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Antagonistic properties of seagrass associated *Streptomyces* sp. RAUACT-1: A source for anthraquinone rich compound

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ABSTRACT

Objective: To identify the antibacterial potential of seagrass (*Syringodium isoetifolium*) associate microbes against bacterial pathogens. **Methods:** Eumeration of microbial associates were analyzed with leaf and root samples of *Syringodium isoetifolium*. MIC and MBC were calculated for bacterial pathogens with microbial associates. Phylogenetic and GC-MS analysis were calculated for *Actinomycetes* sp. (Act01) which was the most potent. **Results:** Of the isolated microbial associates phosphatase producing bacterial isolates were identified as maximum [(261.78±35.09) CFU×10^4/g] counts in root sample. Of the selected microbial isolates *Actinomycete* sp (Act01) showed broad spectrum of antibacterial activity against antibiotic resistant and fish bacterial pathogens. Phylogenetic analysis of Act01 showed maximum identities (99%) with the *Streptomyces* sp. (GU5500072). The 16s rDNA secondary structure of Act01 showed the free energy values as -366.3 kkal/mol. The GC-MS analysis Act01 showed maximum retention value with 23.742 RT and the corresponding chemical class was identified as 1, 4-dihydroxy-2-(3-hydroxybutyl)-9, 10-anthraquinone 9, 10-anthrac. **Conclusions:** In conclusion, *Streptomyces* sp. (GU045544.1) from *Syringodium isoetifolium* could be used as potential antibacterial agent.

1. Introduction

Infectious diseases remain one of the leading causes of death due to antibiotic resistant microorganisms. The frequency of resistance in microbial pathogens continues to grow at an alarming rate throughout the world[1]. Decreased efficacy and resistance of pathogens to antibiotics has necessitated development of new alternatives[2]. To overcome these problems, the development of effective newer drugs without any side effects is an urgent need. In general, marine plants such as mangroves, seaweeds, sea grasses and marines sponges are extensively studied for antiviral, antiplasmodial, antibacterial, antifungal, hepatoprotective, anti ulcer properties[3,4]. But, the studies related to the associated microbes from marine plants are too limited. In view of this the present investigation was started to identify the antibacterial potential of seagrass associated microbes against bacterial pathogens.

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2. Materials and methods

2.1. Isolation of associated microorganisms

Fresh, young leaves and root samples of *Syringodium isoetifolium* were collected from Thondi coastal area (Lat 9° 44′ N and Long 79° 10′ E), Tamilnadu, India, and the samples were surface sterilized by using 2% sodium hypochlorite (containing 0.1% of Tween 20) and the samples were rinsed 5 times with the sterile distilled water. After that, 1 g of each samples were aseptically weighed and serially diluted with 50% of seawater up to 10^{-6} dilutions. One millilitre of serially diluted sample was plated in various selective mediums such as starch casein agar (HIMEDIA) for actinomycetes, Pikovskaya's agar (HIMEDIA) for phosphate solubilising bacteria, Winogradsky medium (HIMEDIA) for *Azotobacter* sp. and phophatase producing bacteria was isolated by using the ingredients (Peptone 3 g, K_2HPO_4 0.2 g, $MgSO_4$ 0.05 g, $FeCl_3$ 0.001 g, soluble casein 0.5 g, agar 15 g and 1 litter of 50% sea water). After that, all the plates were incubated at (28±3) ℃ to attain the visible growth of microbial cultures. The total number of bacterial counts was expressed as colony forming unit per mL or gram of sample.

2.2. Test organisms

Bacterial pathogens such as antibiotic resistant pathogens *viz*., *Streptococcus pneumoniae (S. pneumoniae), Klebsiella* sp., *Pseudomonas aeruginosa (P. aeruginosa)*, *Streptococcus* sp., and *Streptococcus aureus (S. aureus)* obtained from Vinayaka mission hospital, Salem, Tamilnadu, India, fish pathogens *viz*., *Bacillus subtilis (B. subtilis), Serratia* sp., *Aeromonas hydrophila (A. hydrophila), Vibrio harveyi (V. harveyi)* and *Vibrio parahaemolyticus (V. parahaemolyticus)* obtained from Central Institute of Fish Disease Diagnosis and Control, Cochin, Kerela, India.

2.3. Primary screening (Cross streak assay)

Cross streak assay was carried out with morphologically different bacterial isolates against the bacterial pathogens, in brief, single streak of isolated strain was done on sterile Mueller Hinton agar plate followed by overnight culture of different bacterial pathogens perpendicular to the original streak of the isolate and incubated overnight at (37±2) ℃. Bacterial isolate showed maximum inhibitory activity was selected for secondary screening[3].

2.4. Secondary screening

The most potent *actinomycetes* strain (Act01) was selected for the minimum inhibitory concentration assay (MIC) and minimum bactericidal concentration (MBC) assays. In brief, the actinomycete isolate (Acto1) was mass cultivated by using ISP5 medium and was filtered by using Millipore filter. Filtrate was mixed with equal volume of ethyl acetate (v/v) in separating funnel and shaken well and then allowed to stand without any disturbance for 15 minutes. After that, the lower aqueous phase was discarded and the upper solvent phase was concentrated in a vacuum evaporator at room temperature for 24 h to obtain powder form of crude extract and stored in a refrigerator for further analysis. MIC assay was carried out by using 0.5 mL of various concentrations 512, 256, 128, 64, 32, 16 and 8 μg/mL) of the crude extracts were prepared with dimethyl sulphoxide and mixed with 50 μL of 24 h old bacterial pathogens individually and incubated at 37 ℃ for 48 h individually. To calculate the MIC, turbidity due to bacterial growth was observed in each concentration. To avoid the possibility of misinterpretations due to the turbidity of insoluble compounds, MBC was determined by sub culturing the MIC dilutions on to the sterile agar plates. The lowest concentration of the extracts which inhibits the growth of tested bacteria were observed and tabulated.

2.5. 16S rDNA amplification and sequencing

Genomic DNA was isolated for the most potent actinomycetes isolate (Act01) by using standard method[5] and amplified by PCR with forward primer-F243 (5′-GGATGAGCCCGCGGCCTA-3′) and reverse primer R513GC-(5′-CGGCCGCGGCTGCTGGCACGTA-3′). The reaction mixture contains 25 to 50 ng of DNA, Ex Taq PCR buffer, 1.5 mM $MgCl_2$, 10 mM deoxynucleoside triphosphate mixture, 50 pmol of each primer, and 0.5 U of Ex-Taq polymerase. PCR conditions consisted of an initial denaturation at 94 ℃ for 5 min; 30 cycles at 94 ℃ for 1min, annealing 63 ℃ for 1 min and 72 ℃ for 1 min; and final 5 min extension at 72 ℃. The amplification products were examined by agarose gel electrophoresis and purified by using a QIA quick PCR clean up kit with the protocol suggested by Qiagen Inc. The partial 16Sr DNA was sequenced by using the PCR products directly as sequencing template with above mentioned primers. All sequencing reactions were carried out with an ABI 377 automated DNA sequencer.

2.6. Construction of phylogenetic tree

The retrieved gene sequences were compared with other bacterial sequences by using NCBI BLAST search for their pair wise identities, further, the phylogenetic tree was constructed with MEGA 4.0 software (http://www.megasoftware.net) by using the UPGMA neighbor-joining (NJ) method with 1 000 replicates as bootstrap value and NJ belongs to the distance-matrix method[5]. The 16S rDNA sequence was submitted to the GenBank. The RNA secondary structure of the isolates was predicted by using Genebee online software (http://www.genebee.msu.su/services/rna2_reduced.html).

2.7. GC-MS analysis of Streptomyces sp. RAUACT-1 crude extract

About 10 mg of the most potent *Streptomyces* sp. crude extract was dissolved in 1 mL of ethyl acetate. From that, 0.1 μL was injected in to GC-MS (GC 17A, Japan) with standard specification (column size 0.25 mm× 25 m, Carrier gas-Helium, Column-5% phenyl polysiloxane, flow rate 0.4 m/min, sample injection temperature 25 ℃, acceleration and reflector temperature 10 ℃/min, initial temperature 70 ℃). The maximum percentages of compounds obtained from the extracts were identified by chemical library search (TUTOR.LIB, WILEY139.LIB).

3. Results

The result of the present study suggested that, the maximum [(261.78±35.09) CFU×10^4/g] counts of phosphatase producing bacteria were observed in root samples. However, the minimum counts *Actinomycetes* sp. [(1.00±0.00) CFU×10^4/g] was noticed in the root samples (Table 1). The results of the present study revealed that, the *Actinomycete* sp. (Act01) showed broad spectrum of antibacterial activity against all the tested pathogens and minimum level (32 μg/mL) of MIC value was identified against *K. pneumonia* and *P. aeruginosa* bacterial pathogens. However, the maximum level (256 μg/mL) of MIC value was identified against *B. subtilis* and *S. aureus*. Further, the results of MBC were also varied between 64 μg/mL to 512 μg/mL concentrations (Table 2). The results of the BLAST analysis showed that, the most potent *Actinomycete* sp. (Act01) showed maximum similarity indexes (99%) with the *Streptomyces* sp. and the sequence (1 514 base pairs) was deposited in NCBI data bank (Accession No: JN083780; Name: *Streptomyces* sp. RAUACT-1) (Figure 1). Further, the results of the 16s rDNA phylogenetic analysis showed that, the most potent actinomycete (Act01) isolate was showed maximum identities (99%) with the *Streptomyces* sp.

(GU5500072). The structural prediction of 16s rDNA analysis also carried out with the available sequences of *Streptomyces* sp. from NCBI databank (first 5 best hits from BLAST results) and the results showed that, the minimum (−391.4) free energy with the *Streptomyces* sp. (AM889485.1) and maximum free energy with the *Streptomyces* sp. (GU045544.1) (Figure 2). The GC−MS result of the most potent ethyl acetate extract of *Streptomyces* sp. was showed 49 numbers of peak values with different time intervals, of that the maximum retention time value was identified 23.742RT and the chemical class was identified as 1, 4−dihydroxy−2−(3−hydroxybutyl)−9, 10−anthraquinone 9, 10−anthrac (Figure 3A, 3B and 3C).

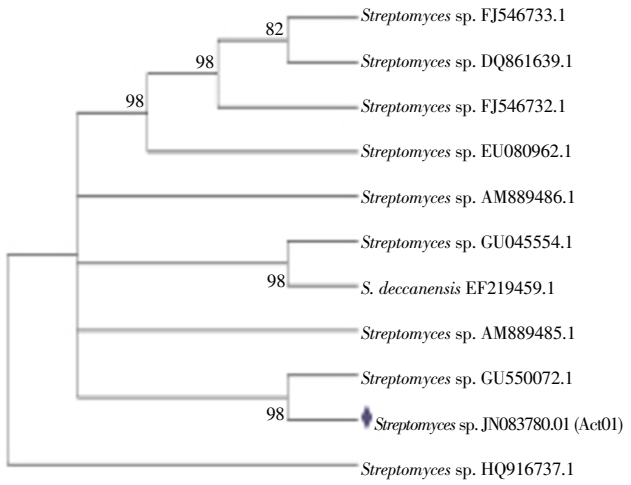


Figure 1. Phylogenetic analysis of *Streptomyces* sp. RAUACT−1 by using Niebuhr joining method with 1 000 replicates.

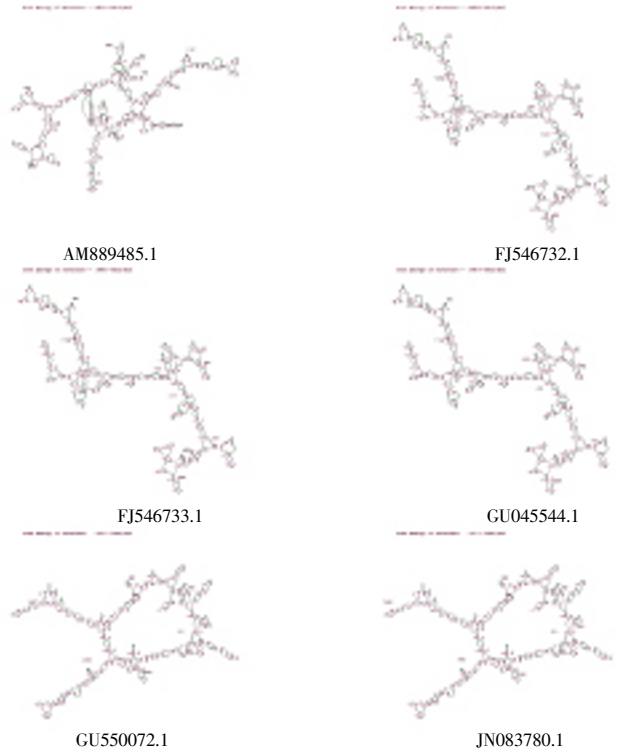
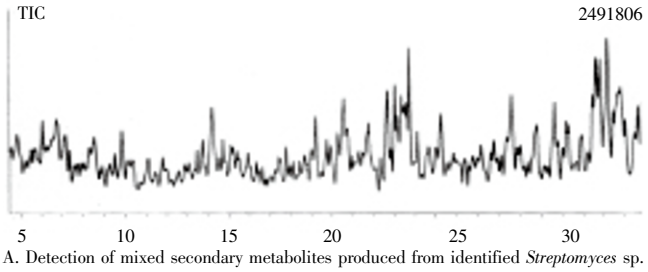
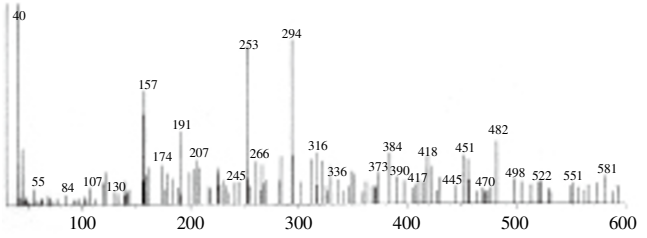


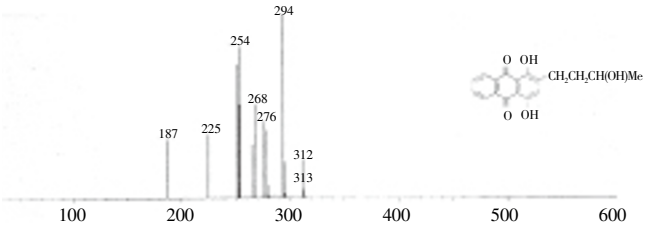
Figure 2. Secondary structure analysis of *Streptomyces* sp. RAUACT−1 with the most similar actinomycete isolates.



A. Detection of mixed secondary metabolites produced from identified *Streptomyces* sp. RAUACT−1.



B. Peak separation at the retention time of 23.742 ; base peak 39.95.



C. Identified chemical constituents [1, 4−dihydroxy−2−(3−hydroxybutyl)−9, 10−Anthraquinone 9, 10−anthrac] present in the crude extract of *Streptomyces* sp. RAUACT−1 (MW 312).

Figure 3. GC−MS result of most potent ethyl acetate extract of *Streptomyces* sp.

Table 1

Counts of different microbial groups associated with *Syringodium isoetifolium* (CFU×10^4/g).

Microbial groups	Number of counts	
	Root	Leaf
Phosphatase producing bacteria	261.78±35.09	189.93±27.09
Phosphate solublizing bacteria	175.28±38.98	120.54±18.92
Azotobacter sp.	26.46±4.09	15.90±5.20
Actinomycetes sp.	3.00±1.00	1.00±0.00

Data represents the mean ±SD values of three replicates.

Table 2

MIC and MBC of the crude extracts obtained from *Syringodium isoetifolium* associated microbes (μ g/mL).

Pathogens		Concentration of Act01 extract	
		MIC	MBC
Fish pathogens	*A. hydrophylla*	128	256
	B. subtilis	256	512
	Serratia sp.	128	256
	V. harveyi	128	128
	V. parahaemolyticus	128	128
Antibiotic resistance pathogens	*S. aureus*	256	512
	K. pneumonia	32	64
	S. epidermis	128	128
	P. aeruginosa	32	64
	S. pneumonia	128	128

4. Discussion

Marie environment is frequently recognized as the largest potential source of biodiversity and it is being increasingly searched for novel chemicals with useful bioactivity[6,7]. In view of this, the present investigation was made an attempt to identify the marine seagrass associated microbes for the possible utilization as antibacterial drugs. The results of the present study suggested that, the maximum counts of colony forming units were identified in the root samples than the leaf samples and this might be due to the quantity and quality of root derived carbon which supports the biomass expansion[8]. As roots grow through the soil, it releases photosynthetically generated carbon in to the soil in a variety of soluble and insoluble form, the totality of which is referred to as rhizodeposition[9]. Of the selected 7 different microbial isolates, the *Actinomycetes* sp. (Act01) showed broad spectrum of antibacterial activity against the selected bacterial pathogens and this might be presence of high content of anthraquinone compounds[10]. Similar, reports are also identified with other researcher findings[11-15]. Further, the results of the present study also suggested the MIC and MBC values between 128 and 512 μg/mL concentrations. Ravikumar *et al*[2] reported the MIC and MBC values between the ranges of 125 to 1 000 μg/mL concentration against different antibiotic resistant pathogens isolated from seagrass endophytes. Moreover, the results of the MBC values were found higher than the MIC values and this might be due to the endospore formation of the bacterial cells which are highly resistant to conditions to which vegetative cells are intolerant[16]. Molecular identification (16s rDNA) of the most potent actinomycete strain was identified as *Streptomyces* sp. In the present study the Act01 strain (JN083780) was proved to have potential antibacterial activities. The most nearest *Streptomyces* sp. (GU550072) from the phylogenetic tree was also proved to have potential antimicrobial activities from Xucheng soil sample (NCBI data bank-unpublished data). Similar reports of phylogenetic analysis was also identified in the marine sediment *Streptomyces* sp.[5]. The secondary structure of the most potent *Actinomycete* sp. (Act01) showed different free energy values, loop structures which might be due to the variations of the GC contents[17]. Hence, the lowest free energy values of the rDNA secondary structures provides the high relationship with the most primitive organisms, and highest free energy indicates the less stability during the evolutionary period[18]. It can be concluded from the present findings that, the actinomycete strain from the root samples of *Syringodium isoetifolium* could be used as potential putative source for the development of antibacterial drugs.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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References

[1] Schmitz FJ, Verhoef J, Fluit AC. Prevalence of resistance to MLS antibiotics in 20 European University hospitals participating in the European SENTRY surveillance program. *J Antimicrob Chemother* 1999; **43**: 783-792.

[2] Ravikumar S, Thajuddin N, Suganthi P, Jacob Inbaneson S, Vinodkumar T. Bioactive potential of seagrass bacteria against human bacterial pathogens. *J Environ Biol* 2010; **31**: 387-389.

[3] Ravikumar S, Ramanathan G, Subakaran M, Jacob Inbaneson S. Antimicrobial compounds from marine halophytes for silkworm disease treatment. *Int J Med Med Sci* 2009; **1**(5): 184-191.

[4] Ravikumar S, Jacob Inbaneson S, Suganthi P, Gnanadesigan M. *In vitro* antiplasmodial activity of ethanolic extracts of mangrove plants from South East coast of India against chloroquine-sensitive *Plasmodium falciparum*. *Parasitol Res* 2011; **108**: 873-878.

[5] Ravikumar S, Fredimoses M, Gnanadesigan M. Anticancer property of sediment actinomycetes against MCF-7 and MDA-MB-231 cell lines. *Asian Pac J Trop Biomed* 2012; **2**(2): 92-96.

[6] Jacob Inbaneson S, Ravikumar S. *In vitro* antiplasmodial activity of marine sponge *Hyattella intestinalis* associated bacteria against *Plasmodium falciparum*. *Asian Pac J Trop Biomed* 2011; **S1**: S100-S104.

[7] Ravikumar S, Jacob Inbaneson S, Suganthi P, Venkatesan M, Ramu A. Mangrove plant source as a lead compounds for the development of new antiplasmodial drugs from South East coast of India. *Parasitol Res* 2011; **108**: 1405-1410.

[8] Ravikumar S, Ramanathan G, Subha N, Jeyaseeli L, Sukumaran M. Quantification of halophilic *Azospirillum* from mangroves. *Indian J Mar Sci* 2002; **31**(2): 157-160.

[9] Barassi CA, Sueldo RJ, Creus CM, Carrozzi LE, Casanovas EM, Pereyra MA. *Azospirillum* spp., a dynamic soil bacterium favorable to vegetable crop production. *Dynamic Soil, Dynamic Plant* 2007; **1**(2): 68-82.

[10] Li S, Tian T, Niu S, Zhang W, Chen Y, Zhang H, et al. Pseudonocardians A - C, new diazaanthraquinone derivatives from a deap-sea actinomycete *Pseudonocardia* sp. SCSIO 01299. *Mar Drugs* 2011; **9**: 1428-1439.

[11] Ravikumar S, Jacob Inbaneson S, Uthiraselvam M, Rajini Priya S, Ramu A, Beula Banerjee M. Diversity of endophytic actinomycetes from Karangadu mangrove ecosystem and its antibacterial potential against bacterial pathogens. *J Phar Res* 2011; **4**(1): 294-296.

[12] Ravikumar S, Syed Ali M, Ajmalkhan M, Dhinakaraj M. Antibacterial activity of *Cymodoceae serulata* root extract against chosen poultry pathogens. *Indian J Sci Technol* 2011; **4**(2): 98-100.

[13] Ravikumar S, Sugnathi P, Fredi Moses M. Crude bioactive compounds of actinomycetes from Manakkudy mangrove sediments. *J Phar Res* 2011; **4**(3): 877-879.

[14] Valan AM, Asha KRT, Duraipandiyan V, Ignacimuthu S, Agastian P. Characterization and phylogenetic analysis of novel polyene type antimicrobial metabolite producing actinomycetes from marine sediments: Bay of Bengal India. *Asian Pac J Trop Biomed* 2012; **2**(10): 803-810.

[15] Ravikumar S, Fredimoses M, Gnanadesigan M. Anticancer property of sediment actinomycetes against MCF-7 and MDA-MB-231 cell lines. *Asian Pac J Trop Biomed* 2012; **2**(2): 92-96.

[16] Laulloo SJ, Bhowon MG, Ravikumar S, Raja M, Gnanadesigan M, Kalaiarasi A, et al. Antibacterial activity of schiff base ligands containing pyridine and disulfide moieties. *J Pharm Res* 2010; **3**(8): 2012-2014.

[17] Peng H, Yang Y, Li X, Qiu G, Liu X, Huang J, et al. Structure analysis of 16S rDNA sequences from strains of *Acidithiobacillus ferrooxidans* school of minerals. *J Biochem Mol Biol* 2005; **39**(2): 178-182.

[18] Somvanshi P, Seth PK. Predicted RNA secondary structures for the conserved regions in dengue virus. *Bioinformation* 2010; **3**(10): 435-439.