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SCREENING OF ANTIOXIDANT ACTIVITY OF MARINE BACTERIA ISOLATED FROM MARINE SOIL OBTAINED FROM NORTH-WEST COASTAL REGION OF INDIA

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ABSTRACT

The present study aims at enriching and isolating potential antioxidant producing microorganisms from marine soil obtained from coastline of North-West coastal region of India. A total of 156 bacterial isolates were obtained, which were screened for their potential to produce extracellular antioxidants. Several non-pigmented and pigmented isolates obtained which exhibited good antioxidant producing capabilities. Amongst the seven pigmented organisms exhibiting superior antioxidant activity, two isolates viz. 6-3 and 8-1 demonstrated a varied TLC banding pattern along with a significant DPPH scavenging activity. These isolates were identified morphologically, biochemically and with 16S rRNA identification studies as *Janibacter melonis* and *Pseudomonas stutzeri* respectively. The gene sequence of both the organisms was submitted to NCBI and the strains were named *Janibacter sp. TKB-1* and *Pseudomonas sp. KKB-1* respectively.

Key words: Antioxidants, DPPH assay, TLC, *Janibacter melonis, Pseudomonas stutzeri*

INTRODUCTION

Exposure to ultraviolet light, ionizing radiations and chemicals results in generation of reactive oxygen species (ROS). Reactive oxygen species are also produced in cells during normal physiological conditions. Although many organisms posses endogenous antioxidant system comprising of enzymes like

catalase, superoxide dismutase and glutathione and repair systems to protect them against oxidative damage, these systems are insufficient to prevent the damage [1]. Overproduction of these reactive oxygen species and/or inadequate presence of antioxidants increase oxidative stress and can cause deleterious effects associated to various disease conditions like diabetes, cancer, neurodegeneration, Alzhiemer's etc. [2].

Microorganisms have a great potential to produce secondary metabolites which could have pharmaceutical importance. Different terrestrial environments e.g soil, sewage, garbage etc. have been explored to isolate such potential microorganisms [3-5]. Marine environment, a rich source of marine bacteria is shown to produce novel secondary metabolites [6, 7]. These microbes are found in different niches in the ocean viz. planktonics, sponge-associated bacteria, deep ocean sediments, deep sea water samples, etc. [1, 8-15].

Marine habitats constantly expose marine organisms to different stresses viz. combination of photosynthesis, symbiont oxygen production, intense sunlight intensities leading to UV induced free radical production etc. [16]. So it is very likely that these organisms have an effective antioxidant mechanism to combat stress. So marine bacteria are likely to be a potential source of novel antioxidant compounds.

The present study aims at enriching, isolating and screening of potent antioxidant producing bacterial strains from marine soil samples obtained from North-West coastal region of India.

MATERIALS AND METHODS

Sample collection

The marine soil samples near sea coast were collected. Sample collection was done mainly from North-West coastal region of India viz. Thane, Vashi, Virar, Goa, Kerala, Kanyakumari and Konkan coastal region (Tarkarli and Ganpatipule beaches). Sufficient quantity of soil samples were placed in sterile containers and stored at refrigeration temperature until further processing.

Enrichment and Isolation of bacterial isolates

For enrichment of marine bacterial microflora, 1 gm of each soil sample was inoculated in nutrient medium comprising of varying concentrations of NaCl (1%, 2%, 3%, 5%, 10%, 15% and 20%) and incubated at room temperature from 24 h upto 4 days until visible growth was observed. Each of the enriched samples were then isolated on the nutrient media containing the respective concentrations of NaCl. The isolates obtained were

maintained on the sterile nutrient agar (NA) slants (containing respective concentration of NaCl) and further screened for the production of antioxidants.

Antioxidant assay

Every isolate obtained was inoculated in nutrient media (consisting respective concentration of NaCl) and incubated on shaker at RT for 24 h. The bacterial cell mass was separated by centrifugation and cell-free supernatants were lyophilised and stored at 4°C. 100 µg of these lyophilised crude extracts were reconstituted in sterile distilled water, filtered through Millipore filter and used for screening the production of antioxidants using DPPH radical scavenging assay. Two ml of extract was mixed with 2ml of freshly prepared DPPH solution (0.03 mM prepared in methanol) and incubated at room temperature for 30 minutes in dark**.** The absorbance was measured at 517 nm [17]. Ascorbic acid was used as positive standard [18]. The percent radical scavenging activity (RSA) was calculated as:

$RSA\ (\%) = (A_0 - A_1/A_0) \times 100$

Where, A_0 is the absorbance of control and A_1 is the absorbance of test sample.

Analysis of extracts by Thin-layer chromatography (TLC)

For determining the pattern of antioxidant bands by TLC, 500 µg of the lyophilised crude extracts were reconstituted in 2ml distilled water and filtered through Millipore membrane. 04µl aliquots were loaded onto the activated silica gel plates (10 x 20 cm). Ascorbic acid (1mg/ml) was used as the standard along with the test samples [19]. The samples were developed with n-butanol: methanol: water (25:15:10**)** as the solvent system [19]. For detecting the presence of phyto-chemical constituents, the plates were air dried, observed under UV transilluminator and later sprayed with 0.02% DPPH reagent. The presence of the antioxidants in the crude bacterial extracts was evident by appearance of white to yellowish spots.

Identification of the isolates

The isolates viz 8-1 and 6-3, exhibiting superior antioxidant activity and varied banding profile in TLC were characterized further by morphological, biochemical and 16S rRNA sequencing. Bacterial cells were subjected to Gram staining and the morphology was studied under light microscope. Biochemical tests were done as described in Bergey's manual of systematic bacteriology. The protocols for gram staining and various biochemical tests were followed as described in [20]. Molecular identification of the isolates was done using 16S rRNA sequencing at Eurofins Genomics India Pvt. Ltd.

RESULTS

Enrichment and isolation

The microorganisms from enriched soil samples were isolated on nutrient media containing respective concentration of NaCl. Altogether 156 isolates were obtained out of which a few isolates were found to produce orange yellow to greenish coloured water soluble pigments. All these isolates were sub-cultured and maintained on sterile NA slants at 4°C for further use (Table 1).

Screening by DPPH

The antioxidant producing capability of the isolates was checked by DPPH assay. Growth of most of the isolates maintained at high concentration of NaCl (10%, 15% and 20%) was very slow and did not show any significant antioxidant activity, hence no further studies were done on these isolates (data not shown). The percent radical scavenging activity for non pigmented isolates ranged from 30% to 51% whereas for pigmented isolates ranged from 28% to 69% (Table 2). Isolates 6-3 and 8-1 exhibited maximum radical scavenging activity viz. 69% and 63% respectively and moreover both these isolates were pigment producers.

Analysis of extracts by Thin-layer chromatography

Isolates showing enhanced antioxidant activity were shortlisted and the banding pattern of antioxidants in crude extracts was analyzed by TLC method. Crude extracts of 6-3 and 8-1 isolates showed a varied pattern of the bands when observed under UV light (Fig. 1).

Identification of the bacterial isolates

The morphological and the biochemical characteristics of isolate 6-3 and isolate 8-1 are summarized in table 3 and 4. Isolate 6-3 showed 1mm circular colonies with entire margin and a characteristic fluorescent greenish yellow pigment. The Gram nature of this isolate was reported as Gram positive coccoid. The 16S RNA sequencing identified the bacterium as *Janibacter melonis.* The gene sequence was submitted to Genebank with accession number KU174194 and the strain was named as *Janibacter sp. TK-1.* The isolate 8-1 showed 1mm, circular colonies with irregular margin and brownish yellow pigment. Its gram nature was gram negative bacterium and by 16S RNA sequencing the isolate was identified as *Pseudomonas stuzeri.* The Genebank accession number is KU174195 and the strain was named as *Pseudomonas sp. KKB-1.*

Table 1: Number of bacterial isolates obtained from different marine sources

	% NaCl in nutrient medium	Source of soil sample	Isolates	% Inhibition of DPPH activity
Non pigmented isolates	1%	Kerala (Ermakullam)	8-3	35.56 ± 0.16
	1%	Tarkarli	6-2	30.44 ± 0.31
	2%	Kerala(Ermakullam)	8-2	29.65 ± 0.33
	2%	Kanyakumari	12-3	36.74 ± 0.08
	5%	Kanyakumari	12-3	45.60 ± 0.09
	5%	Tarkarli	6-1	47.23 ± 0.15
	5%	Ganpatipule	5-3	51.71 ± 0.25
Pigmented isolates	1%	Kerala (Eramallor)	S – 9	36.18 ± 0.02
	1%	Kerala (Eramallor)	S – 1	28.96 ± 0.21
	2%	Kerala (Ermakullam)	8-1	63.61 ± 0.14
	2%	Kerala (Eramallor)	7-3	38.70 ± 0.28
	2%	Kerala (Eramallor)	S-4	28.94 ± 0.22
	2%	Kanyakumari	12-1	33.29 ± 0.22
	5%	Tarkarli	6-3	69.49 ± 0.33

Table 2: Antioxidant activity of crude bacterial extracts

Sr. No	Soil sample	No of isolates obtained	Sr. no	Soil sample	No of isolates obtained
1	Thane creek	11	7	Kerala (Eramallor)	12
2	Vashi creek	12	8	Kerala (Ernakulam)	15
3	Goa	14	9	Virar sea shore	12
4	Juhu beach	12	10	Salt from salt pan	12
5	Ganpatipule	14	11	Kerala (Kovalum beach)	14
6	Tarkarli	15	12	Kanyakumari	13

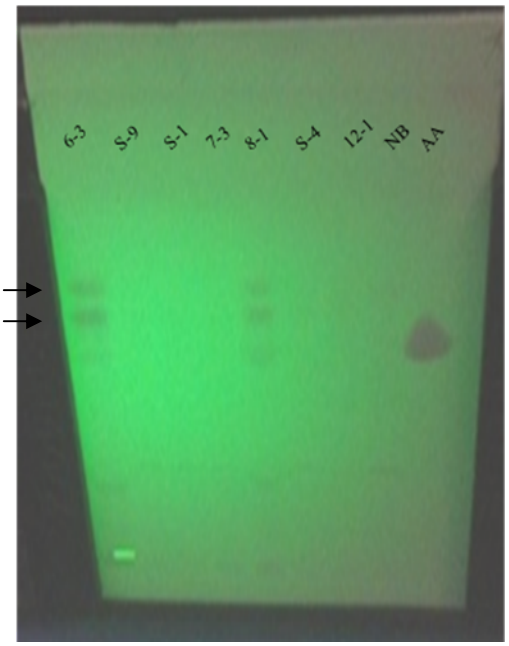

Fig 1: TLC analysis of crude lyophilised extracts of pigmented isolates

Table 3: Biochemical tests of *Janibacter melonis*

Name of test	Results
Indole	-
MR	-
VP	-
Citrate	-
Esculin hydrolysis	Slightly +
PYR	-
ONPG	-
Arginine utilization	+
Glucose utilization	+
Adonitol utilization	-
Arabinose utilization	-
Lactose utilization	-
Sorbitol utilization	-
Mannitol utilization	-
Rhamnose utilization	-
Sucrose utilization	-
Raffinose utilization	-
Catalase	+
Oxidase	-
Starch hydrolysis	-
Motility	Motile
Gelatine hydrolysis	-

Table 4: Biochemical tests of *Pseudomonas stutzeri*

Name of test	Results
Citrate utilization	+
Lysine utilization	-
Ornithine utilization	-
Urease	+
Phenylalanine deamination	-
Nitrate reduction	-
H2S production	-
Glucose utilization	+
Adonitol utilization	-
Lactose utilization	-
Arabinose utilization	-
Sorbitol utilization	-
Catalase	+
Oxidase	+
Starch hydrolysis	-
Motility	Dartingly motile
Gelatine hydrolysis	-

DISCUSSION

In the present study bacteria from marine soil samples producing antioxidants were isolated. A total of 156 bacterial isolates were obtained which were screened for antioxidant production using DPPH assay. Isolates growing at with 10%, 15% and 20% NaCl concentration exhibited poor growth as well as very less antioxidant activity. Isolate 6-3 obtained from Tarkali and isolate 8-1 obtained from Kerala (Ernakulum) exhibited significant antioxidant activity viz. 69% and 63% respectively. Both the isolates showed pigment production and showed varied banding profile in TLC analysis.

Morphological, biochemical characterization and 16S RNA identification studies showed that the isolate 6-3 was *Janibacter melonis* strain

whereas isolate 8-1 was *Pseudomonas stutzeri* strain. Most of the *Janibacter species* have been isolated from marine environment [21-24] and have been reported to be able to degrade pentachlorophenol [23] or express a novel cold-active lipase [25]. Our study reports *Janibacter melonis* as the first marine bacteria capable of producing natural antioxidants. Hamed *et al.,*(2015) reported marine bacteria *Bacillus brevis* isolate 20B to show antioxidant activity and proved to be first recorded for antioxidant bioassay [1]. *Bacillus sorensis* isolated from sea sediment samples [26] and *Brevibacterium spp* and *Corynebacterium spp* isolated from sand sample collected from sea grass area also showed antioxidant activity [12].

Many *Pseudomonas* species have been isolated from marine environment and has also been reported to have potential applications. *Pseudomonas putida* isolated from the sponge *M. microsigmatosa* has shown to produce a powerful antimicrobial substance, active against multidrug-resistant bacteria [27]. Enhanced production of biosurfactants by marine *Pseudomonas aeruginosa* was reported by Gehan and Abou-Elela, 2011[28]. *Pseudomonas stutzeri Nt-1* was shown to be associated with reduction of selenium [29]. Zerrad et al (2014) reported melanin pigment extracted from *Pseudomonas balearica* strain U7, isolated from the marine green alga *Ulva lactuca* possessed efficient free radical scavenging activity [30]. Thus our marine isolate, *Pseudomonas stutzeri,* seems to be a novel strain producing natural antioxidant.

CONCLUSION

Marine soil bacteria are promising source of various natural active metabolites. In the present study two novel isolates obtained from marine soil viz. *Janibacter melonis* and *Pseudomonas stutzeri* exhibited antioxidant activity. However, further studies are required to elucidate the structure of these antioxidant compounds and also their potential application in pharmaceutical industry.

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