ANTIOXIDANT POTENTIAL OF EXTRA POLYMERIC SUBSTANCES FROM MARINE ACTINOBACTERIA *Streptomyces* SPECIES

Running Title: EPE potential of Acinetobacteria Species.

ABSTRACT :

Introduction : The Actinobacteria are a phylum of Gram-positive bacteria. They can be terrestrial or aquatic. They are of great economic importance to humans because agriculture and forests depend on their contributions to soil systems. They are more abundant in soils than other media, especially in alkaline soils and soils rich in organic matter, where they constitute an important part of the microbial population.

Material and Methods : The sediment sample was collected from the Gulf of Mannar, Tamilnadu. The collected sample was sun dried for 48 hrs and turned into fine powder by mortar and pestle.The colour of the mature sporulating aerial mycelium was recorded in naked eye.

Results: The antioxidant potential of actinobacterial EPS was determined on the basis of their scavenging activity of the stable 1,1- diphenyl-2-picryl hydrazyl (DPPH) free radical. Different concentrations (0.5-3mg/ml) of samples were mixed with 2.9ml diphenylpicrylhydrazyl (DPPH) solution (120µM) in methanol and incubated in darkness at 37°c for 30 minutes. The absorbance was recorded at 517 nm. Data were implied as mean ± SEM.

Conclusion : The produced melanin pigment from the Actinobacteria of *streptomyces* species was found to have potent antioxidant potential activity. Further characterisation and bio active properties should be done in the further studies, and more articles in future are yet to come in various properties of melanin pigment .

Key words : Actinobacteria, *Streptomyces* species, Antioxidant activity, EPS, Characterization
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INTRODUCTION :

The Actinobacteria are a phylum of Gram-positive bacteria. They can be terrestrial or aquatic. They are of great economic importance to humans because agriculture and forests depend on their contributions to soil systems.hey are more abundant in soils than other media, especially in alkaline soils and soils rich in organic matter, where they constitute an important part of the microbial population. Actinobacteria can be found both on the soil surface and at depths of more than 2 m below ground. They produce a number of enzymes that help degrade organic plant material, lignin, and chitin.Antioxidant activity is defined "as an limitation of the oxidation of proteins, lipids, DNA or other molecules that occurs by blocking the propagation stage in oxidative chain reactions" and primary antioxidants directly scavenge free radicals, while secondary antioxidants indirectly prevent the formation of free (1,2)- (3).

Streptomyces is the largest genus of actinobacteria and the type genus of the family streptomyceae. Over 500 species of *Streptomyces* bacteria have been described.As with the other Actinobacteria, streptomycetes are gram-positive, and have genomes with high GC content. Found predominantly in soil and decaying vegetation, most streptomycetes produce spores.They produce over two-thirds of the clinically useful antibiotics of natural origin Streptomyces is the largest antibiotic-producing genus, producing antibacterial, antifungal, and antiparasitic drugs, and also a wide range of other bioactive compounds, such as immunosuppressants.(4), (5), (6). Almost all of the bioactive compounds produced by Streptomyces are initiated during the time coinciding with the aerial hyphal formation from the substrate mycelium. Microbial EPS are biosynthetic polymers (biopolymers). EPS were defined (GEESEY 1982) as "extracellular polymeric substances of biological origin that participate in the formation of microbial aggregates". Another definition was given in a glossary to the report of the Dahlem Workshop on Structure and Function of Biofilms in Berlin 1988 (CHARACKLIS AND WILDERER 1989). Here, EPS were defined as "organic polymers of microbial origin which in biofilm systems are frequently responsible for binding cells and other particulate materials together (cohesion) and to the substratum (adhesion)" (7), (8) (6,9).

The vast majority of microorganisms live and grow in aggregated forms such as biofilms and flocs ("planktonic biofilms"). This mode of existence is lumped in with the somewhat

inexact but generally accepted expression “biofilm”. The common feature of all these phenomena is that the microorganisms are embedded in a matrix of extracellular polymeric substances (EPS). The production of EPS is a general property of microorganisms in natural environments and has been shown to occur both in prokaryotic (Bacteria, Archaea) and in eukaryotic (algae, fungi) microorganisms (10), (11). Biofilms containing mixed populations of these organisms are ubiquitously distributed in natural soil and aquatic environments, on tissues of plants, animals and man as well as in technical systems such as filters and other porous materials, reservoirs, plumbing systems, pipelines, ship hulls, heat exchangers, separation membranes, etc. EPS are mainly responsible for the structural and functional integrity of biofilms and are considered as the key components that determine the physicochemical and biological properties of biofilms (12), (13). Our team has extensive knowledge and research experience that has translated into high quality publications (14)-(15,16)

The present study focuses on the antioxidant property of extracellular polymerics which is derived from the marine Actinobacteria *streptomyces* species. Previous studies on antioxidants and the other properties had shown that extracellular polymeric has a potent antioxidant and cytotoxic effects. These properties of extracellular polymerics had increased the use of extracellular polymeric substances over industries and all over the country.

MATERIALS AND METHODS:

Antioxidant potential of EPS from marine Actinobacteria *Streptomyces* sp.

Sample collection and preparation

The sediment sample was collected from the Gulf of Mannar, Tamil Nadu. The collected sample was sun dried for 48 hrs and turned into fine powder by mortar and pestle.

Isolation of actinobacteria

Isolation and enumeration of actinobacteria were carried out in Kuster’s agar medium (KUA) supplemented with 0.5% (W/v) NaCl. To minimize the fungal and bacterial contamination, KUA medium was supplemented with cycloheximide (10 µg/ml) and nalidixic acid (10 µg/ml) respectively (Kathiresan *et al.,* 2005). Collected sediment samples were serially diluted and inoculated on KUA medium and incubated at 36°C for 7 days. The colonies were counted and the population density has been expressed as colony forming units per gram (CFU/g) of sediment. Morphologically distinct colonies were selected and pure cultures were obtained.

Identification of actinobacteria

Aerial mass colour: The colour of the mature sporulating aerial mycelium was recorded in naked eye. When the aerial mass colour fell between two colours series, both the colours were recorded. If the aerial mass colour of a strain to be studied showed intermediate tints, then also,

both the colour series were noted. The media used were Yeast Extract-Malt Extract Agar and Inorganic-Salt Starch Agar.

Melanoid pigments: The grouping was made on the production of melanoid pigments (*i.e.* greenish brown, brownish black or distinct brown, pigment modified by other colours) on the medium. The strains were grouped as melanoid pigment produced (+) and not produced (-). In a few cases, the production of melanoid pigments was delayed or weak, and therefore, it was not distinguishable. This is indicated as variable (V). This test was carried out on the media ISP-1 and ISP-7 (Appendix I), as recommended by the International *Streptomyces* Project (Shirling and Gottlieb, 1966).

Reverse side pigments*:* Reverse side pigment production of the isolate was determined on ISP7 medium. The pigment production was noted as distinctive (+) and not distinctive or none (-). In case, a colour with low chroma such as pale yellow, olive or yellowish brown occured, it was included in the latter group (-).

Soluble pigments: Soluble pigment production of isolate was observed on ISP7 medium. The diffusible pigment production other than melanin was considered positive (+) and not produced (-). The colour was recorded (red, orange, green, yellow, blue and violet).

Spore chain morphology: Spore morphological characters of the strains were studied by inoculating a loopful of one week old cultures into solidified agar medium containing sterile glass slide. The cultures were incubated at 28 ± 2^0 C and examined periodically for the formation of aerial mycelium, sporophore structure and spore morphology.

Chemotaxonomical characteristics

Hydrolysis

Hydrolysis was done for releasing amino acids. Harvested cells of each strain weighing 20 mg (fresh) were placed in an ampo bottle and 1 ml of 6 N HCl was added and sealed with alcohol blast burner. The samples were kept at 121^0 C for 20 h in a sand bath. The bottles were cooled by keeping them at a room temperature of 28 ± 2^0C. Hydrolysis was also done for releasing sugars. Harvested cells of each strain weighing 50 mg (fresh) were placed in an ampo bottle and 1 ml of 0.5N HCl was added and sealed with alcohol blast burner. The samples were kept at 110^0 C for 2 h. The bottles were then cooled by keeping them at a room temperature of 28 ± 2^0 C.

Thin Layer Chromatography (TLC): Spotting of the whole cell hydrolysates was made carefully on TLC plate using a microliter pipette. Spots were of 5-10 mm in diameter. This was done by multiple applications on the same spot of very small portions of the sample, which were dried by a hand drier.

Amino acids: Each sample (3 µl) was applied on the baselines of TLC plate (20 cm x 20 cm). Adjacent to this, 1µl of DL-diaminopimelic acid (an authentic material mixture of DAP isomers)

and 1µl of amino acetic acid (glycine) were spotted as standards. TLC plate was developed with the solvent system containing methanol: pyridine: glacial acetic acid: H_2O (5: 0.5: 0.125: 2.5 v/v). It took approximately more than 4 h for development. The spots were visualized by spraying with 0.4% ninhydrin solution in water-saturated n-butanol, followed by heating at 100^0 C for 5 min. Spots of amino acids ran faster than DAP. The sample spots were immediately compared with the spots of the standards since spots gradually disappeared in few hours.

Whole-Cell sugars: On a cellulose TLC plate (20 cm x 20 cm), 5µl of samples was spotted along with 3 µl of sugar solutions as standards on the same plates. Galactose, arabinose, xylose and madurose were the sugars, which were used as standards. TLC plate was developed with the solvent mixture containing ethyl acetate: pyridine: acetic acid: distilled water (8: 5: 1: 1.5 v/v). The development time was more than 4 h. Spots were visualized by spraying with aniline phthalate reagent (3.25 g of phthalic acid dissolved in 2 ml of aniline and made upto 100 ml with water saturated n-butanol). The sprayed plate was heated at 100^0 C for 4 min. Hexoses appeared as yellowish brown spots and pentoses, as maroon coloured spots.

Assimilation of carbon source: The ability of the actinobacterial strain in utilizing various carbon compounds as source of energy was studied, following the method recommended by International Streptomyces Project (Shirling and Gottlieb, 1966). Chemically pure carbon source certified to be free of admixture with other carbohydrates and contaminating materials were used for this purpose. Carbon sources for this test were Arabinose, Xylose, Inositol, Mannitol, Fructose, Rhamnose, Sucrose and Raffinose. These carbon sources were sterilized by ether sterilization without heating. The media and plates were prepared and inoculated according to the convention of ISP project (Shirling and Gottlieb, 1966). For each of the carbon sources, utilization is expressed as positive (+), negative (-), or doubtful (±). In the 'doubtful ' strains, only a trace of growth slightly greater than that of the control was noticed.

EPS Production and quantification: The production of EPS from potential marine actinobacteria was estimated by the method of Sivaperumal et al., 2018. In brief, well grown actinobacteria was inoculated in yeast extract broth at room temperature. After 120hr of inoculation the EPS was separated by centrifugation at 12,000 rpm for 50 min at 4°C. The pellet was suspended with 95% ice cold ethanol and allowed to stand for 24hrs at 4°C. After incubation the EPS precipitate was collected by centrifugation at 15000 rpm for 30 min at 4°C and rinsed thrice with ice cold ethanol. Then the sample was freeze dried and stored for further analysis.

Estimation of EPS components: Total carbohydrate in EPS was estimated by phenol sulfuric acid method with glucose as a standard (Dubois et al., 1956). The protein content was determined by bicinchoninic assay (BCA) with Bovine Serum Albumin (BSA) as a standard (Smith et al., 1985).Nucleic acid content was estimated by the method of Sheng et al., (2005).

Total antioxidant activity: Total antioxidant activity of the crude EPS was determined by following method: 0.3 ml of sample was prepared in different concentrations (0.5 – 3mg/ml)

with 3ml of reagent solution (0.6 M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). Reaction mixture was incubated at 95ºc for 90 minutes in a water bath. Absorbance of all sample mixtures was measured at 695 nm. Total antioxidant activity has been expressed as the number of equivalents of ascorbic acid.

DPPH Assay : The antioxidant potential of actinobacterial EPS was determined on the basis of their scavenging activity of the stable 1,1- diphenyl-2-picryl hydrazyl (DPPH) free radical. Different concentrations (0.5-3mg/ml) of samples were mixed with 2.9ml diphenylpicrylhydrazyl (DPPH) solution (120µM) in methanol and incubated in darkness at 37°c for 30 minutes. The absorbance was recorded at 517 nm. Inhibition of free radical by DPPH in percentage (I %) was calculated with the following equation:

Percentage of Inhibition (I %) = $(A_{blank} - A_{sample}) / A_{blank} \times 100$

Where, A_{blank} is the absorbance of the control reaction and A_{sample} is the absorbance of the test compound. The values of inhibition were calculated for the various concentrations of the sample. Ascorbic acid was used as positive control (Kamala et al., 2015) and all the tests were carried out in triplicate.

Scavenging of nitric oxide: The reaction mixture (3ml) containing 10 mM sodium nitroprusside and the sample (0.5-3mg/ml) in benzene: chloroform was incubated at 25ºc for 150 min. After incubation, 0.5ml of the reaction mixture was mixed with 1ml of sulfanilic acid reagent (0.33% sulfanilic acid in 20% glacial acetic acid) and allowed to stand for 5min for complete diazotization. Then, 1ml of naphthyl ethylenediamine dihydrochloride (0.1%) was added and allowed to stand for 30 min at 25ºC. A pink coloured chromophore was formed and the absorbance was measured at 540 nm against the blank solution. Percentage of scavenging of NO was calculated as stated above.

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

RESULTS :

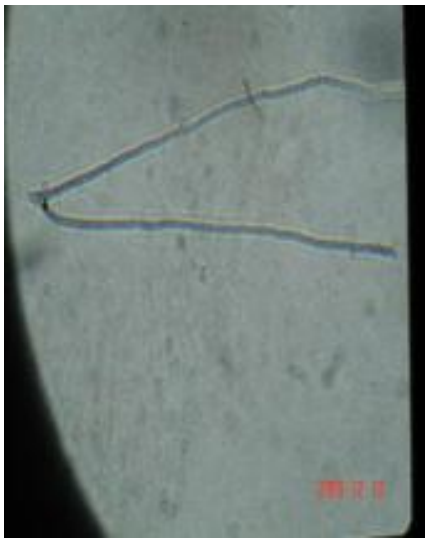

Fig-1 : The figure shows the *Streptomyces* culture and their spore chain morphology

TABLE 1: The table shows the cultural characteristics of Actinobacteria.

Color of aerial mycelium	White
Melanoid pigment	-
Reverse side pigment	-
Soluble pigment	-
Spore chain	RF
Assimilation of carbon source	
Arabinose	+
Xylose	+
Inositol	-
Mannitol	+
Fructose	+
Rhamnose	±
Sucrose	-
Raffinose	±

The colour was found to be white with RF spore chain mosphology. melanoid pigment and the other pigments like soluble pigment and reverse side pigment were absent. Assimilation of carbon source has shown the presence of xylose and Arabinose among all the carbon compounds mentioned above. These results helped in the isolation of *streptomyces* species.

TABLE 2: The table shows the cell wall characteristics of Actinobacteria.

Cell wall amino acids			Cell wall sugar		Cell Wall type	Index
LL-DAP	MesoDAP	Glycine	Arabinose	Galactose		

+	-	+	-	-	I	*Streptomyces* sp.

LL-DAP and Glycine were found and the other cell wall amino acids mentioned above were absent and cell wall sugars i.e., arabinose and galactose were absent. The cell wall belongs to type 1. This shows an index for *streptomyces* species.

TABLE 3: the table shows EPS components obtained from streptomyces sp.

EPS components	%
Carbohydrates	49
protein	27
Nucleic acid	16
Unidentified	8

TABLE 4: The table shows the total antioxidant activity of extracellular polymeric substances that is derived from the Streptomyces species. AAE- Ascorbic acid Equivalent. Ascorbic acid as a standard.

TAA	AAE
25µg/ml	32.71 ±1.217
50 µg/ml	49.63 ±1.302
75µg/ml	61.08 ±0.812
100µg/ml	83.26 ±1.225
125µg/ml	96.34 ±1.314
150µg/ml	114.81 ±1.286

TABLE 5:The table shows the DPPH assay of extracellular polymeric substances that are derived from marine actinobacteria streptomyces species.

DPPH	%	Std
25µg/ml	11.39 ±1.27	37.3 ±1.27
50 µg/ml	34.74 ±1.31	62.7 ±

75µg/ml	42.85 ±1.28	78.52 ±1.28
100µg/ml	56.91 ±0.78	83.59 ±0.78
125µg/ml	65.72 ±1.26	92.4 ± 1.26
150µg/ml	85.76 ±1.24	98.6 ±1.24

TABLE 6: The table shows the nitrogen scavenging assay of EPS obtained from streptomyces sp.

NO	%	Std
25µg/ml	11.37	30.38 ±1.127
50 µg/ml	32.64	51.92 ±1.164
75µg/ml	47.52	70.34 ±1.512
100µg/ml	58.39	82.17 ±1.231
125µg/ml	69.57	90.53 ±1.204
150µg/ml	74.59	95.68 ±1.168

the results showed that the colour was found to be white with RF spore chain mosphology. melanoid pigment and the other pigments like soluble pigment and reverse side pigment were absent. Assimilation of carbon source has shown the presence of xylose and Arabinose among all the carbon compounds mentioned above. These results helped in the isolation of *streptomyces* species. The cell wall characteristics of Actinobacteria are discussed as LL-DAP and Glycine were found and the other cell wall amino acids mentioned above were absent and cell wall sugars i.e., arabinose and galactose were absent. The cell wall belongs to type 1. This shows an index for *streptomyces* species.

DISCUSSION

The filamentous, aerobic, soil-dwelling, gram-positive Streptomyces bacteria have been found residing in soil samples collected from many countries. The traditional practice regarding the isolation of Streptomyces from soil samples, have over the years resulted in the rediscovery of compounds which slowly exhausted the supplies of new compounds. It has been suggested that understudied ecosystems hold Streptomyces species which can meet the growing demand of the drug discovery and development industry (17),(11,18) Researchers

who made an effort to study Streptomyces from less explored ecosystems such as mangrove forests were able to discover novel Streptomyces species and Streptomyces strains showing potent antioxidant activities.Evidence from ear-lier animal studies have noted synthetic antioxidants as potentially unsafe for human consumption, since higher doses and prolonged exposure can induce carcinogenesis.

Nowadays, industries prefer searching for safer and better antioxidant remedies among natural sources by utilizing a variety of antioxidant assays (19), (11,18,20) Antioxidant activity of culture filtrate, lyophilized culture filtrate and ethyl acetate extract of *Streptomyces* species was determined by various *in vitro* assays such as ferric reducing power assay, phosphomolybdenum reduction, DPPH and ABTS radical scavenging activities.

The results revealed that the culture filtrate of *Streptomyces* species effectively scavenged DPP and ABTS radicals in a concentration dependent manner when compared with the IC50 values. (17), (21). Production of protease from the strain of streptomyces was simple and it will be easy to scale up, as this actinobacteria grows on simple media with feathers as a sole source of carbon, nitrogen, and energy, thus allowing its enzyme production from an inexpensive substrate and a commercial potential with low productivity.

CONCLUSION :

The present study revealed that the derived extracellular polymerics from marine actinobacteria streptomyces species were found to have potent antioxidant potential activity(22-31). Further characterisation and bio active properties should be done in the further studies, and more articles in future are yet to come in various properties of extracellular polymeric substances. Streptomyces derived from extreme and understudied ecosystems such as the mangrove forests are potential sources of biologically active and therapeutically useful compounds. (4), (32)

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