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Physiochemical Studies on Bio-active Marine Organism Streptomyces *fradiae*

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Bio-active marine organism Streptomyces.*fradiae* is studied for its various physicochemical parameters by adopting methods like Gram's staining, Acid fast staining, Motility test, Endospore staining, Indole production, Methyl Red and Voges-Proskauer (MR-VP) test, citrate utilization, Urease production, Carbohydrate fermentation, Triple sugar iron (TSI) test, Oxidase test, Catalase test, Nitrate reduction test , Melanin production, Xanthine/hypoxanthine/esculin/arbutin hydrolysis,Gelatin/casein/cellulose/ starch/tween20/tween 80 hydrolysis, Chitin/pectin hydrolysis,Haemoglobin hydrolysis. These studies prove that marine S.*fradiae* showed both positive and negative results for the above physiochemical studies.

Key words: Bio-active organisms, physicochemical parameters, primary screening of culture.

Actinomycetes are known to produce bioactive substances, especially antibiotics that are effective against phytopathogenic fungi[1]. Bio-control with beneficial bacteria is one promising alternative to fungicides[10]. Hydrolases such as chitinase contribute to degradation of fungal cell walls[2]. Chitin is the second most abundant polysaccharide in nature and a major component of fungal walls, insect exoskeletons and crustacean shells. Chitinase secreted by a BCA is likely to be effective against pathogenic fungi, the cell walls of which are mainly Chitin. Several species have been isolated and screened from the soil in the past decades. Marine actinomycetes are efficient producers of new secondary metabolites that show a range of biological activities including antibacterial, antifungal, anticancer, and insecticidal and enzyme inhibition. Bioactive compounds from marine actinomycetes possess distinct chemical structures that may form the basis for synthesis if new drugs that could be used to combat resistant pathogens.[8]To investigate important characteristics of bioactive organisms like Streptomyces.*fradiae*, many beneficial results may be obtained[9]. Hence physiochemical studies are conducted.

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MATERIALS AND METHODS

Various physiological and biochemical tests were carried out according to the methods of Shirling and Gottlieb (1966)[7], methods outlined in the Bergey's Manual of Systematic Bacteriology[3] and in the Laboratory Manual for Identification of Actinomycetes (IMTECH, 1998).

Gram's staining

Solution A	
Crystal violet	2.0 g
Ethanol	20.0 ml
Solution B	
Ammonium oxalate	0.8 g
Distilled water	80.0 ml
Gram's iodine	
Iodine	1.0 g
Potassium iodide	2.0 g

Distilled water	50.0 ml
Safranine	
Safranine	0.25 g
Ethanol	50.0 ml
Distilled water	100.0 ml

To a clean glass slide, a thin smear of *S.fradiae* was made, air-dried and heat fixed. The slide was flooded with crystal violet for a min and rinsed with tap water. Then Gram iodine solution was added, kept for a min and the slide was rinsed with tap water. The smear was decolorized with Gram decolorizer and immediately rinsed with tap water. The slide was then flooded with safranine for a min thin rinsed with tap water, blot dried and observed under a light microscope.

Acid fast staining

A uniform thin smear of *S.fradiae* was prepared on a clean slide, air-dried and heat fixed. The slide was flooded with concentrated carbolfuchsin and steamed at intervals to prevent the stain from drying. It was continued for 3-5 min by constantly adding the stain. Then the slide was washed with water and decolorized with 1% sulphuric acid. The slide was again washed with water and flooded with methylene blue counter stain for 2 min. The slide was then rinsed in water, blot dried and observed under a light microscope.

Motility test

The hanging drop method was used to examine the motility of *S.fradiae*. The cover glass and cavity slide were dipped in alcohol and cleaned properly. At the four corner of cover slip, a thin film of Vaseline was placed and a loopful of log phase culture of *S.fradiae* was placed in the centre of cover slip. The cavity slide was inverted and placed over the cover slip and pressed so that the Vaseline adhered to the slide. The slide was carefully turned upside down so that the drop hangs in the cavity and motility of the isolate was observed using a light microscope.

Endospore staining

Sporulation medium

Peptone	5.0 g
Beef extract	10.0 g
$MnSO_4$	0.005 g
Agar	20.0 g
Seawater	1000.0 ml
pH	7.0
Malachite green reagent	
Malachite green	5.0 g
Distilled water	100.0 ml
Safranine solution	
Safranine	0.25 g
Ethanol	10.0 ml
Distilled water	100.0 ml

S.fradiae was grown on the above sporulation medium for 7 days. Then a loopful of the sporulated culture was taken, a uniform smear was prepared on a clean slide and heat fixed. The slide was flooded with malachite green reagent and steamed intermittently for 5 min. The slide was then washed under running tap water, counter stained with safranine and observed under a light microscope.

Indole production

Indole broth

Peptone	20.0 g
Sodium chloride	5.0 g
Seawater	1000.0 ml
pH	7.0
Kovac's reagent	
p-dimethlyaminobenzaldehyde	5.0 g
Isoamyl alcohol	75.0 ml
Con. HCl	25.0 ml

A loopful of *S.fradiae* culture was used to inoculate the indole broth and incubated at room temperature for 7 days. The production of indole derivatives by *S.fradiae* was determined by the addition of Kovac's reagent. The formation of a red colored ring in the inoculated indole broth tubes indicated a positive reaction

Methyl Red and Voges-Proskauer (MR-VP) test

MR-VP medium

Peptone	7.0 g
Dextrose	5.0 g
K_2HPO_4	5.0 g
Seawater	1000.0 ml
pH	7.0
Methyl Red reagent	
Methyl red	1.0 g
Ethanol	300.0 ml
Distilled water	200.0 ml
VogesProskauer (VP test)	
Barrits A reagent	
a-naphthol	5.0 g
Ethanol	95.0 ml
Barrits B reagent	
KOH	40.0 g
Creatine	0.3 g
Distilled water	100.0 ml

S.fradiae was inoculated in the tube containing 5 ml of MR-VP medium and incubated for 7 days at room temperature. The volume of culture in the tube was divided equally between 2 tubes. To one of the tubes, few drops of methyl red reagent was added and to the other, Barrits A and Barrits B (for VP) were added in the ratio of 6:1 The appearance of red color is due to the addition of MR reagent was a positive reaction and indicated the production of acids by *S.fradiae*. Development of Crimson's red to ruby pink is due to the addition of VP reagent indicated a positive reaction for VP test.

Citrate utilization

Simmons citrate agar

NH_4HPO_4	1.0 g
K_2HPO_4	1.0 g
NaCl	5.0 g
$MgSO_4.7H_2O$	0.2 g
Bromothymol blue	0.08 g
Sodium citrate	2.0 g
Agar	18.0 g
Seawater	1000.0 ml
pH	6.8

S.fradiae was streaked on Simmon's citrate agar in slants, incubated at room temperature for 48 h and the change in medium colour was observed. The color change from green to Persian blue indicated the ability of *S.fradiae* to utilize the citrate as carbon source.

Urease production

Christensen's urea agar

Peptone	1.0 g
Glucose	1.0 g
NaCl	5.0 g
K_2HPO_4	2.0 g
Phenol red	0.12 g
Agar	18.0 g
Seawater	1000.0 ml
pH	6.8

The production of urease by *S.fradiae* on Christensen's urea agar medium was tested. Urea solution of 40% was prepared in distilled water and sterilized. About 5.0 ml of the aliquot of urea solution was added to 100 ml of molten medium and poured into test tubes. *S.fradiae* culture was streaked on to slants, incubated at room temperature for 48 h and the changes in medium colour were observed. The change of medium color from orange to deep pink indicated the production of urease.

Carbohydrate fermentation

Peptone water

Peptone	10.0
NaCl	10.0
Phenol red	0.08
Seawater	1000.0
pH	7.2

Durham's tubes were carefully introduced into test tubes containing 5 ml of peptone water and sterilized at 121°C for 20 min at 15 psi. To peptone water, filter sterilized 1% carbohydrate solutions *viz*., glucose, maltose, sucrose, glycerol, lactose, sorbitol, galactose, fructose, mannitol, and starch were added separately. The tubes were inoculated with *S.fradiae*, incubated for 96 h at room temperature and observed for bubble formation. The presence of air bubble within the inverted Durham's tube indicated the gas production by *S.fradiae* and change in colour of the medium from red to yellow indicated acid production.

Triple sugar iron (TSI) test

The TSI agar (Himedia, Mumbai, India) was prepared as per the instructions of the manufacturer and sterilized by autoclaving at 121°C for 20 min at 15 psi. *S.fradiae* was inoculated in the TSI slants by stabbing the butt and streaking in the slants. The tubes were incubated at room temperature for 5 days and examined for acid, gas and H_2S production. Acid butt, alkaline slant (yellow butt, red slant) indicated the glucose fermentation. Alkaline butt, acid slant (red butt, yellow slant) indicated that no fermentation occurred. Acid butt, acid slant (yellow butt, yellow slant) indicated lactose or sucrose fermentation. Blackening of the butt indicated H_2S production and appearance of bubble in the butt indicated gas production.

Oxidase test

A loop full of *S.fradiae* culture was rubbed over the oxidase disc (Himedia, Mumbai, India) with the help of a clean glass rod. Then the color change in the oxidase disc were observed. The color change to blue or purple of the oxidase disc within 5-10 sec indicated the production of oxidase.

Catalase test

A loop full *S.fradiae* culture was transferred from agar plate to a clean glass slide. Immediately a drop of 3% hydrogen peroxide was

added onto the culture and rapid evolution of air bubbles were observed which indicated the production of catalase by the organism.

Nitrate reduction test

Tryptone nitrate broth

Tryptone	20.0 g
Dextrose	1.0 g
KNO_3	1.0 g
Agar	1.0 g
Seawater	1000.0 ml
pH	7.2
Solution A	
Sulphanilic acid	0.008 g
5 N Acetic acid	10.0 ml
Solution B	
a-naphthylamine	0.005 g
5 N Acetic acid	10.0 ml

S.fradiae was grown in tryptone nitrate broth for 5 days, to which, 0.5 ml of sulphanilic acid and a-naphthylamine reagent were added and the development of red color was observed which indicated the ability of organism to reduce the nitrate.

Melanin production

Nutrient agar	100.0 ml
Tyrosine	0.5 g

The above medium was prepared and poured into Petriplates. A loopful of *S.fradiae* culture was streaked on the surface of medium and incubated at room temperature. After 14 days, the plates were observed for production of brown colored melanin.

Xanthine/hypoxanthine/esculin/arbutin hydrolysis

Nutrient agar	100.0 ml
Xanthine/ hypoxanthine/esculin/ arbutin	0.5 g

The above medium was poured into Petriplates, a loopful of *S.fradiae* culture was streaked on the surface of medium and incubated at room temperature. After 14 days, the plates were observed for hydrolysis of different substrates.

Gelatin/casein/cellulose/starch/tween20/tween 80 hydrolysis

Peptone	10.0 g
Beef extract	10.0 g
NaCl	5.0 g
Gelatin/ casein/ cellulose/ starch/ tween20/ tween 80	10.0 g
Agar	18.0 g
Seawater	1000.0 ml
pH	7.0

The above medium was poured into Petriplates, a loopful of *S.fradiae* culture was streaked and incubated for 7 days at room temperature. The indicator solutions and the description were mentioned earlier for gelatin/ casein/cellulose/starch/tween20/tween 80 hydrolysis**.**

Chitin/pectin hydrolysis

Peptone	10.0 g
Beef extract	10.0 g
NaCl	5.0 g
Colloidal chitin/ pectin	30.0 g
Agar	18.0 g
Seawater	1000.0 ml
pH	7.0

A loopful of *S.fradiae* was streaked on to the medium and incubated for 7 days at room temperature. The plate was flooded with methyl red (0.5%) solution for 3-5 min and observed. The clear zone around the colony against reddish white background indicated the hydrolysis of chitin/ pectin.

Haemoglobin hydrolysis

Peptone	10.0 g
Beef extract	10.0 g
NaCl	5.0 g
Blood	5.0 ml
Agar	18.0 g
Seawater	1000.0 ml
pH	7.0

A loopful of *S.fradiae* was streaked on to the above medium and incubated for 5 days at room temperature. The clear zone formed around the colony confirmed the haemolysis.

S rRNA sequencing

Genomic DNA isolation and PCR analysis

Genomic DNA was extracted from overnight grown cultures of the selected bacterial isolates using QIAGEN DNA isolation kit [5]suspended in 100µl of elution buffer (10mM/L Tris-HCl, pH 8.5) and quantified by measuring OD at 260nm. PCR amplification was performed using a 20µl reaction mixture containing 100ng of template DNA, 20µmol of 16S rRNA primers, 200µM of dNTPs, 1.5mM of $MgCl_2$, 1U of *Taq*DNA polymerase (MBI Fermentas) and 2µL of 10x *Taq*polymerase buffer. The sequences of 16S rRNA primers used were as follows.

FP 5'-AGAGTTTGATCCTGGCTCAG-3'

RP 5' -ACGGCTACCTTGTTACGACTT -3'

Amplification was carried out with an initial denaturation at 95°C for 5min followed by 35 cycles of denaturation at 94°C for 45sec, annealing at 56°C for 45sec, extension at 72°C for 1min and final extension at 72°C for 5min using a thermo cycler .PCR products were analyzed on 1% agarose gel for 16S rRNAamplicons in 1x TBE buffer at 100V.

Cloning and sequence analysis of PCR products

The 16S rRNA amplified fragments were purified using the QIA quick gel extraction kit (Qiagen, Valencia, CA) from the agarose gel and sequenced using automated DNA sequencer (Model 3100, Applied Bio systems, USA). The sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) software (http://www.ncbi.nlm.nih.gov/blast) against the 16S ribosomal RNA sequence database and submitted in GenBank.

Phylogenetic analysis

The sequences of these 16S rRNA genes were compared against the sequences available from GenBank using the BLASTN program and were aligned using CLUSTAL W software. Distances were calculated according to Kimura's two-parameter correction. Phylogenetic trees were constructed using the neighbor-joining method. Bootstrap analysis was done based on 1000 replications. The MEGA5 package [5] was used for all analyses.

RESULTS AND DISCUSSION

Results of the various physiological and biochemical tests carried out as outlined in the Bergey's Manual of Systematic Bacteriology[6] and the Laboratory Manual for Identification of

Table 1. Physiochemical characteristics of S. *fradiae*

S.No	Physiochemical test	Result
1.	Gram's reaction	+
2.	Motility	-
3.	Acid- fast	-
4.	Endospore	-
5.	Aerial mycelium	+(pink)
6.	Substrate mycelium	+(Brown)
7.	Melanin production	+
8.	Diffusible pigment	-
9.	Spores	Chain and coil
10.	Indole	-
11.	Methyl Red(MR)	-
12.	Vogesproskauer(VP)	-
13.	Nitrate Reduction	+
14.	Triple sugar Iron(TSI)	H_2S, Gas, Alkaline butt, AlkalinSlant
15.	Urease	+
16.	Catalase	+
17.	Oxidase	+
18.	Gelatin liquefaction	+
19.	Citrate	-
20.	Blood hydrolysis	+
21.	Tyrosine	+
22.	Xanthine	-
23.	Hyphoxanthine	+
24.	Esculin	-
25.	Arubtin	-
26.	Tween 20/80	+
27.	Phenol (1.5%)	-
28.	Crystal violet (0.05%)	-
29.	Sodium azide (0.001%)	-
30.	Lysozyme (0.005%)	ND

Actinomycetes (IMTECH, 1998) are presented here under. The marine *S.fradiae* P-311 is a Gram positive, non-acid fast, non-motile and non-endospore-forming organism. Results of biochemical tests showed positive reaction for nitrate reduction, urease, H_2S, TSI (alkaline butt and alkaline slant with gas formation), oxidase, catalase, blood hydrolysis, hypoxanthine, tween 20/80 and melanin pigmentation on tyrosine agar. *S.fradiae* P-311effectively liquefied the gelatin. However, it showed negative reactions for indole, MR, VP and citrate (Table. 1).

Moreover, the isolate marine *S.fradiae* P-311 grew well between 25 and 37°C at pH range of 7.0 - 9.0. It is tolerant to NaCl up to 14% concentration and is sensitive to phenol (1.5%), crystal violet (0.05%) and sodium azide (0.001%). However, its growth was not determined in lysozyme at 0.005% concentration (Table. 1).

16S rRNA sequence:

The 16S rRNA sequence of *S.fradiae* P-311 was generated for a total of 1384 nucleotide base pairs and submitted to the GenBank data base. This sequence was compared with 7 different reference species of *Streptomyces* and 2 different species of *Pseudomonas* available in the GenBank database. The *S.fradiae* P-311 showed closest match of 99% similarity with *S.fradiae* NR043485. The phylogenetic tree constructed by neighbor joining analysis is presented in Fig.1

Streptomyces *fradiae*

TGCAAGTCGAACGATGAACCCGCTTCGGTG
GGGGATTAGTGGCGAACGGGTGAGTAACAC
GTGGGCAATCTGCCCTGCACTCTGGGA
CAAGCCCTGGAAACGGGGTCTAATACCGGAT
ACGACCACTTCAGGCATCTGATGGTGGTG
GAAAGCTCCGGCGGTGCAGGATGAGCCCGC
GGCCTATCAGCTAGTTGGTGA GGTAACGG
CTCACCAAGGCGACGACGGG TAGCCGGC
CTGAGAGGGCGACCG GCCACACTGGG
ACTGAGACACGG CCCCGTAGGC GGCTT
GTCACGTC GGTTGTGAAAG CCCGGGGCTTA
ACCCCGGGTCTGCAGTCACGC CGTAAAC
GTTGGGAACTAGGTG TGGGCGAC ATTCCA
CGACGTC CGTGCCG CAGCT AACGCAT
TAAGTTCC CCGCCTGG GGAGTA CGGCCGCA
AGGCTAAAACTC AAAGGA ATTGACGGGGGC
CCGCACAAGCGGCGGAGCATGTGG
CTTAATTC GACGCAACG

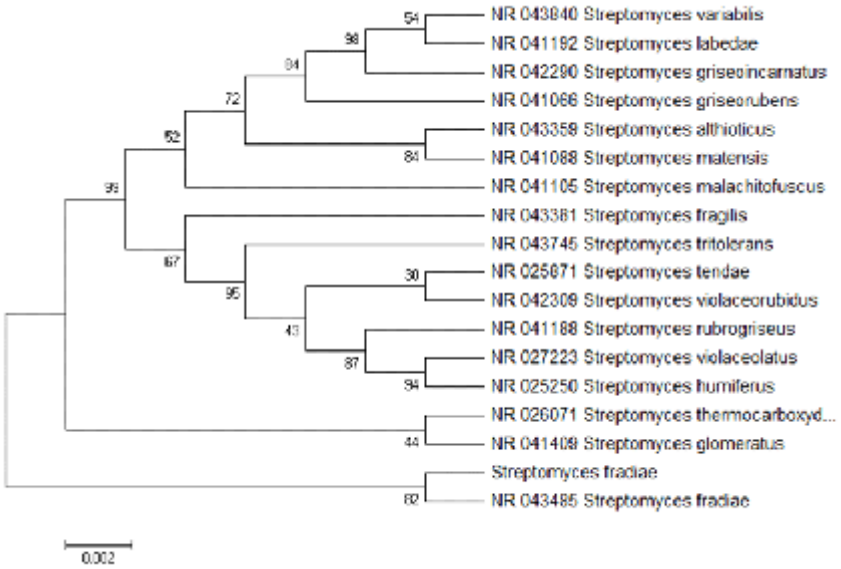

Fig. 1. Phylogenetic tree

REFERENCES

1. Crawford DL , Lynch, JM, Whipps JM, Ousley MA., Isolation and characterization of actinomycycete antagonistis of a fungal root pathogen. *Applied and environmental microbiology* 1993; **59**: 3899- 3905. Crawford, D.L., US patent 55275226,1995.

2. Karlsson, A., Saravia-Otten, P., Tegmark, K., Morfeldt, E. and Arvidson, S., Decreased

Amounts of Cell Wall-Associated Protein A and Fibronectin-Binding Proteins in Staphylococcus aureus sarAMutants due to Up-Regulation of Extracellular Proteases", *Infection and immunity.* 2001; **69**(8): 4742-4748.

3. Williams, S.T., Lanning, S. & Wellington, E.M.H. Ecology of actinomycetes In The Biology of the Actinomycetes, eds. Goodfellow, M., Mordarski, M. & Williams, S.T. 1984; 481-528. London: Academic Press, Inc. ISBN 0-12-289670-x.
4. Qianqun G, Jia L, Chengbin C, Tianjiao Z, Yuchun F, Hongbing L, Weiming Z., Recent researches of bioactive metabolites in marine organisms—associated microorganisms. *J Ocean univ China* 2004; **3**(2):150-156. DIO:10.1007/S 11802-004-0026-7
5. Lem, P., spiegelan, J., Toye,B. and Ramotar , K. "Direct Detection of mecA, nuc and 16s RNA genes in BacT/Alert blood culture bottles", Diagnostic microbiology and infectious Disease. 2001; **41**: 165-168.
6. Williams ST, Sharpe ME and Holt JG., Bergey's manual of systematic bacteriology, 1989; 4
7. Shilling E.B. and Gottlieb, D., Method for characterization of streptomycetes species. *Int J. Ssyst. Bacteriol.*, 1996; **16**: 313 – 340
8. Bernan VS, Montenegro DA, Maiese WM, Steinberg DA & Green stein M., Bioxalomycine, new antibiotics produced by the marine streptomycens sp. LL-31F508: taxonomy and fermentation. *J Antibiot* 1994; **47**:1417-1424
9. Hedge VR, Puar MS. Dai P, Pu H, Patel M, anthcs JC, Richard C, Terracciano J.Das PR, Gullo V., A family of depsi-peptide fungal metabolite, as selective and competitive human tachykinin receptor (NK2) antagonists: fermentation, isolation and physic-chemical properties, and biological activity. *J antibiot* 2001; **54**:125-135.
10. Bressan W., Biological control of maize seed pathogenic fungi by use of actionomycetes. *Biocontrol* 2003; **48**:233-240.