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Identification of an Antibacterial Potential Marine *Streptomyces sps* from *Sargassum fluitans*

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ABSTRACT

The discovery of new broad antibiotics is an urgent need to combatfrequently prompting diseases for social welfare. The marine environment posses most diverse environment from which novel secondary metabolites can be derived. The various number of bioactive compound has been identified from marine samples. To address this issue, there is chance for identifying bioactive compounds from marine actinomycetes. The aim of study was to isolate and identify secondary metabolites producing actinomycetes from marine algae (*Sargassumfluitans*.). The isolate SW A4 were partially characterised by morphological and biochemical methods. Their antibacterial activity werefurther analysed by preliminary and Disc diffusion assays. Interestingly, the SW A4 isolate showed excellent inhibition activity aganist certain disease causing human pathogenic bacteria. Here the methanolic extract showed efficient activity of 12,17,13,12 mm zoneaganist S.typhi, K.pneumonia, E.coli, B.cereus respectively.

***Keywords*—** AIA (Actinomycetes Isolation Agar), ISP Medium (International Streptomyces Project), MHA (Muller Hinton Agar).

I. INTRODUCTION

Actinomycetes are aerobic, gram-positive bacteria are very widely distributed on terrestrial and marine environment. It has been estimated that approximately two-third of naturally occurring antibiotics are isolated from actinomycetes. Due to environmental adaptation of marine actinomycetes it produces novel bioactive metabolites having applications in human medicine with pharmacological properties against various infections. As an alternative source of antibiotics, endophytic actinomycetes from algae were used as they utilize the nutrients, sugar moieties, carbohydrate polymers, amino acids and peptides from plants with different concentrations. Pyronone, taxol, camtothecin, phenylproponoid like compounds, Alnumycin, munumbicins A to D, coronamycins and anthraquinones, lupinacidins A and B are some of the natural compounds that were isolated earlier form marine endophytic actinomycetes. Epiphytic symbiotic microbes from marine algae were reported for bio fouling and bio surfactant properties. Unique properties of marine eukaryotes to provide symbiotic habitat for colonizing microbes induces competion between them. Chemically mediated interaction between the host may results microbial diversity due to a daptation. Among these algae, the studies on *Sargassum* revealed the abundance of epiphytic colonies on *Sargassumfluitans* and *Sargassumnatans.* The distribution of epiphytic communities are not same within an individual *Sargassum* floats. Newer portions (younger blade) of algae have few epiphytes and older portions are heavily colonized. It occurredby the release of phenolic compounds from younger blades than older blades, thereby epiphytic colonization became delayed[1]. These adaptations leads to diversities for secondary metabolite productions. The Sargassum isolates were reported for antimicrobial, anticancerous and other agronomic applications[2-5].With the increasing need of drug discovery, marine epibiotic bacteria is an unexplored area.

II. MATERIALS AND METHODS

2.1 Sample Collection and Isolation of Actinomycetes

Fresh seaweed *Sargassumfluitans* were collected from Thirumullavaram Beach, Kollam, Kerala, India using sterile polythene bags in saline condition at sub zerotemperature. They were rinsed by sterile seawater to remove associated sands, microorganisms, salts and other suspended particles. The microbes were isolated by swabbing the surface of the algae with a sterile cotton and spread it on petriplate containing fresh media Actinomycetes Isolation Agar with Antibiotic/Antifungal media(Gibco)50mg/ml(11580486). The plates were incubated for 7-14 days at 28°C[6].

2.2 Phenotypic Characterisation

Characterisation of the SWA4 isolate was carried out according to the method recommended by International Streptomyces Project (ISP).Spore chain and sporphore morphology of mature colony was determined under light microscope by Slide culture technique[7]. Growth of actinomycetesin different temperature and pH were analysed.

2.2.1 Utillization of Different Carbon Sources and other Biochemical Tests

The ability of the test strain on four different carbon sources for energy utilization were examined in carbon utilization media(SRL laboratories)(REF 14517).The inoculated test were incubated to 7-20 days at 28°C and the color change was recorded[8],[9].

2.2.2 Cultural Characterisation of SWA4 Isolate

In generally, physiological and morphological characters of actinomycetes may varies depends upon various nutrient factors. In order to realise, the morphological characters of isolate was cultured on different ISP Medias(ISP 1-ISP7 Media)and AIA Medium.

2.3 Molecular Characterisation and Identification

The 16SrRNA gene of TSA5 strain were amplified by PCR using forward 27F-(5'AGATTTGATCTGGCTCAG3') and reverse 1492R-(5'TACGGYTACCTTGTTACGAT3' Y=C:T) primers (Biogene). The PCR mixture contain 20µl of reaction mixture with 1X PCR Buffer (100mM Tris HCl,pH8.3,500mM KCl), 0.2mM each dNTPs (dATP,dCTP,dGTP and dTTP), 2.5Mm MgCl2,1 unit of AmpliTaq Gold DNA polymerase enzyme,0.1mg/ml BSA,4%DMSO,5 pmol of forward, reverse primers and template DNA. PCR program involved 35 cycles of denaturing at 95°C for 5 minutes, primer annealing at 60°C for 40sec, and extension at 72°C for 60 sec. Sequencing was performed by DNA Sequencer ABI 3730 DNA Analyser (Applied Biosystem, USA), 16SrDNA sequence was searched for similarities with known sequence in Gen Bank database (National Center Biotechnology Information) using BLAST search program.

2.4 Preliminary Assay

Small inoculum of SW A4 isolate was crossly streaked on nutrient agar plate and incubate them for 7 days[10].After seven days, the test organisms were perpendiculary streaked and incubated for 18-24hr at 32°C.

2.5 Extraction and Purification of Active Compounds

The active isolate SW A4 were inoculated to 100ml of Starch casein broth media at 28-30°C in a rotary shaker at (150-200 rpm) for seven days of incubation. After incubation the cell free extract were obtained by centrifugation at 10000 rpm for 10 minutes. The supernatant were filtered and aseptically transferred into a screw capped bottle and stored at 4°C for further assays. The extracts were prepared by equal concentration of solvent and condensed by evaporation. The dried residue were reconstituted in 2ml of respective solvent.

2.6 Antibacterial Disc diffusion Assay

The test organisms used for bioassay are S. typhi, K. pneumonia, E. coli and B. cereus. Freshly prepared MHA media poured in sterile plates were allowed to cool. The respective test organisms spreaded in each plates.20µl of crude extract was loaded on sterile disc(6mm) and placed them to inoculated petriplates. Incubated the plates for 18-24hrs at 32°C in an inoculation chamber[11].

III. RESULTS AND DISCUSSIONS

The discovery of new antibiotics is an urgent need to combat frequently mutating resistant microbes for social welfare. This is an attempt to solve this need by isolating secondary metabolites producing bacteria from marine algae. The algae samples were collected from Thirumullavaram beach region, Kollam, Kerala and are partially characterised by physiological, biochemical and morphological methods. The physiological and biochemical properties of SW A4 isolate was presented on Table 1.The colony grow abundantly on 28°C at the range of pH 7-8.3 and could hydrolyse starch and casein.

Table 1: Physiological and Biochemical Properties of A4 Isolate

Reactions	Response	Results
Starch Hydrolysis	Zone appeared	Positive
Caesin Hydrolysis	Zone appeared	Positive
Ureases	Phenol red to Yellow	Negative
Temperature		
20°C	+	Poor growth
25°C	++	Moderate growth
28°C	+++	Good growth
30°C	++	Moderate growth
50°C	-	No growth

90°C	-	No growth
Ph		
6.0	+	Poor growth
6.5	+	Poor growth
7.0	++	Moderate growth
7.5	+++	Good growth
8.0	++	Moderate growth
8.5	+	Poor growth
9.0	-	No growth

The morphological characters of the isolate were studied by Slide culture technique. The characteristics of spore bearing hyphae and spore chain structure were observed by direct microscopic examinations. The ability of the isolated strain to utilize various carbon energy sources were assayed. The isolate grew better in sucrose and maltose, moderate in lactose and poorly ingalactose (Table 2), Figure 1.The optimum growth of SWA4 isolate was analysed on different ISP Medias. ISP 4 identified as a good media. The morphological characters of the isolate was observed as given in Table 3. The 16r DNA sequencing of SWA4 isolate yielded 1500bp , it was searched through the NCBI database for similarity and the isolate shows similarity towards some Streptomyces sps.

Table 2: Utilization of Carbon Sources

Utilization	Carbon sources
Positive	Sucrose,Maltose
Moderate	Lactose
Poor	Galactose

Figure 1: Carbon Energy Source Utilization of SWA4. (a) Sucrose, (b) Galactose, (c) Lactose, (d) Maltose, (e) Control

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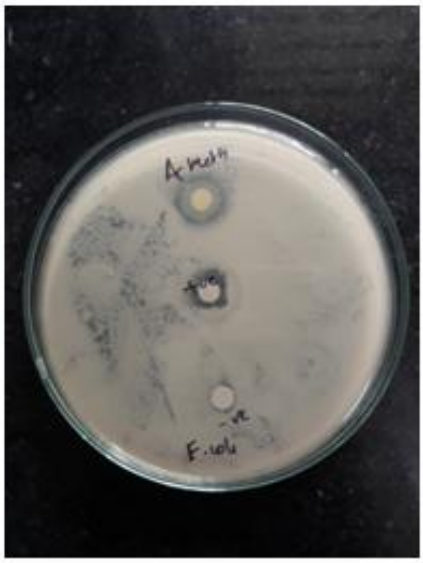
Table 3: Growth of SWA4 Isolate on Different ISP Medium

Medium	Growth	Aerial Mycelium	Substrate Mycelium	Soluble pigment
ISP1(Tryptone-Yeast extract Agar)	Pointed	White powdery	Cream	Nill
ISP2(Yeast Extarct –Malt extract Agar)	Good	White powdery	Yellowish	Yellowish
ISP3(Oat meal Agar)	Faint	White powdery	Cream	Nill
ISP4(Inorganic salts-Starch Agar)	Good	White powdery	White	Nill
ISP5(Glycerol-Asparagine Agar)	Good	White powdery	Yellowish	Yellow
ISP6(Peptone-yeast extract Agar)	Good	White powdery	Bright Yellow	Crimson
ISP7(Tyrosine Agar)	Good	White powdery	Yellow	Nill
AIA(Actinomycetes Isolation Agar)	Good	White powdery	Cream	Nill

The antibacterial efficacy of the isolate was tested by methanolic extract. Methanol extract showed the maximum zone of Inhibition (17mm), Pencillin G provided as positive control(1mg/ml). Various solvent were used for isolation of antibiotics from actinomycetes by many workers[12]. Using methanol was reported to be the best solvent for the extraction of bioactive compounds[13][14][15]. Ethylacetate extract exhibited moderate inhibition against the test pathogens. Correspondingly methanolic extract revealed maximum zone of inhibition, 12,17,13,12mm aganist *S.typhi*, *K. pneumonia*, *E. coli*, and *B. cereus* respectively. It showed that the methanolic crude extract is a strong antagonist for gram negative bacteria Figure 2.

The result of this study indicates that the extract from marine algae are potent antibacterial agent. Actinomycetes are identified as additional genera for the search of antibacterial compounds that can be used in human health care and related applications. More and extended studies for dectecting the molecular identity and purification of active components are undertake in the lab.



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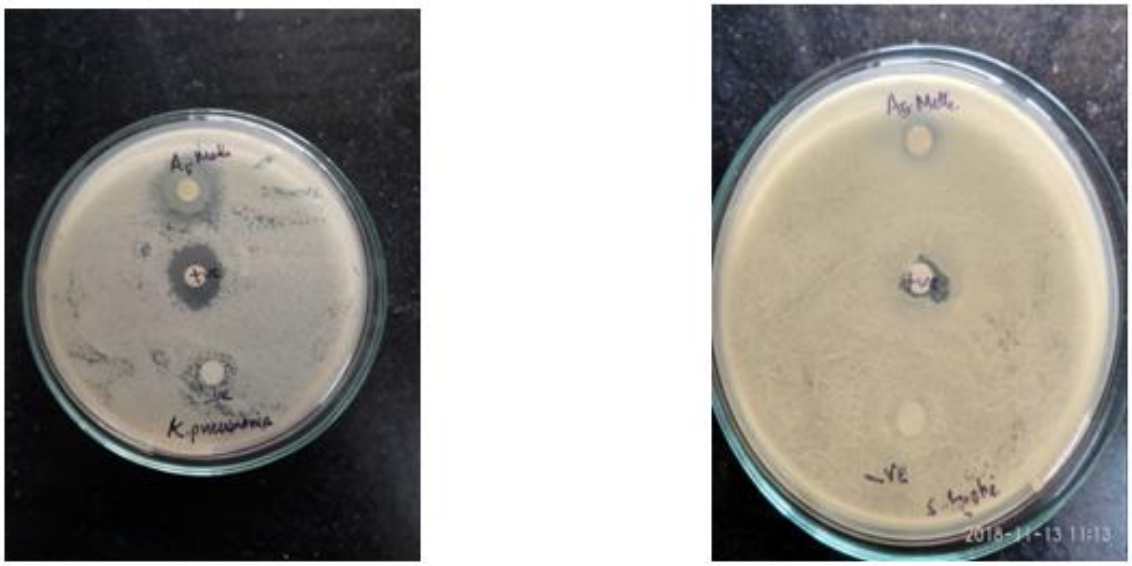

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Figure 2: Antibacterial activity SWA4 methanolic extract on (a) *E.coli*, (b) *B.cereus*, (c) *K.pneumonia*, (d) *S.typhi*.

Table 4: Zone of Inhibition in mm

SW A4 Extract	Zone of Inhibition(mm)			
	S.typhi	*K.pneumonia*	*E.coli*	*B.cereus*
Methanolic extract	12	17	13	12

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