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COMPARATIVE EVALUATION OF *IN VITRO* ANTIOXIDANT POTENT OF THE MARINE ACTINOBACTERIA FROM GULF OF MANNAR BIOSPHERE RESERVE

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ABSTRACT

The present study was carried out to evaluate the *in vitro* antioxidant activity of actinobacteria isolated from the sediments of coral reef environment of the Gulf of Mannar Biosphere Reserve, India. Among 86 strains, five actinobacterial strains (PM15, PM16, PM17, PM18 and PM19) were tested for antioxidant potential, using standard methods. The most active strain PM17 was identified using chemotaxonomical and molecular taxonomical methods. Out of 6 different isolates, PM17 (*Streptomyces sp.*) showed higher antioxidant activity with an ascorbic acid equivalent of 204.16 ±0.408. Total reducing power was 81.66±0.577 µg/ml and scavenging of hydrogen peroxide and nitric oxide 66.29% and 60.30% respectively. Quantitative analysis of the scavenging assays of the strains assured the presence of antioxidant activity of the respective strains. The most active strain PM17 was taxonomically identified as a species of *Streptomyces*.

KEY WORDS: Coral reef, Marine actinobacteria, *Streptomyces* sp. and Antioxidant

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INTRODUCTION

Actinobacteria are gram-positive, free-living, saprophytic bacteria, widely distributed in soil and water. They show marked chemical and morphological diversity but form a distinct evolutionary line of organisms[1]. They are found in various vertically delineated marine habitats, which are spread globally across oceanic realms, separated geographically and influenced by varying geophysical parameters such as temperature, salinity, underlying geochemistry and ocean currents. Regional ecosystems of salt marshes and wetlands, estuaries, continental shelves, open oceans and deep seas are all influencing the actinobacterial distribution[2]. However, only a few reports are available pertaining to the actinobacterial diversity in mangroves, seagrasses and coral reefs [3, 4, 5]. Due to their great metabolic diversity, actinobacteria have vast biotechnological potential for the production of pharmaceuticals and for conversion of waste materials into useful chemicals. In this regard, various antimicrobial substances from actinobacteria have been isolated and characterized including amino glycosides, anthracyclines, glycopeptides, beta-lactams, macrolides, nucleosides, peptides, polyenes, polyester, polyketides, actionomycins and tetracyclines [6]. There is also an increasing interest in studying natural antioxidants from microbes, particularly in scavenging ability of free oxygen radicals in various diseases. These pathological and clinical backgrounds have prompted to investigate novel and potent antioxidant compounds from microorganisms, which are of therapeutic use. Hence, the present study to isolate and identify the actinobacteria from the coral reef environment of Poomarichan Island, Gulf of Mannar Biosphere Reserve, to study the *in vitro* antioxidant activity.

MATERIALS AND METHODS

Sample collection, pretreatment and isolation of actinobacteria

Sediment sample from the coral reef environment of the Poomarichan Island of the Gulf of Mannar Biosphere Reserve, south east coast of India (09°14'53.70N, 79°10'48.31E) was collected with a corer. The collected sample was transferred to a sterile polythene bag and taken immediately to the laboratory. The sample was air-dried aseptically and pretreated by incubating it at 55ºC in a hot air oven for ten minutes. After the pretreatment, the sediment was serially diluted and plated in the Kuster's agar supplemented with nystatin (20mg/l) and cycloheximide (50mg/l) to prevent fungal and gram negative bacterial growth, respectively. After incubation for 1–3 weeks at 30ºC, the actinobacterial colonies that developed on the plates were counted and expressed in colony forming units (CFU). The colonies were picked out and purified before being stored in ISP-2 slants.

Fermentation and extraction of secondary metabolites

Spore suspension of the actinobacterial isolates (PM15, PM16, PM17, PM18 and PM19) was inoculated in the ISP2 medium and incubated for 5 days on a rotary shaker (200 rpm) at 35°C. After fermentation, the cultures were centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was extracted twice with equal volume of ethyl acetate and concentrated under the reduced pressure[7]. The dried extract was dissolved in phosphate buffer (pH 7.4) and stored at 4 ºC until use.

Antioxidant activity of actinobacterial extract

Total antioxidant activity

Total antioxidant activity of the actinobacterial extracts were determined by Ashwini et al. [8]. Total antioxidant activity has been expressed as the number of equivalents of ascorbic acid in microgram per gram of extract.

Total reducing power

Total reducing power of the actinobacterial extracts were determined by the method of Pandimadevi et al., [9]. Ascorbic acid (20-100µg/ml) was used as positive control.

Increased absorbance of the reaction mixture indicated the increasing reducing power.

Scavenging of Hydrogen peroxide

Ability of the actinobacterial extracts to scavenge H_2O_2 was determined according to the method of Vaijanathappa et al., [10] against a blank solution containing phosphate buffer without H_2O_2. The percentage of scavenging of H_2O_2 was calculated as:

$$\text{Scavenging effect (\%)} = \frac{A_{cont} - A_{test}}{A_{cont}} \times 100$$

Scavenging of Nitric oxide (NO)

NO generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which were measured by the Griess reaction [11]. Ascorbic acid (20-100µg/ml) was used as positive control. The NO scavenging activity of the actinobacterial extract has been reported as % inhibition.

Identification of actinobacterial strain

Whole cell sugar and cell wall amino acids of marine actinobacteria were studied using thin layer chromatography[12]. General morphology and cultural characters were determined on ISP2 agar[13]. Spore chain morphology was determined by direct microscopic examination using the 15 days old cultures by observing them under a light microscope[13].

16S rDNA sequence analysis

Genomic DNA was extracted from cultures grown on ISP 2 using the method of Cook and Meyers [14]. Each 50 µl amplification reaction contained 1 µl template DNA (50–200 ng), 5 µl 10x PCR buffer, 1 µl each PCR primer (20 mM) (27F, 1492R), 1 µl dNTP mix (10 mM), 2.5 U Taq DNA polymerase, 5 µl DMSO and 35 µl sterile MilliQ water. The reaction conditions were initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 90 seconds. A final extension was performed at 72 °C for 10 min. Reaction products were electrophoresed on a 1% agarose gel and checked with ethidium bromide under UV light, and then purified and sequenced directly using a Taq Dye Deoxy Terminator Cycle Sequencing Kit and an ABI Prism 3730 automated DNA sequencer (Applied Biosystems). Both strands were sequenced as a cross-check by using forward and reverse sequencing primers. Sequence similarity was made for the 16S rDNA sequence of all isolates by applying their sequence to BLAST search of the NCBI (National Centre for Biotechnological Information, USA) Phylogenetic analysis was performed using the software package MEGA (Molecular Evolutionary Genetics Analysis) version 4 (Tamura et al.)[15] after multiple alignment of data by CLUSTAL_X (Thompson) [16]. A phylogenetic tree was constructed using neighboer-joining method of Saitou and Nei [17] from Knuc values (Kimura) [18]. The topology of the phylogenetic tree was evaluated by using the bootstrap resampling method of Felsenstein[19] with 1000 replicates.

RESULTS

In the present investigation, actinobacterial colonies were enumerated from the sediment samples of coral reef environment in the Poomarichan island. Population density of the actinobacteria in Kuster's medium was 0.5×10^2 CFU/g.

Total antioxidant activity

Fig.1 shows that the total antioxidant activity of the actinobacterial extracts equivalent to ascorbic acid (100µg/ml) at 695nm. Among the five extracts, PM17 showed maximum activity compared to the other strains, with the ascorbic acid equivalent (204.16 ± 0.408 µg) followed by

PM16 (165.83 ± 0.866 µg) and PM18 (146.93 ± 0.759). Total antioxidant activity of the strains PM15 (12.3 ± 0.249 mg ascorbic acid equivalent) and PM19 (6.06 ± 0.249 mg ascorbic acid equivalent) was very negligible. Considering this, PM16, PM17 extracts have shown higher activity.

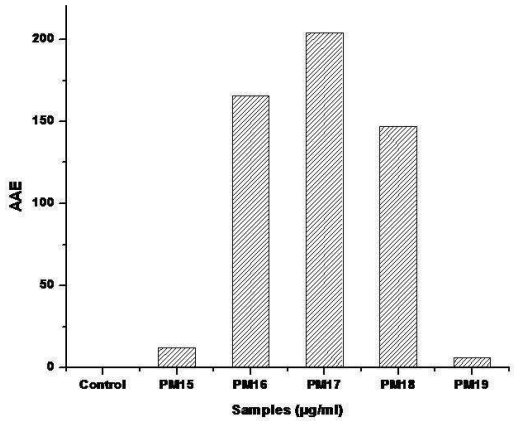

Figure 1
Total antioxidant activity of actinobacterial extracts (100µg/ml). (AAE – Ascorbic Acid Equivalents).

Total reducing power

The reducing capacity of the actinobacterial extracts equivalent to the ascorbic acid was tested at 700 nm (Fig. 2). Among the five extracts, PM17 showed a strong activity of 81.66±0.577µg/ml, equivalent to the standard ascorbic acid whereas, the strain PM16 (38.3±0.60) and PM18 (16.0±0.05) showed moderate activity followed by the least activity of the strain PM15 (7.94±0.26) and PM19 (2.01± 0.076). However, the total reducing power indicates the potential antioxidant ability, PM17 (81.97 ± 0.050 mg AAE) can be taken as a potent source of antioxidant followed by PM16 and PM18.

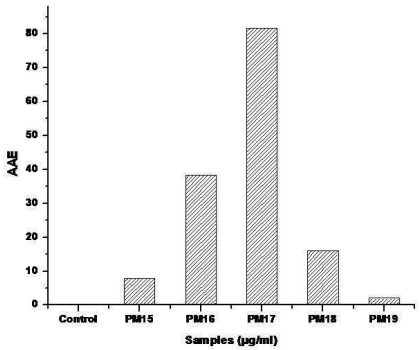

Figure 2
Total reducing ability of actinobacterial extracts (100µl/ml).

Scavenging of hydrogen peroxide

The percentage scavenging activity (Fig. 3) of the five actinobacterial extracts (PM15, PM16, PM17, PM18 and PM19) with the standard ascorbic acid (20-100µg/ml). Among them, PM17 exhibited a

maximum H_20_2 scavenging activity (99.48 ± 0.46147%) followed by PM16 (98.48 ± 0.59%) which are significantly higher than the standard L-ascorbic acid whose scavenging effect is only 61.3%.

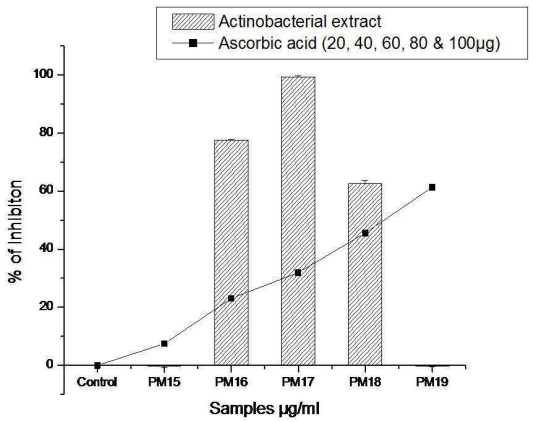

Figure 3
Comparison of percentage scavenging of hydrogen peroxide radical by different actinobacterial extracts (100µg/ml) with standard ascorbic acid

Nitric Oxide Radical (NO) Scavenging Activity

The Extracts of PM15 to PM19 showed varying NO scavenging activity. The phosphate buffer extract of the strain PM17 at 100µg/ml exhibited 60.83 ± 0.496% followed by PM18 (41.70 **±** 1.90%) and PM16 (36.90 **±** 1.6 %), significant decrease in the NO radical due to the scavenging ability of the actinobacterial extracts and ascorbic acid (Fig. 4). These results suggest that PM17 is a potent and novel source of therapeutic agents for scavenging NO and regulating the pathological conditions caused by excessive generation of NO.

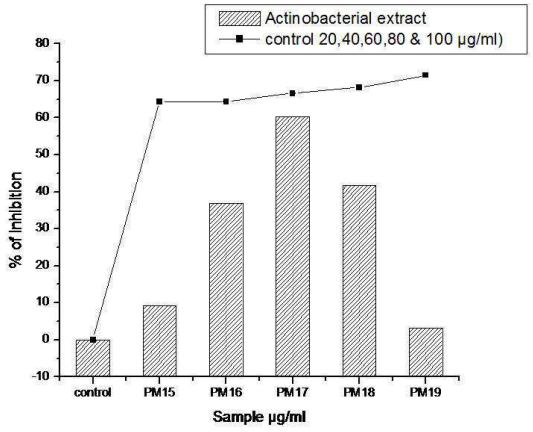

Figure 4
Scavenging effect of actinobacterial extracts (100µg/ml) and standard ascorbic acid on Nitric Oxide radical.

Taxonomic investigation of the most active isolates, PM17

Chemotaxonomical results showed that the cell wall of the strain possesses LL-DAP with glycine, indicating that it belongs to the cell wall chemo type I. Comparison of the 16S rRNA gene sequence of the strain PM17 (1453 bp) with previously obtained sequences of *Streptomyces* species deposited in the GenBank (HQ331481) (Fig. 5) indicated that this organism is phylogenetically related to the members of the genus *Streptomyces*.

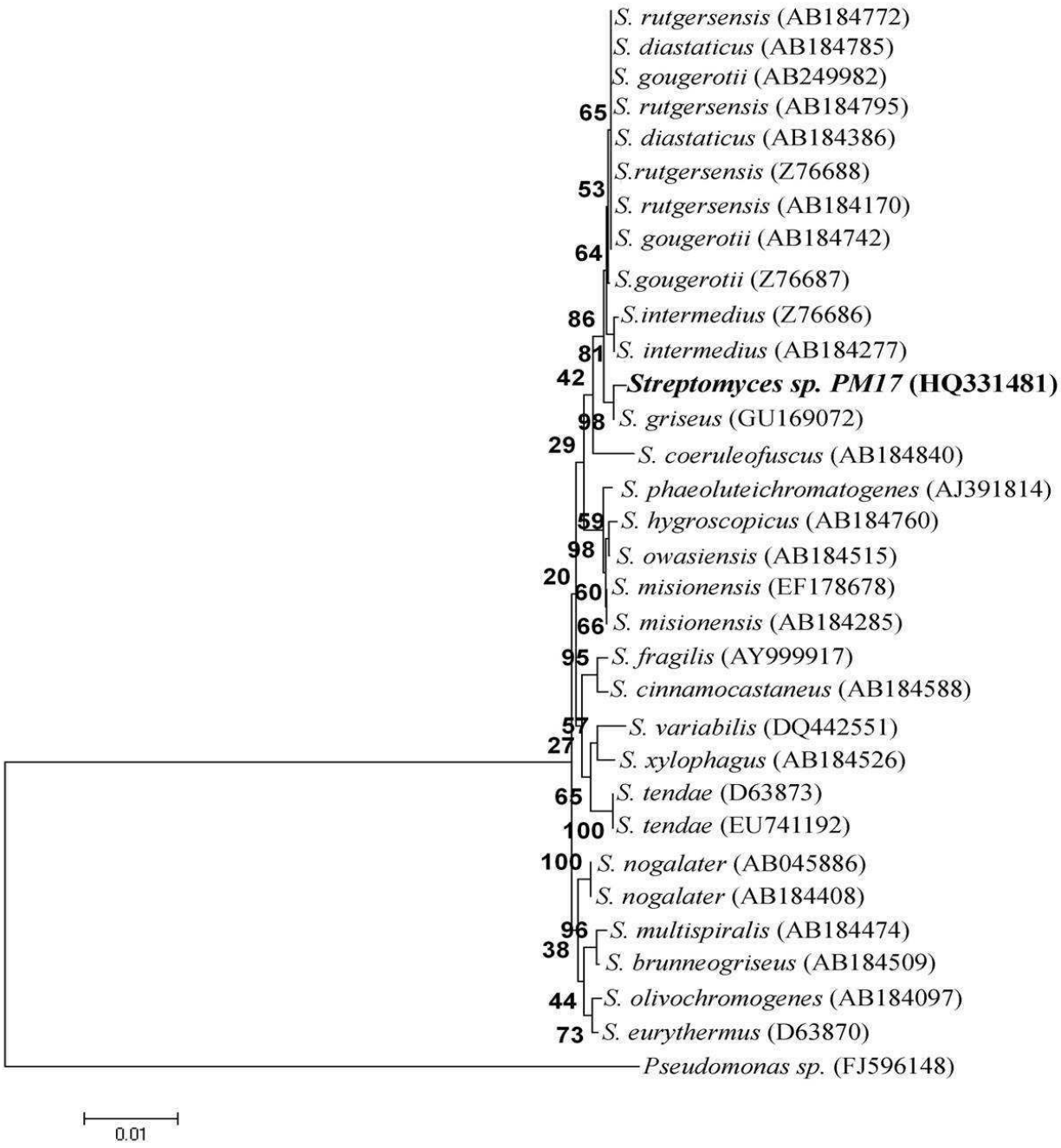

Figure 5

Neighbour – joining tree based on 16Sr DNA sequences, showing the relationship between the strain PM17 and Streptomyces species (Pseudomonas sp. incorporated as out group).

DISCUSSION

Arrays of powerful cellular antioxidants protect cells from excessive oxidation from oxidative damage by inactivating ROS. These pathological and clinical backgrounds have prompted to investigate novel and potent antioxidant compounds from microorganisms which are ultimately of therapeutic use. The secondary metabolites produced by actinobacteria have a broad spectrum of biological activities such as antibacterial, antifungal, antiviral, antiparasitic, immunosuppressive, antitumor, insecticidal, anti-inflammatory, antioxidant, enzyme inhibitory, diabetogenic and others. The compounds Cladoniamides (A–G) are the potent free radical scavengers isolated from *Streptomyces uncialis* [20]. In the present study population density of the actinobacteria in Kuster's medium was 0.5 x 10^2 CFU/g. The obtained result was less when compared to that of the mangrove environment (2.1 to 6.4×10^4 CFU/g) and seagrass environment [3], [4]. Soils were considered as excellent sources for the isolation of actinobacteria with diverse potential [21,22]. Vijayabaskara setubathi[23] studied the actionobacterial density (5×10^3CFU/g) from the coral reef sediments of the Great Nicobar, which was higher when compared to that of the present study.

Total antioxidant activity

Antioxidant activity of the strains PM15 to PM19 was measured in different systems of assay viz. total antioxidant activity, total reducing power, hydrogen peroxide scavenging assay and nitric oxide scavenging assay. Total antioxidant activity was estimated based on the reduction of MO (VI) to MO (V) by the sample and the subsequent formation of a green phosphate /MO (V) complex at acidic pH [24]. However, Chandini *et al*., [25] have reported the total antioxidant activity in the range of 9. 65 to 39.62 mg ascorbic acid equivalent for 1g extract of seaweed. The formation of green colour indicated the total antioxidant activity of the actinobacterial extract. Among the different actinobacterial strain, PM17 showed the maximum activity indicates a good source of antioxidant.

Total reducing power

Reducing ability of the actinobacterial extract depends on the presence of reductons in the extract which exhibit antioxidative potential by breaking the free radical chain by donating a hydrogen atom. This property could be associated with the presence of reductons in the extract, which was reported to be terminators of free radical chain reaction by reducing Fe^{3+} to ferrous ions (Fe^{2+}) more effectively. Similar trend has been reported by Pandimadevi *et al*; Meenakshi *et al.,* [9, 26] in the alcoholic extract of the seaweeds. Present study suggests that the actinobacterial extract of PM17 are acting as potent source of antioxidant compounds.

Scavenging of hydrogen peroxide

Hydrogen peroxide can cross cell membrane rapidly and react with Fe^{2+} and possibly Cu^{2+} to generate extremely reactive oxygen species, including hydroxyl free radical and this may be the origin for many toxic effects[27]. Unlike superoxide, which can be detoxified by superoxide dismutase, the hydroxyl radial cannot be eliminated by the enzymatic reaction[28]. The ability of actinobacterial extract to scavenge H_2O_2 could also reflect its ability to inhibit the formation of hydroxyl radical *in vivo* and it was determined according to the method of Dimitrios [11]. Thus, the present study suggests that the actinobacterial extracts of PM17, PM16 and PM18 can act as better antioxidant agents for removing H_2O_2.

Nitric Oxide Radical (NO) Scavenging Activity

Nitric oxide was an essential bioregulatory molecule required for several physiological processes like regulation of blood pressure, prevention of aggregation and adhesion of platelets, assisting the immune system to kill a

wide variety of pathogens and block viral replication, promotion of certain types of cancer[29]. However, elevation of the NO results in several pathological conditions including cancer. NO generated from the terminal guanido nitrogen atom of L-arginine by various NADPH-dependent enzymes called NO synthases (NOS), and their toxicity multiplies only when they react with O_2 radicals to form peroxynitrite and damage biomolecules like proteins, lipids and nucleic acids[30]. Nitric oxide is generated when sodium nitroprusside reacts with oxygen to form nitrite. Actinobacterial extracts inhibit nitrite formation by competing with O_2 to react with nitric oxide directly. In present study, suggest that PM17 is a potent and novel source of therapeutic agents for scavenging NO and regulating the pathological conditions caused by excessive generation of NO.

Taxonomic investigation of the most active isolate, PM17

The isolate PM 17 produced white aerial mass colour in ISP 2 medium. The substrate mycelium was branched and the spores in the substrate mycelium were formed in chains. The isolate belonged to the wall type – I (Lech). Phylogenetically, the isolate PM 17 exhibited 99.9% similarity to *S. griseus* (FJ596148) and 99.7% similarity to *S.intermedius* (AB184277, Z76686).Micromorphological,chemotaxonomical and molecular studies of the strain PM 17 showed that the isolate is speices of *Streptomyces*.

CONCLUSION

From the present findings, it was conformed that the marine actinobacteria (*Streptomyces sp.* PM17) have strong antioxidant activity, which can be utilized in preventing or slowing down the progress of various oxidative stresses related disorders. The present study clearly demonstrates that isolated *Streptomyces* sp strain PM17 from the coral reef environment possess an antioxidant activity.

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