short bacillus that is motile due to its peritrichous flagella [2]. For over 100 years, this group of bacteria has been known to infect humans. The most common species that infects humans thorough contaminating food are *Salmonella enteriditis* and *Salmonella typhimurium*. Salmonella infection, known as salmonellosis, normally results in abdominal cramping, diarrhea and fever after 12 to 72 hours of ingestion. Few strains of these bacterium have become resistant to treatment with antibiotic. Antibiotics, such as Amoxicillin, chloramphenicol, ampicillin, trimethoprim-sulfamethoxazole, and ciprofloxacin, have been used to treat typhoid fever in many developed countries [3]. From year 1989, strains of *Salmonella typhi* resistant to chloramphenicol, ampicillin, and trimethoprim (i.e., multidrug-resistant [MDR] strains) have been responsible for many outbreaks in countries like the Indian subcontinent, Southeast Asia, and Africa. Mortality rates related with typhoid fever may vary from region to region with the highest reported from Indonesia, Nigeria and [4]. CDC estimates that over 1 million cases occur annually, according to a 2011 report [5] of these cases, approximately 20,000 results in hospitalization and 378 results in death. This shows that *Salmonella* accounts for almost 30 percent of food borne illness-related deaths each year (CDC report).

Antioxidant compounds have an ability to inhibit the free radicals; these free radicals play a major role in the oxidization of nucleic acids, proteins, lipids which triggers the degenerative disease. Highly reactive free radicals and oxygen species are present in biological system. Antioxidants can prevent disease like cancer, coronary heart disease and also have a wide range of applications as dietary supplements [6,7,8]. Along with these antioxidants have various industrial uses, such as in cosmetics, to prevent degradation of rubber, gasoline etc. and also as food preservatives [9].

Marine microorganisms have become important in the search for novel microbial products. Many marine microorganisms contain substances that have antimicrobial, antiviral, anticoagulant and cardioactive properties. Some of these substances have chemical structures that are unlike any compounds found in terrestrial species. These active compounds may serve as models in discovery of new drugs [10]. In the present world the production of antibiotics against some particular disease causing organism is very important. It is to control the wide spreading of life- threatening diseases such as Typhoid fever. Actinobacteria are well known as secondary metabolite producers and hence of high pharmacological and commercial interest [11]. They have a complex life cycle which is widely distributed in terrestrial ecosystems. They are producers of a large number of natural products, many of them with clinical, pharmaceutical [12]. The marine environment is a potential source for new Actinobacteria and novel antibiotics. Streptomyces, Micromonospora and Nocardia are the three most common genera in the marine environment. Most of the microbial metabolites were isolated from Actinobacteria especially from Streptomycetes. Actinobacteria have contributed for more than 70% antibiotics or bioactive compounds [13] in clinics today, such as Erythromycin, Chloramphenicol, Gentamicin and so on. Actinobacteria still hold the key position in the discovery of new antibiotics. This study is carried out to screen the anti-typhoid and antioxidant activity of marine actinobacteria.

MATERIALS AND METHODS

Chemicals

All the chemicals and media used in the study were purchased from Hi Media chemicals, Mumbai, India.

Isolation of Actinobacteria

Marine sediments were collected from the Chennai region (Latitute: 11º00'N Longitute: 78º00'E), Tamil Nadu, India. Isolation of actinobacteria was done using Starch-Casein agar and actinobacteria isolation agar by spread plate technique after serial dilution in 50% sea water. All agar plates were supplemented with 50 µg/ml of nystatin to avoid fungal contamination and incubated at room temperature for one month. Powdery and leathery colonies were isolated, purified and stored at 4ºC until further use [14].

Clinical Culture

Salmonella typhi culture was collected from Narayani Hospital Sripuram, Vellore, Tamil Nadu

Fermentation media

All six isolates were separately inoculated in 50 ml of production media (soluble starch 25 g, glucose 10 g, yeast extract 2 g, $CaCO_3$ 3 g, trace elements 1 ml, distilled water 1000 ml) in 100ml conical flask at 37°C in a shaker for 1 week at 200-250 rpm. After incubation the broths were centrifugation 10000rpm for 10min and the supernatant was collected to carry out the activities

Screening of actinobacteria crude extract against *Salmonella typhi*

Screening against *Salmonella typhi* was done by using agar well diffusion method. The supernatant obtained after fermentation was loaded at different concentration (20-100µg/ml) into wells bored in agar plated swabbed with test culture. The plates were incubated at 37°C for 24 h and examined. After incubation the zone of inhibition was measured.

Solvent Extraction

Extraction of antimicrobial compounds is done by using different solvents. The selected antagonistic actinobacteria isolate was inoculated into production medium and incubated at 37°C in a rotary shaker (200-250 rpm) for seven days. After incubation the broth was filtered through Whatman No.1 filter paper and then through Millipore filter (Millipore Millex-HV HydrophilicPVDF 0.45 µm). The filtrate was transferred aseptically into a conical flask and stored at 4°C for further assay. To the culture filtrate, equal volume of various solvents (hexane, chloroform, ethyl acetate and butanol) was added separately and kept in a rotatory shaker for 24 hours to extract the anti typhoid

compound [15]. The compound obtained from each solvent was tested for their activity against *Salmonella typhi* by agar well diffusion method. After incubation the zone of inhibition was measured.

Anti-oxidant assays
The potent isolate VSKB-3 was assessed by performing anti-oxidant assays.

DPPH assay
The potential isolate VSKB-3 was centrifuged at 5000 rpm for 10 min. Supernatant was collected for anti-oxidant assay. In test tubes, 1ml of supernatant 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 [16].

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 [17] 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°C for 20 min. 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 50ºC for 10 min. Absorbance was measured at 700 nm [18].

Metal chelating assay
The metal chelating activity was measured using the protocol of Zhao et al [19]. 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 min 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- (A1-A2)/A0] * 100
Where, A0 is absorbance of control, A1 is reaction mixture; A2 is absorbance without $FeCl_2$.

Identification of potential marine actinobacterial
The genus of the isolate (VSKB-3) with good anti- typhoid and antioxidant activity was identified using cell wall composition analysis and micromorphological studies [20]. The species was identified based on methods described by Shirling and Gottlieb in 1966 [21] and the key of Nonomura in 1974 [22].

RESULTS AND DISCUSSION

Isolation of Actinobacteria and screening against *salmonella typhi*
A total of 6 marine actinobacteria were isolated from marine sediment samples collected from Chennai based on colony morphology and microscopic appearance. The Actinomycetes isolation agar recovered more actinobacteria colonies, when compared to starch casein agar. They were designated as VSKB -1 to VSKB- 6. Bhaskaran *et al.*, in 2011 [23] reported that starch casein agar, Kuster's agar were effective media for the isolation of marine actinobacteria. However, in this study the maximum numbers of colonies were obtained in Actinomycetes isolation agar. All six isolated marine actinobacteria were tested for their antagonistic activity against *Salmonella typhi*. Among these six isolates the maximum inhibition activity was noticed with strain VSKB-3 (Fig 1) with a zone of 18mm (Table 1).

Table 1 Zone of inhibition against *salmonella typhi* at different concentration

Strains	Zone of inhibition against *salmonella typhi*				
	20µg/ml	40µg/ml	60µg/ml	80µg/ml	100µg/ml
VSKB 1	-	-	-	-	2mm
VSKB 2	-	-	-	2mm	5 mm
VSKB 3	5mm	9mm	11mm	15mm	18mm
VSKB 4	-	-	-	1mm	3mm
VSKB 5	-	-	-	-	-
VSKB 6	-	-	-	1mm	4mm

(- = Negative)

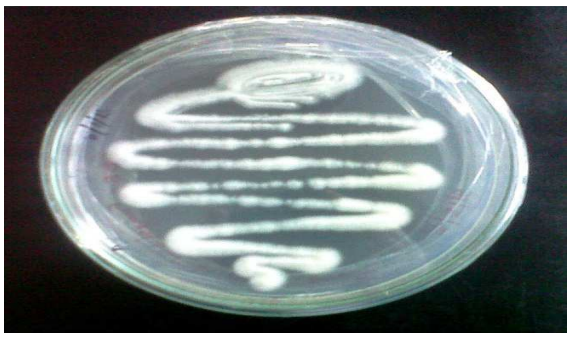

Fig. 1 Potential actinobacterial strain VSKB-3

Screening using solvent extracted compound

In primary screening potential marine actinobacteria strain VSKB-3 was selected for further studies, i.e., solvent extraction method. The isolate VSKB-3 showed maximum zone of inhibition (22mm) in butanol as solvent against the test organism *Salmonella typhi* (Table 2). Here in this study butanol was found as the best solvent for extraction of anti-typhoid compounds from marine actinobacteria. Ogunmonyi et al in 2010 [24] reported ethyl acetate as suitable for solvent extraction from marine actinobacteria, but our results indicates that Butanol can also be used for solvent extraction to enhance the activity.

Table 2 Screening of crude extract against *salmonella typhi* using different solvents

Solvents	Zone of inhibition (100µg/ml)
Hexane	11mm
Chloroform	9mm
Ethyl acetate	11mm
Butanol	22mm

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, the antioxidants present in the sample will turn the free radical from violet to yellow colour. This change of colour indicates a positive test. The isolate VSKB-3 has high free radical scavenging activity and the results are depicted in Fig.2.

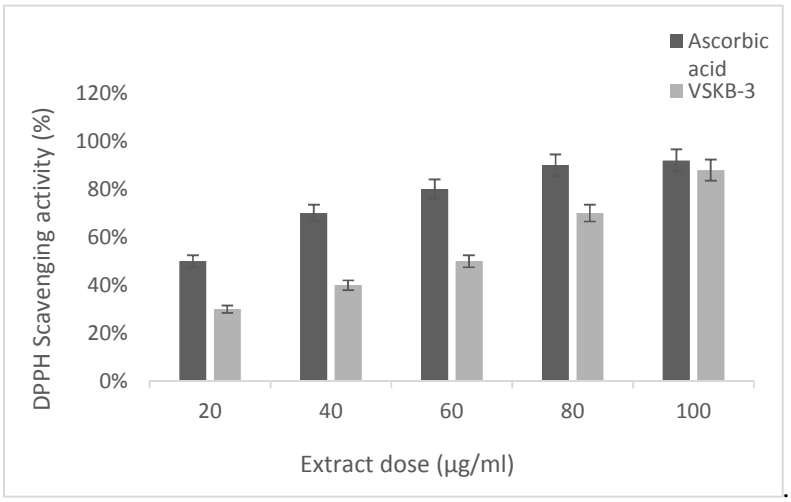

Fig. 2 DPPH assay using potentail strain VSKB 3

Reducing Power Assay

The electron donating capability of an antioxidant is measured by the reducing power assay. When reducers are present they convert Fe3+ (ferricyanide) complex to the ferrous form, which is a significant indicator of antioxidant activity. Green colour indicates positive test. Increase in the absorbance of the reaction mixtures indicates an increase in reducing power. Potent isolate VSKV-3 showed maximum reducing power (Fig. 3).

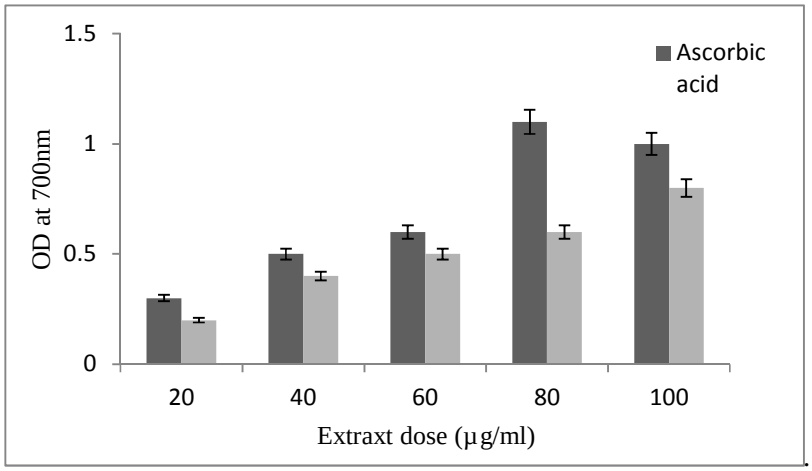

Fig. 3 Reducing power Assay using potential strain VSKB 3

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 a reduction in the red colour. The Chelating activity of the sample is determined by measurement of colour. The transition metal ion Fe2+ has the ability to move electrons, from this it can allow propagation and formation of radical reaction. The potent actinobacterial isolate VSKB-3 showed high metal chelating activity (Fig. 4).

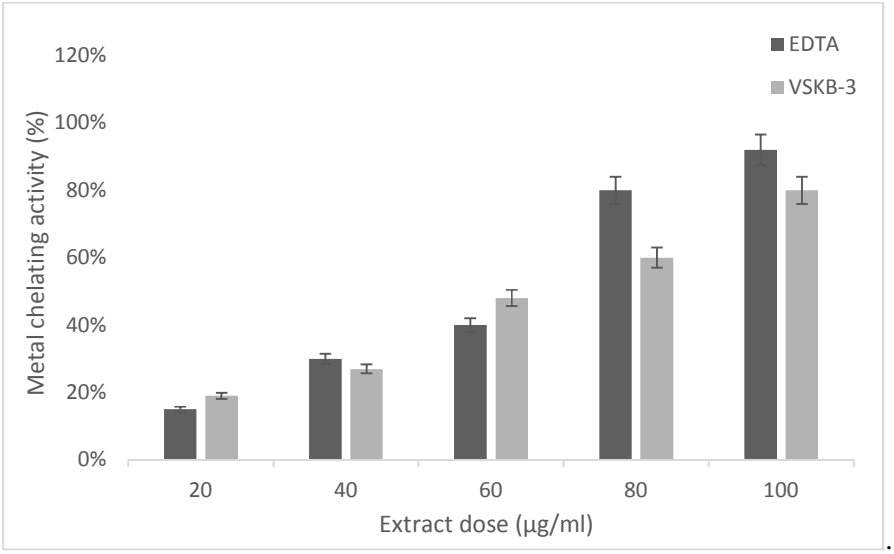

Fig. 4 Metal chelating Assay using potential strain VSKB 3

Identification of Actinobacteria

The isolate VSKB-3 possesses LL-DAP and contains glycine in its cell wall. Presence of LL-DAP along with glycine indicates the cell wall chemotype I. The micromorphological observations of the isolate VSKB-3 reveal that the strain belongs to the genus Streptomyces. The physiological characteristics of the isolate VSKB-3 was given in Table 3 and compared with those of the Streptomyces species given in the key of Nonomura. Short, compact spiral shaped spore chains were observed under microscope (100x) (Fig 5). Thus, VSKB-3 was identified as *Streptomyces bluensis*. Other researchers have also demonstrated that most of the actinobacteria producing potent antibiotics belong to the genus *Streptomyces*[25,26]. According to Kutzner in 1972 [27] for proper identification of genera and species of actinomycetes, besides morphological and physiological properties, various other biochemical properties such as cell wall chemo type, whole-cell sugar pattern, peptidoglycan type, phospholipids type and G+C% of DNA should be determined.

Table 3 Morphological characteristics of isolate VSKB-3

Characteristics	VSKB-3	*S. bluensis*	Characteristics	VSKB-3	*S. bluensis*
Aerial mass colour	Brownish white	W (B)	Utilization of sole carbon sources		
Melanoid pigment	--	--	Arabinose	+	+
Reverse side pigment	--	--	Xylose	+	+
Soluble pigment	--	--	Inositol	+	+
Spore chain	Spiral	Spiral	Mannitol	+	+
			Fructose	+	+
			Rhamnose	+	+
			Sucrose	+	+
			Raffinose	+	+

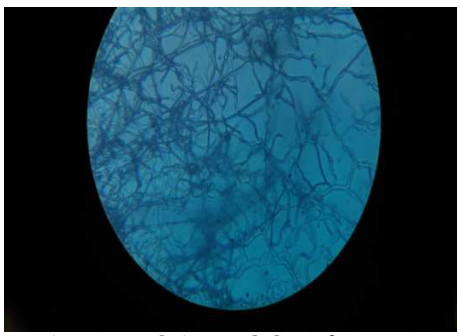

Fig. 5 Spore chain morphology of VSKB -3

CONCLUSION

Streptomyces bluensi isolated from Chennai marine sediments was found to be a promising source of anti typhoid compounds. This potential strain can be used for the production drugs against *salmonella typhi*. The isolated also showed good antioxidant activities like reducing power, metal chelating and free radical scavenging activity. Characterization and identification of anti typhoid and antioxidant compound from marine *Streptomyces bluensi* and to increase the activity against *salmonella typhi* will be focused in future.

Acknowledgment
We are thankful to VIT University, Vellore, and Tamilnadu, India for supporting this study.

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