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Exploration of Culturable Marine *Streptomyces* from Southern Coastal Region of India with Antibacterial Properties

Deepika Thandayuthapani[1], Nivetha Chinnappa[1], Arjunan Annavi[1] and Muthusevam Manickam[1*]

[1]Department of Biotechnology, Bharathidasan University, Tiruchirappalli-620020, Tamil Nadu, India.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The Southern coastal region is a geographically positioned area with rich source of microbial diversity, of which producing broad spectrum of bioactive compounds. The marine sediments are collected from the various sites of south east coastal region of Tamil nadu and are processed. Out of 78 isolates, the 4 strains of actinomycetes are showing broad spectrum activity against *Klebsiella pneumoniae* (MTCC 1687), *Proteus vulgaris* (MTCC 3160), *Salmonella typhi* (MTCC 3231), *Shigella dysenteriae* (MTCC 3642) and *Vibrio cholerae* (MTCC 3906). The bioactive isolates are proceeded for further morphological features like growth pattern and mycelial coloration, biochemical and polyphasic taxonomical characterisation were documented. Isolates are investigated for abiotic stress condition to study the growth rate of isolates. Finally, the 16S rRNA molecular identification and phylogenetic analysis of the isolates were explored. Further, bioactive isolates may be potential source for discovery of molecules with industrial applications.

Keywords: Chemotaxonomy; phylogenetic analysis; International Streptomyces Project (ISP); MH agar.

**Corresponding author: E-mail: muthuphd@gmail.com;*

1. INTRODUCTION

The advent of new antibiotics are on rise due to the rapid spread of antibiotic resistant pathogenic organism causing life threatening infections and side effects as most of the current drugs are chemically synthesized [1]. Thus, the drug discovery from natural sources are immense important to overcome the aforementioned problems [2].

Marine environment is the largest resource for taxonomically diverse bacterial groups with potential and novel secondary metabolites, which are unique in nature as they are extremities (Cross, 1981); [3]. The unique properties and compounds that are observed in marine environment microbes are not exhibited in the terrestrial sources [4,5]. So, the research has been focused on the screening of marine sediment derived microorganisms [6], Numerous of antibiotics are isolated from marine source, of which one third of drug are isolated from Actinomycetes [7].

The organism has shown to produce secondary metabolites with novel properties, which can be potentially used as such as antimicrobial, anticancer, and anti inflammatory agents.

Though isolation of marine actinomycetes and cultivating in the laboratory is a complex process, optimizing the culture conditions will provide us efficient source and ample scope for the investigation of the biologically active metabolites. *Streptomyces* are the most valuable soil inhabiting Gram positive bacteria, with vast source of novel biologically active compounds with unique structure and properties [8,9,10,11]. The new techniques are arrived for isolation, cultivation and screening to increase the diversity of cultivable isolates. Various pre-treatment was employed to enrich the isolation of actinomycetes from other genera. Air dry treatment of soil is a universal method to increase the number of mycelium forming actinomycetes sampling sites, sampling strategy. Geographic and habitat plays are essential role in the isolation of Actinomycetes (Zhang et al, 2006).

2. MATERIALS AND METHODS

2.1 Sampling and Processing

Marine sediment was collected from four different sites of Dhanuskodi, Rameswaram, Pamban, and Mandapam of Tamil nadu, India. The samples were collected at maximum depth of 40m. Each 500 g of soil samples were collected in a clean sterile zip lock polythene bag, stored at 4^0C, and transferred to the laboratory under sterile condition for further analysis. Different pre-treatment methods such as mechanical, physical and chemical are generally used for the selective isolation of Actinomycetes. In this study, samples were air dried for 15-20 days to inhibit the growth of other genera and stimulate the growth of actinomycetes. Then the sediments were passed through a 2 mm sieve to remove gravel and debris.

2.2 Physico - Chemical Analysis of Soil

2.2.1 Temperature, pH and Electrical conductivity

The air dried soil sample 10 g was dissolved in 100 mL of distilled water after noting the temperature. A suspension of 1:10 weight/volume dilution was made and the pH and electrical conductivity were noted. Quantification of Carbon, Nitrogen source [12], Phosphorous, Potassium, Calcium and Sodium (Murugesan and Rajakumari, 2005) were also determined.

2.2.2 Isolation of actinomycetes

One gram of air-dried sample was added to 9 mL of distilled water in a sterile test tube and shaken vigorously, and the sample was further diluted to 10^6 using sterile distilled water. A volume of 100 µL of diluted sample was inoculated by spreading with sterile glass rod in Starch Casein Nitrate Agar medium (Starch -10 g/L, Casien - 0.3 g/L, K_2HPO_4 - 2 g/L, KNO_3 - 2 g/L, Nacl - 2 g/L, $MgSo_4.7H_2O$ - 0.05 g/L, $CaCO_3$ - 0.02 g/L, $FeSo_4.7H_2O$ - 0.01 g/L, Agar -15 g/L). Further, the medium was supplemented with nystatin 30 µg/mL and nalidixic acid at 10 µg/mL to inhibit the fungal and bacterial contamination. The inoculated plates were incubated at 30°C for 10 days (Mincer et al, 2002). Individual colonies were streaked on International Streptomyces Project (ISP) agar medium and further incubated for 7 days.

2.3 Screening for Antibacterial Activity

2.3.1 Agar well diffusion method

Antibacterial activity of 78 isolates were screened by Agar well diffusion method, The well (6 mm diameter) were made on Mueller-Hinton (MH) agar using sterile cork borer. Bacterial pathogens

(100 µL of 8 hours old culture broth such as *Klebsiella pneumoniae* (MTCC 1687)*, Proteus vulgaris* (MTCC 3160), *Salmonella typhi* (MTCC 3231)*, Shigella dysenteriae* (MTCC 3642) and *Vibrio cholerae* (MTCC 3906) were spread on the surface of agar plate using the cotton swab. Further, 100 µL of cell free supernatant of actinomycete isolates were added into the wells and incubated at 37°C for 24 hours. The zone of inhibition was measured after the intended time. Ampicillin and DMSO was used as positive and negative controls [13].

2.4 Morphological Characterisation

2.4.1 Culture characteristics

Morphological and cultural characters such as colony characteristics, type of areal hyphae, substrate hyphae and spore formation were observed by inoculating in various ISP medium (Shirling and Gottlieb, 1996) The electronic image of bioactive isolates was captured in Scanning Electron Microscope. The isolates were subjected to various biochemical characterisation such as Urease test, Nitrate reduction, Gelatin, Catalase, H_2S, Starch Hydrolysis, IMVIC test, according to Berby manual.

2.5 Physiological Characterstics

2.5.1 Effect of temperature and pH

To optimize the best growth conditions of isolates, the culture were grown in ISP2 liquid broth at various pH of acidity and alkalinity, temperature at 28°C for 7 days and agitated at 200 rpm, as described by Xu et al. [14] and Xu et al. [15].

2.5.2 Carbon and Nitrogen utilisation test

Utilization of carbon source was observed in minimal salt medium, (M9 Salt Na_2HPO_4 - 6 g/L, K_2HPO_4 - 3 g/L, NaCl – 0.5 g, NH_4Cl - 1 g/L), supplemented with 1 mL of 1M $MgSo_4$ and 1 mL of 1M $CaCl_2$ and 1g of various carbon (fructose, glucose, sucrose, maltose, lactose) and nitrogen source (alanine, aspargine, glutamine, tyrosine, urea) W/V maintained at 30°C for 7 days at pH 7 (Anurag *et al.,* 2005).

2.5.3 Determination of salt tolerance

Salt tolerance of the isolate was determined on ISP2 agar medium prepared with series of various NaCl concentrations of 1%, 3%, 5%, 7%, 9%,11% and incubated at 28°C for 5 days (Basilino et al., 2003).

2.6 Chemotaxonomy

Cell mass of isolates were obtained by inoculating in ISP2 liquid broth medium at 28°C for 4 days with agitation of 140 rpm. The biomass was centrifuged at 4500 rpm for 10 min and the pellet was washed with methanol and water, and freeze dried at 20°C. Further, the cell mass was hydrolyzed with 5ml of 6M HCl and incubated at 100°C for 18 hours. The filter was evaporated to remove HCl and paper chromatography was preceded with solvent system of methanol, water, 10M HCl and pyridine in the ratio of 80:26:2:5:20. Ninhydrin was sprayed to develop the chromatogram [16].

2.7 Molecular Characterization

2.7.1 Extraction of genomic DNA

The culture was grown in yeast extract/malt extract broth at 28 ˚C for 7 days in shaker at 200 rpm. The mycelia was harvested by centrifuging for 10 min at 10,000 rpm in 4˚C. The SET buffer 25 mM Tris HCl, 25 mM EDTA, pH 8, 0.3M sucrose and 3 µL of lysozyme 20 mg/mL was added and vortexed vigorously, followed by incubation at 37˚C for 45 min at 600 µL of 10%, 3%, 5%, 7%, 9% and 11% incubated at 28°C for 5 days. SDS was added and incubated at 56˚C for 15 min. Later, 3 µL of RNase was added and incubated at 37˚C for 15 min. 2ml of phenol: chloroform: isopropanol at the ratio of 25:24:01, and centrifuged at 10,000 rpm in 4˚C for 10 min. The aqueous layer was transferred carefully to the sterile tube and precipitate with ice cold absolute ethanol. Then the mixture was kept in 80˚C for 30 min. The precipitate was transferred to the sterile tube and centrifuge for 10,000 rpm for 10min. The pellet obtained was washed with 70% ethanol and dried in room temperature. Finally, the pellet was dissolved in 200 µL of TE buffer at 10 mM Tris, 1M EDTA and pH 8. On the sequence, it was transferred and the DNA was checked in 1% Agarose.

2.7.2 PCR amplification of 16S rRNA

The extracted DNA molecule was amplified by using two universal primers 27F 5´AGATTTGATCMTGGCTCAG 3´, 1492R 5´GGTTACCTTGTTACGACTT 3´. PCR reaction was performed with the 50 µl reaction mixture

containing 0.5 µM of each primer, 1 µl extracted DNA 50 ng, 23 µl sterile distilled H_2O and 10% (v/v) of DMSO to the final volume of PCR premix Emerald, Takara, Japan. Amplification were performed with the thermo cycler, the polymerase chain reaction for 5 min at 95°C and followed by 35 cycles of 1 min at 94°C, 1 min at 58°C and 3 min at 72°C, followed by 10 min as final extension at 72°C. The amplified products were analyzed by electrophoresis in 0.8% (w/v) agarose gels and purified by DNA extraction kit Quiagen, India. Sequencing was performed on ABI 310 automatic DNA sequencer using the 27f and 1492r promoter primer.

2.7.3 Phylogenetic analysis

The sequences were aligned using BLAST tool. Multiple alignments was preformed using the CLUSTAL_X and the phylogenetic tree was constructed using MEGA 6.0 software.

3. RESULTS AND DISCUSSION

Soil is one of the valuable property of nature, with essential nutrients andorganic matter that play important role in the distribution of microorganisms [17,18]. On the soil characteristics of the sample from the location site Arichimunai (A1), Rameswaram (R1), Pamban (P1), Mandapam (M1) were observed p^H of the samples A1, R1,P1, M1 to be 7.46, 7.52, 7.31, 7.43, respectively. The electrical conductivity of thesoil samples shows slight difference between each sampling spot and were observed to be 0.41, 0.19, 0.34, 0.29 dSm^1 in A1, R1, P1, M1, respectively. Cation exchange capacity for A1, R1, P1, M1 were 1.97, 0.20, 1.92, 2.14c/mol proton/kg, respectively. Significant amount of potassium was observed, invariably in all the four samples, followed by the nitrogen, calcium and phosphorus. Organic carbon was noted only in P1 and M1 site (Fig. 1).

A sum of 78 actinomycetes were isolated from 4 different soil samples, showing that marine resources are rich inbiologically active actinomycetes, with industrial importantbioactive compound [19] and [14]. Upon the initial incubation, plates showed white chalk powder like dots and turned to thick leathery form after seven days. The culture developed aerial and substrate mycelium and diffused its color around the colony over further period of incubation. Also, the colony exhibited the musty or earthy odour. Actinomycetes isolated from soil samples are reported to exhibit antagonistic activity against pathogenic organisms [20].

The bioassay revealed antibacterial metabolite producing isolate, which enabled to narrow down the best antibacterial metabolite producer. Four isolates from each sampling site was taken for further characterization studies. The isolate D4 from Dhanuskodi sampling site at Arichimunai area spot shows the broad spectrum against pathogens, and P4 isolated from Pamban sampling site exhibits better activity. Isolate M2 from mandapam sampling site has the antibacterial metabolite, and R6 from Rameswaram shows antibacterial activity (Table 1). Marine ecosystem has been reported to be the best source for biologically active metabolite producing Actinomycetes [21], Fiedler et al., 2005. Since the biosynthetic gene cluster seem to play a major role, molecular approach of natural product drug discovery could be explored as a potential tool.

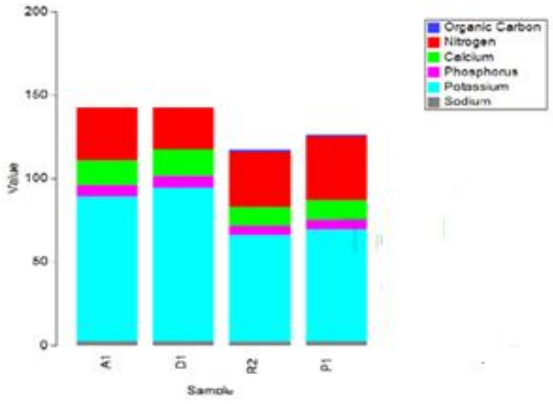

Fig. 1. The Physico-Chemical analysis of Four marine sediment sample collected from Arichimunai (A1), Rameswaram (R1), Pamban (P1), Mandapam (M1)

Table 1. Preliminary screening of isolates against *Klebsiella pneumoniae* (MTCC 1687), *Proteus vulgaris* (MTCC 3160), *Salmonella typhi* (MTCC 3231), *Shigella dysenteriae* (MTCC 3642), *Vibrio cholerae* (MTCC 3906). A: *Streptomyces* R6, B: *Streptomyces* D4, C: *Streptomyces* P4, D: *Streptomyces* M2

TEST ORGANISM	ISOLATE D4	ISOLATE P4	ISOLATE M2	ISOLATE R6
Klebsiella pneumoniae (MTCC 1687)	21 mm	24 mm	24 mm	27 mm
Proteus vulgaris (MTCC 3160)	24 mm	20 mm	18 mm	17 mm
Salmonella typhi (MTCC 3231)	27 mm	28 mm	25mm	18 mm
Shigella dysenteriae (MTCC 3642)	18 mm	21 mm	23 mm	20 mm
Vibrio cholerae (MTCC 3906)	18 mm	17 mm	16 mm	22 mm

Morphological analysis plays an important role in the characterization of Streptomyces species. Three salient features were observed in the microscopic analysis: vegetative mycelium, aerial mycelium bearing spore chain and mycelium with spore formation only [22,23]. The colour of aerial mycelia, substrate mycelia, spores and the pigment diffuse from spores and around the isolates in the culture was notable and were much useful for the differentiation of species. These growth pattern were recorded by Yeast extract-Malt extract agar (ISP 2), Oat meal agar (ISP3), Inorganic salts-starch agar (ISP 4), Glycerol asparagines agar (ISP 5), Peptone yeast extract iron agar (ISP 6) and Tyrosine agar (ISP 7).

The result of cultural characteristics of the *Streptomyces* R6, *Streptomyces* D4, *Streptomyces* P4, *Streptomyces* M2, are shown in Figs. 2 and 5, respectively. Different range of colours were observed in the present study, where the isolates shows whitish grey, white, yellowish grey and grey, representing the production of various pigments. The yellow culture of Streptomyces has the ability to produce antitubercular pluramycins [24]. The growth of *Streptomyces* R6 were observed to be goods in ISP 2, 3, 4, 5, 7 and moderate in ISP 6. The isolate diffused brown pigment in ISP2, ISP3, ISP7 and yellow in ISP4, and showed the moderate sporulation in ISP 4 & 7, and also poor sporulation in rest of the media. The *Streptomyces* D4 shows good growth in ISP 2, 3, 4, 5 and moderate growth in ISP 6 & 7. The isolate diffused yellow pigment in ISP 2, ISP 6, ISP 7 and showed moderate sporulation in ISP 4 & 5, but poor sporulation in rest of the media. The *Streptomyces* P4 showed good growth in ISP 2, 3, 4, 5 and moderate growth in ISP 6 & 7. The isolate diffused grey pigment in ISP 2 & 3, pink in ISP 4, black in ISP 7 and showed the good sporulation in ISP 4, moderate sporulation in ISP 2, 5 & 6 and poor sporulation in rest of the media. *Streptomyces* M2 showed good growth in ISP 2, 3, 5, 6 and moderate growth in ISP 4&7. The isolate diffused yellow pigment in ISP 2 & 7 and showed good sporulation in ISP 2 & 7, moderate sporulation in ISP 3 & 6 and poor sporulation in rest of the media. The overall results of cultural characteristic of strain shows distinct pattern, which differentiates the species.

Fig. 2. Cultural characteristic of *Streptomyces* R6

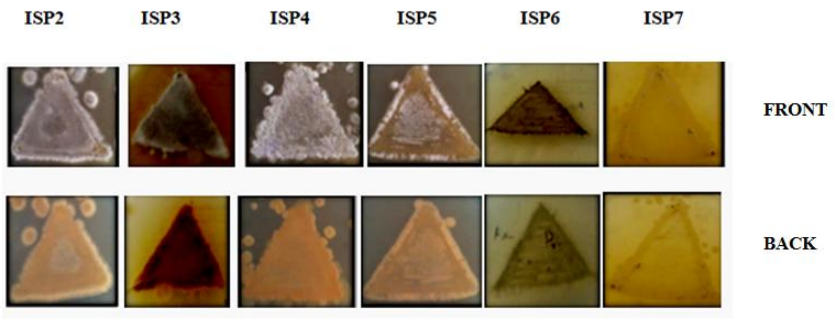

Fig. 3. Cultural characteristic of *Streptomyces* D4

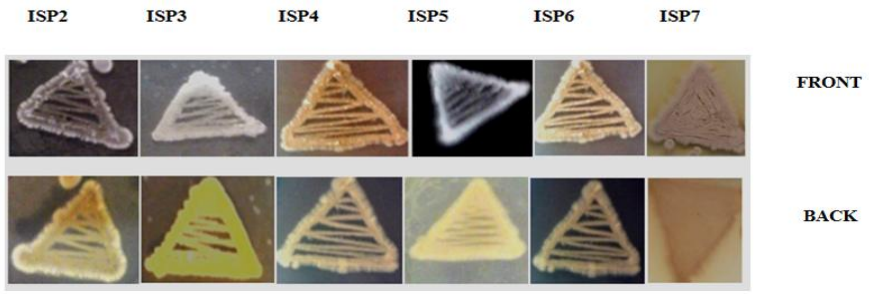

Fig. 4. Cultural characteristic of *Streptomyces* P4

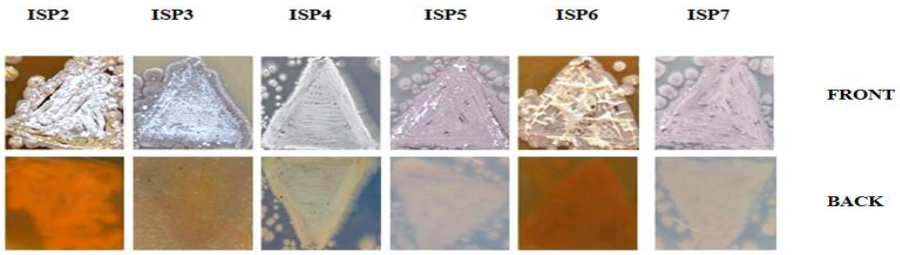

Fig. 5. Cultural characteristic of *Streptomyces* M2

The morphological analysis of spore is shown in Fig. 6. *Streptomyces* R6 was found to bear soft spore chains of hexagonal shape. *Streptomyces* D4 with rectiflexible spore chains, with hairy sheath and *Streptomyces* P4 with spiny shealths in spore chains. Long, soft spore chains showing ellipsoid structure was observed in *Streptomyces* M2. Reports on spore surface morphology shows distinct pattern for every species [25,26]. Thus, the present study reports different spore morphology for different isolate.

Biochemical analysis of all the isolates are shown in the Table 2. All the isolates were found to be aerobic, Gram positive and showed positive results for urea hydrolysis and nitrate reductase, whereas, negative result was observed in gelatin. Citrate production was observed in all isolate except *Streptomyces* P4. Positive result for starch hydrolysis was observed in *Streptomyces* D4, *Streptomyces* P4 and *Streptomyces* M2. *Streptomyces* R6 showed positive result for the Catalase production. Nitrate reductase, H_2S production, gelatin and starch hydrolysis are considered for characterization of *Streptomyces* sp [27] and [28]. Thus, the study reports different biochemical characteristic for different isolate.

Physiological parameters greatly influence the growth rate of *Streptomyces* [29]. The morphology of the isolates was dependent on the temperature and incubation time [30]. The pH plays vital role in production of bioactive metabolite in the culture. The optimal

temperature and pH for the growth of isolate is 30°C and pH 7 (Fig. 7), whereas culture has grown beyond 40°C. The isolates *Streptomyces* R6 and *Streptomyces* D4 utilize the glucose as efficient carbon source to attain maximum growth, whereas *Streptomyces* P4 and *Streptomyces* M2 utilize the sucrose and maltose as carbon source to attain maximum growth. Glutamine is the best nitrogen source for *Streptomyces* R6 and *Streptomyces* M2. The isolates *Streptomyces* D4 and *Streptomyces* P4 utilize Asparagine as nitrogen source (Fig. 8).

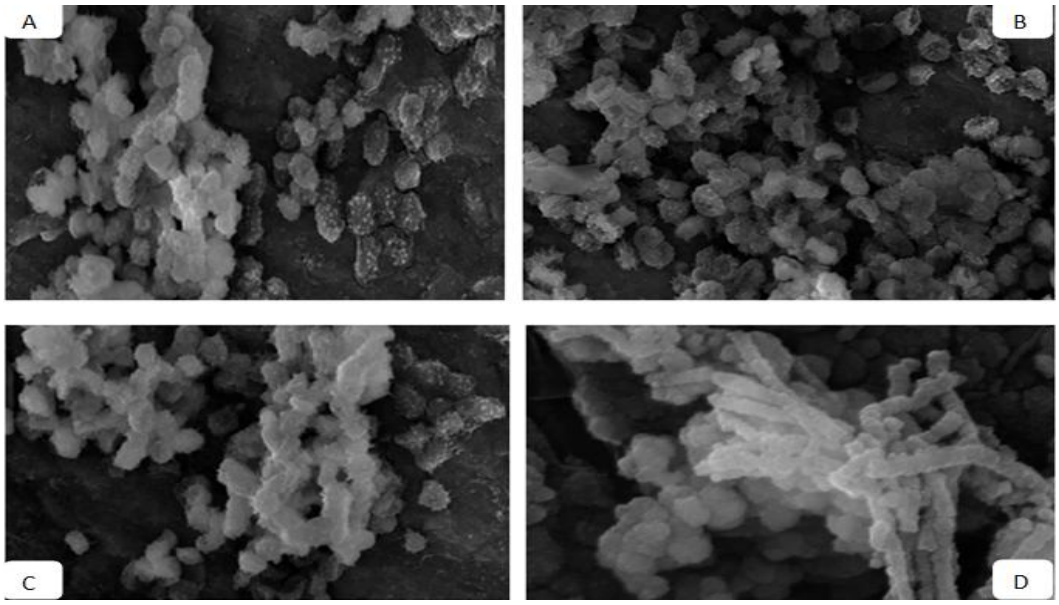

Fig. 6. The SEM image of isolates showing different forms of spore morphology. A: *Streptomyces* R6, B: *Streptomyces* D4, C: *Streptomyces* P4, D: *Streptomyces* M2

Table 2. Biochemical characterization of bioactive isolate

Biochemical test	*Streptomyces* R6	*Streptomyces* D4	*Streptomyces* P4	*Streptomyces* M2
Citrate utilization	+	+	—	+
Urea Hydrolysis	+	+	+	+
Gelatin Hydrolysis	—	—	—	—
Methyl Red	—	—	—	—
Voges Proskauer	—	—	—	—
H_2S Production	—	—	—	—
Nitrate reduction test	+	+	+	+
Starch Hydrolysis	—	+	+	+
Catalase test	+	—	—	—
Gram Stain	+	+	+	+

+ Postive, - Negative.

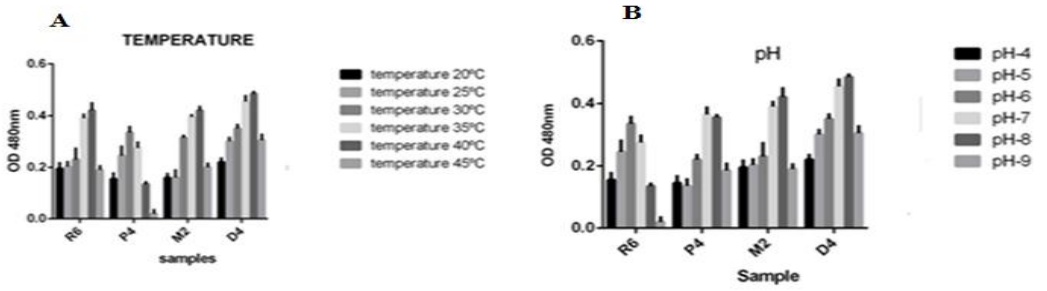

Fig. 7. Optimization of temperature and pH. A: The effect of temperature on the growth of isolates, B: The effect of pH on the growth of isolates.

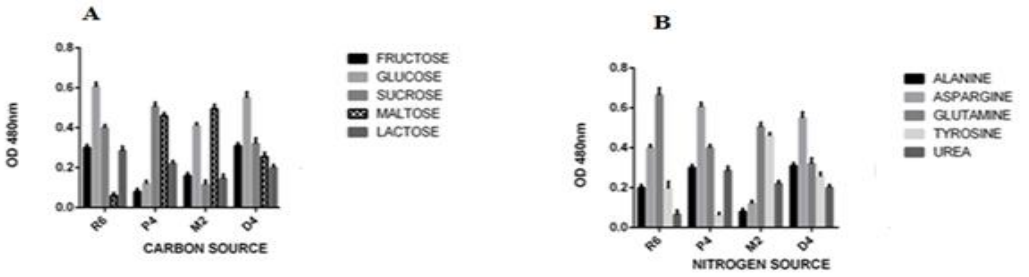

Fig. 8. Optimization of carbon source and nitrogen source. A: The utilization of carbon source for the growth of isolates; B: The utilization of nitrogen source for the growth of isolates

NaCl tolerance have been reported in *streptomyces* taxonomy based on the color and nature of the spore. The yellow or white spore forming colony shows high tolerance to NaCl, whereas the red spotted spore colonies shows lesser tolerance. The spiny spore has much tolerance than the smooth spore [31]. *Streptomyces* P4 and *Streptomyces* M2 exhibit tolerance up to 7% of NaCl concentration, whereas the *Streptomyces* D4 shows high tolerance up to 11%. *Streptomyces* R6 exhibits low tolerance up to 5% (Fig. 9). Thus, the spiny spores studied in the present investigation exhibited strong tolerance when compared to smooth spores.

The result of cell wall analysis using LL-Diaminopimelic acid (LL-DAP) was demonstrated (Fig. 10), showing it contains LL-Diaminopimelic acid and glycine, thereby indicating that strain exhibits cell wall chemotype I. The actinomycetes have a cell wall composition alike to that of Gram positive bacteria and actinomycetes [32]. DAP is widely distributed as a key amino acid and it has optical isomer. The presence of DAP suggest that, bacteria contains one of the isomers, LL form or mess form, which locates at peptidoglycan layer (Sivakumar et al., 2007). In the present investigation the isolate was found to contain LL-DAP, along with glycine, with the presence of sugar pattern, which indicates characteristic of *Streptomyces* genus.

The molecular characterization of streptomyces sp was analysed by PCR amplification of 16S rRNA (Fig. 11) and sequenced using 27f and 1492r primers. The resulting sequence was compared with NCBI using BLAST and the obtained sequence was deposited in the GeneBank in the ID of MG198770, KM925137, MG198770, MG198767. The 16s rRNA sequencing is a molecular revolution in bacterial systematic. The phylogenetic analysis was based on 16S rRNA sequence analysis. *Streptomyces* R6 (Fig. 11) shows that the strain was closely related to the *Streptomyces rochi.* The distinct branch observed from rooted tree using neighborhood joining method. *Streptomyces* D4 strain (Fig. 12) was closely related to the *Actinobacterium dagang* 5. The other isolates *Streptomyces* P4 and *Streptomyces* M4 (Figs. 13 and 14) shows the distinct cluster from rooted suggesting, the strains belong to *Streptomyces* genus. Thus the polyphasic taxonomical studies reports that the isolate belongs to the genus of *Streptomyces.*

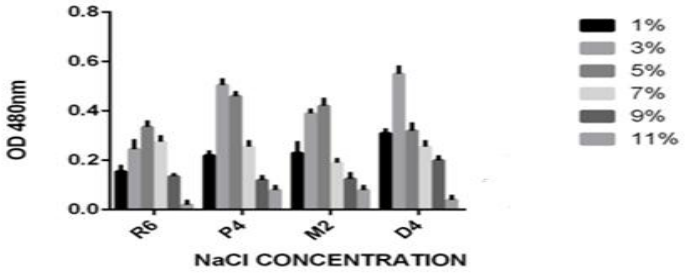

Fig. 9. Differences of salt tolerance of isolates in different concentrations

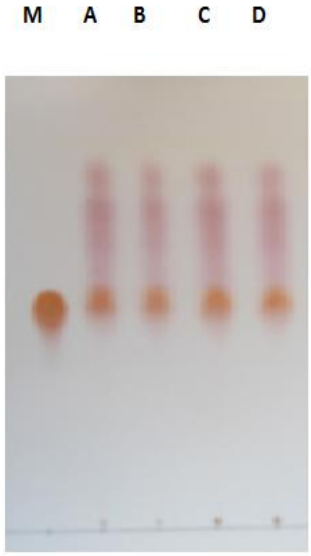

Fig. 10. Separation of A2pm isomers from isolates by thin layer chromatography M: LL-Diaminopimelic acid (0.1M) Standard. A: *Streptomyces* R6, B: *Streptomyces* D4, C: *Streptomyces* P4, D: *Streptomyces* M2

Fig. 11. The Phylogenetic analysis of R6 strain was closely related to the *Streptomyces rochi*

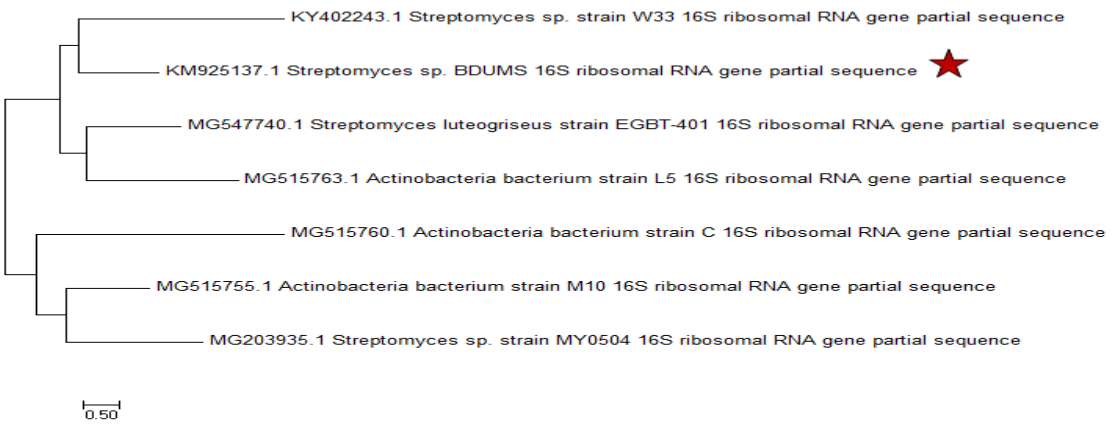

Fig. 12. The Phylogenetic analysis of D4 strain was closely related to the *Actinobacterium*

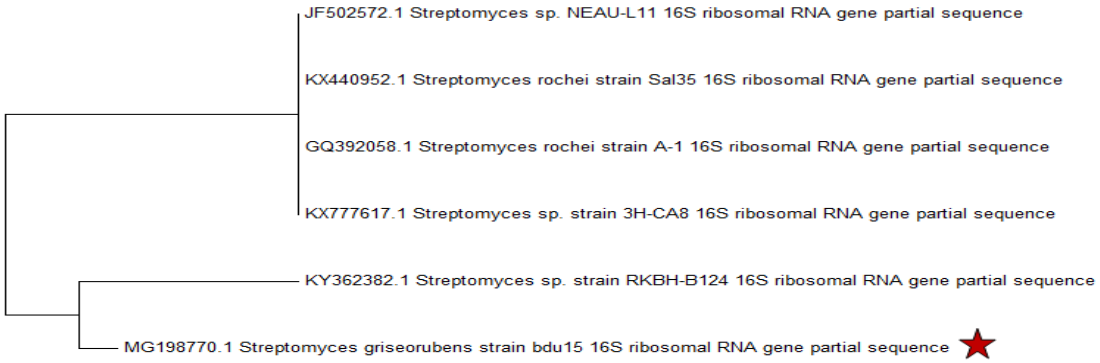

Fig. 13. The Phylogenetic analysis of P4 strain was closely related to the *Streptomyces* sp.

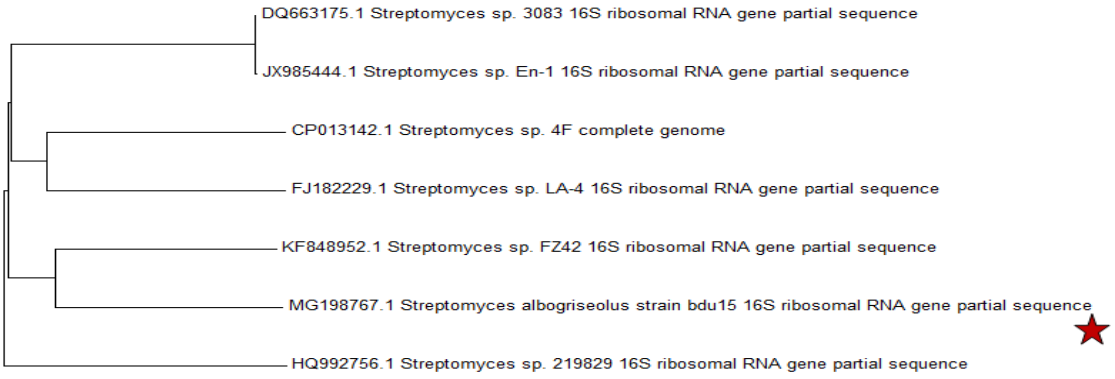

Fig. 14. The Phylogenetic analysis of M2 strain was closely related to the *Streptomyces* sp.

4. CONCLUSIONS

As per literature studies the marine actinomycete are one of the most bioactive compound producers, so the collected marine soil sediments undergone soil analysis and the micro and macro nutrient are ruled out, which helps to frame the blueprint for metabolites. The specific pretreatment are done to aviod specific growth of other genera and the actinomycetes are isolated.

Out of 78, the 4 isolates exhibits the strong antibacterial activity, so the 4 isolate is proceeded for the polyphasic characterisation, several physiological parameters like salinity, pH, carbon and nitrogen source. These parameters helps to set the essential requirement for the growth of actinomycetes. Then, the isolates are moved to next level study; molecular characterisation are phylogenetic analysis that reveals the similar strains which have highly broad specturm antimicrobial activity. The obtained literature data and experimental studies reveals that the isolates are highly potent, out of which *Actinobacterium dagang 5* exhibits the broad spectrum activity among other isolates.

CONSENT

It is not applicable.

ETHICAL APPROVAL

This study does not involve experiments on animals or human subjects.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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