Industrial Application of Marine ActinobacteriaFrom Mangrove Sediment

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ABSTRACT

An ecosystem is a biological environment consisting of all the organisms living in a particular area, as well as all the nonliving, physical components of the environment with which the organisms interact, such as air, soil, and water. Oceans are the home to huge microbial populations and diversity (Stach et al., 2005; Sogin et al., 2006) and they live in every corner of the ocean and their habitats are diverse; they are distributed in open waters, sediments, associated with many organisms, estuaries, hydrothermal vents (Cevera et al., 2005). They are always involved in the important processes of the sea in promoting organic material transformation and mineralization in the sediments and overlying waters (Das et al., 2007). Microbial communities are structured by temporal and spatial variability of physicochemical and biotic parameters (Hewson et al., 2007).

Keywords: Marine Actinobacteria, Mangrove Sediment, Thermostability and Barophilisity

INTRODUCTION

Actinobacteria (actinobacteria) is one of the largest taxonomic units within the Bacterial domain (Niva*et al.,* 2006; Jiang *et al.,* 2012). They are versatile aerobic gram-positive bacteria with a higher amount of guanine plus cytosine (>50mol% G+C) in their DNA (deoxyribo-nucleic acid). They possess a wide range of morphologically and physiologically diverse properties. They have been isolated from a wide variety of environmental sources, where they act as saprophytes, symbionts, chemo-organotrophs, parasites or even pathogens (Niva*et al.,* 2006; Trujillo, 2008). Their morphology ranges from coccoid (e.g. *Micrococcus*), rod-coccoid (e.g. *Arthrobacter*), fragmentinghyphal forms (e.g. *Nocardia*) to those with a highly differentiated branched mycelium (e.g. *Streptomyces*). In the case of filamentous actinobacteria, hyphae that branch repeatedly become attached on the surface of the agar to form tough, leathery and velvety colonies, which highly resemble fungi (Trujillo, 2008).T marine environment represents a largely less tapped source for isolation of new microorganisms including actinobacteria The first actinobacterium isolated from the oceanic sediments was not considered as a marine form. Scientists believed that it came from the spores of terrestrial bacteria that had simply blown into the oceans and remained dormant.Actinobacterial research from Indian mangroves was started in late seventiesBredholt*et al.,* (2008). manyactinobacteria isolated from the ocean sediments were true marine forms. Actinobacteria are ubiquitous in nature (Sethubathi*et al.,* 2013) and play important ecological roles and substantially impact the cycling of complex carbon substrates in the benthic and other ocean habitats (Mincer *et al.,* 2002). Actinobacteria living in the oceans experience a dramatically different set of environmental challenges compared to their terrestrial relatives, it is not surprising that speciation has occurred and unique marine taxa are now being recognized (Jensen *et al.,* 2005). Not only the full extent of marine actinobacterial diversity is yet to be determined, but also their adaptations to the

environmental parameters in the sea are to be understood (Sethubathi*et al.,* 2013).The biological and chemical diversity of the marine environment has been the source of unique chemical compounds with the potential for industrial development as pharmaceutical, cosmetics, nutritional supplements, molecular probes, enzymes, fine chemicals and agrochemicals (Ireland *et al.,* 1993). Especially, marine actinobacteria are efficient producers of innovative secondary metabolites that show a range of biological activities including antibacterial, antifungal, anticancer, antioxidant and insecticidal substances as well as enzyme inhibitors and enzymes (Solanki*et al.,* 2008). Actinobacteria are capable of catalyzing various biochemical reactions with novel enzymes such as cellulase, dioxyribonuclease, lipase and protease (Sivakumar*et al.,* 2007), because they are metabolically active in the marine environment, producing various compounds that are not observed in terrestrial stains (Jensen *et al.,* 1991). Among them *Streptomyces* exhibit remarkable capacity for synthesis of secondary metabolites and use of numerous extra cellular hydrolytic enzymes to degrade organic material in their natural habitat (Morosoli*et al.,* 1997).

Enzymes make ideal catalyst in food industries owing to their specificity, mild reaction condition and non-toxicity. Enzymes from marine microbes have unique protein molecule when compared with terrestrial microbes. Properties like high salt tolerance, thermostability and barophilisity of marine microbes made the scientist to consider these kinds of enzymes in large scale for commercial purpose (Sivakumar*et al.,* 2007).

MATERIALS AND METHODS

Vellar Estuary

The Vellar estuary (Lat. 11°29'N and Long. 79°46'E) on the Southeast Coast of India, originates from the Shervaryan Hills, Salem district, Tamil Nadu . It joins the Bay of Bengal at Parangipettai, and is said to be a 'true estuary' as there is no complete closure of the mouth. This estuary has been demarcated into marine, gradient, tidal and freshwater zones supported salinity characteristics. It is subjected to semi-diurnal tides with maximum tidal amplitude of about 1 m. The average depth ranges between 2.3 and 5.0m. Influence of the neritic water with the estuarine environment promotes an ideal exchange of both biotic and abiotic components and therefore the tidal influence extends over a distance of 16 km upstream of the estuary. It is being subjected not only to alterations in chlorinity, but also to differences due to the season within the amount of freshwater input and consequent circulation of elements between the estuarine system and the neritic waters. It is potential and relatively healthy estuary, because no major sources of pollution other than sewage and agricultural run-off compared to the other estuaries of southeast coast of India. An artificial mangrove, seagrass, oyster bed and continuous fresh water flow of Vellar estuary provided nutrient rich sediment layer along the estuarine edges.

Sample Collection

Mangrove sediment samples were collected from the rhizosphere region of the mangroves of the Vellar estuary, Bay of Bengal, using a sterile spatula. The samples were placed in sterile polythene covers and brought to the field laboratory immediately and after arrival, necessary dilutions were made to carry out further microbiological analysis.

Isolation of actinobacterial strains

Isolation of actinobacteria was carried out in Starch Casein Nitrate Agar (SCNA) (Appendix). The SCNA medium was supplemented with cycloheximide(10 µg/ml) and nalidixic acid (10 µg/ml) respectively (Kathiresan*et al.,* 2005) to minimize the fungal and bacterial contamination. One gram of pretreated sediment samples were serially diluted using sterile seawater and 0.1 ml of serially diluted samples were added to the petriplates containing SCNA and spread using a 'L' shaped glass spreader. The plateswere incubated at 37°C for seven days in an inverted position. The morphologically distinct actinobacterial colonies appeared in the petri dishes were picked up and re-streaked in appropriate media and pure cultures were obtained and pure culture slants were maintained in ISP2 agar slants at 4ºC.

Cellulose enzyme potential

Cellulose enzyme screening

The isolated actinobacterial strains were inoculated on carboxy methyl cellulose agar medium (CMC) (Appendix) and incubated at 37 °C for 5-7 days. After incubation, the plates were flooded with iodine solution (0.1 ml HCl + 5 ml of 1% iodine in 2% KI). Formation of clear halo around the colony against reddish-brown background considered to be cellulose positive (Radhakrishnan*et al.,* 2007). The potential cellulase enzyme producer were selected for further studies.

Cellulase activity

The potential strain was inoculated in 100 ml of CMC liquid medium (Appendix) and the flasks were incubated at 37°C for 5-7 days in shaker incubator at 150 rpm. After incubation, the medium was centrifuged at 7000 rpm for 10 min and the cell free supernatant was used for enzyme assay. The cellulase assay was tested by DNS (3,5-dinitrosalicyclic acid) method (Miller, 1959). To determine the activity, the crude enzyme (0.5ml); 1% (w/v) CMC (0.5 ml) in 110 mM potassium phosphate buffer (pH 7) was mixed and incubated for 20 min. at 70°C; 1% DNS reagent (3 ml) (Appendix) was added to the tube and the reaction mixture was heated at 90°C for 5-15 min to develop red brown colour. Then, 1ml of potassium sodium tartrate (Rochelvle salt solution) was added to stabilize the color. After cooling, absorbance was recorded in a UV-Vis spectrophotometer (UV-2450, Shimadzu, Japan) at 575 nm. The formation of reducing sugar was quantified using glucose as standard.

Amylase enzyme Potential

Amylase enzyme screening

The isolated actinobacterial strains were inoculated on starch-agar medium (Appendix) and were incubated at 37 °C for 5-7 days. After incubation, the plates were flooded with iodine solution (0.1 ml HCl + 5 ml of 1% iodine in 2% KI). Formation of clear halo around the colony against reddish-brown background indicated the amylase activity (Collinas and Lynne, 1980).

Amylase activity

The amylase activity was measured by dinitrosalicylicacid(DNS) method (Harley and Prescott, 2002). The potential actinobacterial strain was inoculated inamylase

production medium (Appendix)and was incubated at 28 °C for 24 hour in shaker incubator at 150 rpm. 1.5 ml of the culture broth was centrifuged in 4500 rpm at 4 °C for 10 min after fermentation(Amoozegaret al., 2003) and one milliliter of the supernatant was transferred to a new sterile test tube. Enzymatic reactionwas initiated by adding 1 ml of 1% (w/v) starch solutionat 40 °C for 30 min. The reaction was stopped by adding1 ml of DNS and absorbance was measured in 540 nm(Smitet al., 1996; Mosbach, 1976). Exact of 0.5 ml of the solutioncontaining enzyme was anesthetize boiling temperature tomake inactive the enzyme so as to organize the blank solutionand then all the steps of enzyme measurement (DNSmethod) were performed according to the sample. The abovementioned tests were administered in triplicate for all samples. The standard curve of the amylase activity was drawn by diluting glucose, DNS method and reading the absorption in540 nm in order to find the rate of the amylase activity (Godfrey and West, 1996).

Identification of potential actinobacteria

Characterization and identification of the potential cellulase positive strain at genus level were made supported the standards of Cummins and Harris (1956), Shirling and Gottlieb (1966), Lechevalier and Lechevalier (1970) and Nonomura (1974).

Morphological characteristics

Aerial mass colour

Colour of the mature sporulating aerial mycelium was recorded in a simple way (white, grey, black, red, blue and violet). When the aerial mass colour fell between two colour series, both the colors were recorded. If the aerial mass colour of a strain to be studied showed intermediate tints, then also, both the color series were noted. The media used were Yeast Extract-Malt Extract Agar (ISP-2) and Inorganic-Salt Starch Agar (ISP-4) (Shirling and Gottlieb, 1966).

Melanoid pigments

The grouping was made on the assembly of melanoid pigments (i.e. greenish brown, brownish black or distinct brown, pigment modified by other colours) on the medium. This test was carried out on the ISP-7 medium as recommended by International Streptomyces Project (Shirling and Gottlieb, 1966).

Reverse side pigments

Strains were divided into two groups, according to their ability to produce characteristic pigments on the reverse side of the colony, namely, distinctive (+) and not distinctive or none (-). In case, a color with low chroma as straw , olive or raw sienna occurred, it had been included within the latter group (-). This test was carried out in the medium ISP7 as recommended by International Streptomyces Project (Shirling and Gottlieb, 1966).

Soluble pigments

Strains were examined for their ability to produce soluble pigments other than melanin: namely, produced (+) and not produced (-). The colour was recorded (red, orange, yellow, blue, green and violet). This test was carried out on the media ISP-1 and ISP-7 as recommended by International Streptomyces Project (Shirling and Gottlieb, 1966).

Spore chain morphology

Characteristics of the spore bearing hyphae and spore chains were determined using direct microscopic examination of the culture surface. Adequate magnification (400X) was wont to establish the presence or absence of spore chains and to watch the character of sporophores viz. rectiflexibiles (RF) and spirales (S). Spore morphological characters

of the strains were studied by inoculating a loopful of 1 week old cultures into 1.5% agar medium contained in test tubes, at 370 C. The actinobacteria were suspended and thoroughly mixed within the semisolid agar medium and 1 or 2 drops of the medium were aseptically pipetted on to a sterile glass slide. A drop of agar was spread well on the slide and allowed to solidify into a thin film so as to facilitate direct observation under the microscope. The cultures were incubated at 28+20 C and examined periodically for the formation of aerial mycelium, sporophore structure and spore morphology.

Assimilation of carbon source

Ability of the actinobacteria strain within the utilization of varied carbon compounds as source of energy was studied, following the tactic recommended by International Streptomyces Project (Shirling and Gottlieb, 1966). Chemically pure carbon source certified to be free of admixture with other carbohydrate and contamination 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. 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 'doubtful' strains, only a trace of growth slightly greater than that of the control was noticed.

Chemotaxonomical characteristics

C.°2± Hydrolysis was made for releasing the amino acids. Harvested cells of each strain weighing 2mg were placed in a culture tube and 2ml of 6N HCl was added. The samples were kept for 4h in a sand bath. The culture tubes were cooled by keeping them at a room temperature of 28.

Thin Layer Chromotography (TLC)

Spotting of the whole cell hydrolysates was made carefully on TLC plates using a micropipette. Spots were of 2-5 mm in diameter. This was done by multiple applications on the same spot of very small portions of the sample, which were dried by hand drier.

Amino acids

l of amino µl of DL-diaminopimelic acid (an authentic material mixture of DAP isomers) and 1µ Each sample (2µl) was applied on the base lines of cellulose precoated TLC plate (20cm X 20cm) and 1 ethanoic acid (glycine) were spotted as standards. TLC plate was developed with the solvent system containing methanol: distilled water: 6N HCl: pyridine (80:26:4:10). It took more than 4 hrs for development. The spots were visualized by spraying with 0.2% Ninhydrin solution in acetone, followed by drying in hot air oven; spot of amino acetic acid 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 TLC plate (procedure as finished DAP), 2 µl of sample was spotted, alongside 1 µl of sugar solutions as standards on the sample plates. Galactose, arabinose, xylose, glucose, rhamnose and mannose were the sugars which were used as standards. TLC plate was developed with the solvent mixture containing n-butanol: distilled water: pyridine: toluene (10:6:6:1 v/v). The developing time was more than four hours. Spots were visualized by spraying with aniline pthalicreagent (2.5g of pthalic acid dissolved in 2ml of aniline and made upto 100ml with water saturated n-butanol). The sprayed plate

was dried in hot air oven. Hexoses appeared as yellowish brown spots and pentoses, as maroon coloured spots.

Molecular identification

The generic status of the strains was also confirmed based on their 16S r DNA gene sequences, with the following methodology.

Genomic DNA isolation

Total genomic DNA was extracted from the actinobacterial broth by phenol- chloroform isoamyl alcohol method, which removes the protein and their cellular components from the macromolecule to get the pure DNA.

Log phase broth actinobacterial culture (2 to 4 ml) was taken and centrifuged at 10,000rpm for 15 minutes at 4°C. Centrifugation was repeated to wash the cells twice with 500 µl of TE buffer (Appendix). The pellet was resuspended in 500 µl of TE buffer and incubated for 1 h and incubated at 37°C until the cells became translucent. Then 30µl of 10% SDS was added followed by the addition of the proteinase K (3µl), shaken well and kept at 50-55°C for nearly 1 hour, by shaking thoroughly at regular intervals; 250 µl of neutral phenol-chloroform-isoamyl alcohol solution (phenol 25: chloroform 24 : isoamyl alcohol 1 v/v) was added and thoroughly mixed for 30 sec, then spinned for 5-10 min in the micro-centrifuge (12,000 rpm) and the supernatant was removed, leaving the white interface behind. This step was repeated twice until the absence of interface. Then 0.1 volume of 3M sodium acetate (pH 4.8) was added and mixed well; 1 volume of isopropanol was added and mixed well again. The thoroughly mixed sample was kept at 0°C to 5°C for 15 min. Then the sample was centrifuged using a microcentrifuge at 10,000 rpm for 10 min at 4°C and 70% ethanol was added to wash the pellet and air dried. After drying, The DNA was resuspended in 100 µl TE buffer and stored at 4°C until it was used (Hapwood*et al*., 1985). DNA sample (10µl) was mixed with 2µl of loading (6X) dye and loaded 1% agarose gel. The separated DNA was visualized by UV transilluminator.

Amplification of 16S rDNA

Each 50µl amplification reaction contained 1µl template DNA (50-200ng), 5µl 10X PCR buffer, 1µl both forward (27F (5'-AGAGTTTGATCMTGGCTCAG-3')) and reverse (1492R (5'-GGYTACCTTGTTACGACTT-3') primers, 1µl dNTP mix (10mM), 6µl $MgCl_2$ (25mM), 2.5 U Taq DNA polymerase, 2.5µl DMSO and 31.5µl sterile water. The reaction conditions were initial denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 90 sec. A final extension was performed at 72°C for 10 min (Karuppiah*et al.,* 2011). Reaction products were electrophoresed on a 1% agarose gel with 2µl ethidium bromide and visualized under UV transilluminator then purified.

16S rDNA sequencing

The purified fragment was directly sequenced using aAmpli Tag FS DNA sequencing Kit (Applied Biosystem). The data were analyzed using applied Biosystem DNA editing and assembly software and sequences comparisons were obtained using the Micro Seq Software.

Phylogenetic analysis

Sequence similarity search was made for the 16S rDNA sequence of all isolate by applying their sequence to BLAST search of the NCBI (National Centre for Biotechnological Information, USA). Final editing of sequence alignment was done using BioEdit (Hall, 1999) and phylogenetic analysis was performed with version 5 of the MEGA (Molecular Evolutionary Genetics Analysis) software package (Tamura *et al.,* 2007). A phylogenetic tree was constructed by using the neighbor-joining tree-making algorithum (Saitou and Nei,

1987). The topology of the phylogenetic tree was evaluated by using the booststrap resampling method of Felsenstein (1985) with 1000 replicates.

RESULTS AND DISCUSSION

Actinobacteria isolation

A total of 10 morphologically distinct actinobacterial strains were selected from the mangrove sediment samples using the SCNA medium. These strains were labeled as VR1, VR2, VR3, VR4, VR5, VR6, VR7, VR8, VR9 and VR10 (Fig. 2 and Fig. 3). Similarly, Sahu*et al.* (2005) isolated maximum number of actinobacteria using this medium from Vellar estuary. Raghavendrudu and Kondalarao (2007) studied that the distribution of actinobacteria in the Gaderu mangroves of Gautami Godavari estuarine system, east coast of India. They used five different agar media for isolation, among them, SCNAmedium was found to be more suitable for the isolation of the genus *Streptomyces*, which was observed very frequently on this medium.

While, Baskaran*et al.* (2011) also reported that SCNA medium was found to be the well supporting medium to marine actinobacterial population. Sethubathi*et al.* (2013) reported that SCNA medium frequently yielded higher counts of actinobacterial colonies and Mohseni*et al.* (2013) isolated 44 actinobacterial strains from the sediments of the Caspian Sea using SCNA medium, as one of the medium.

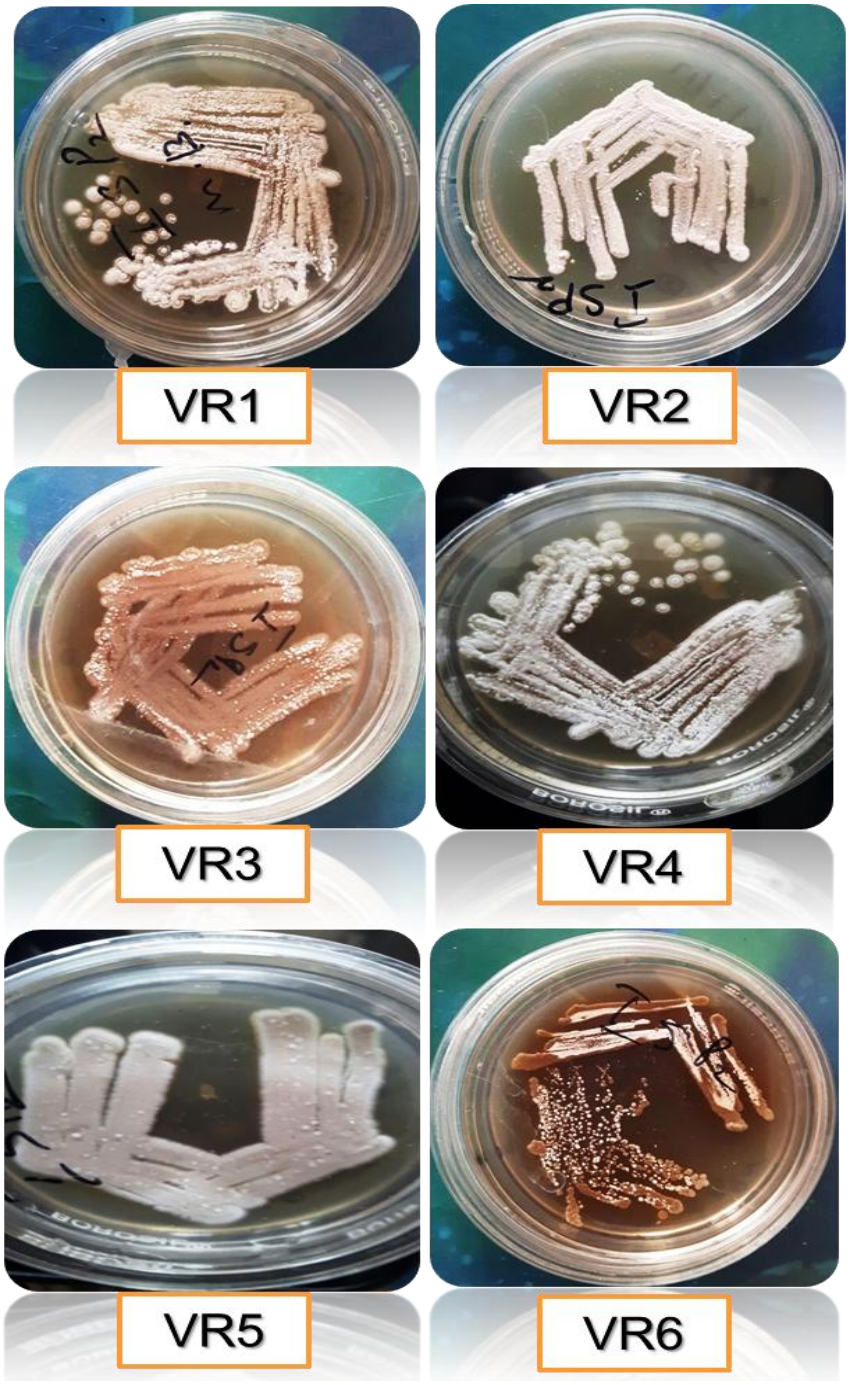

Fig. 2. Agar plates shows the morphologically distinct actinobacterial strains

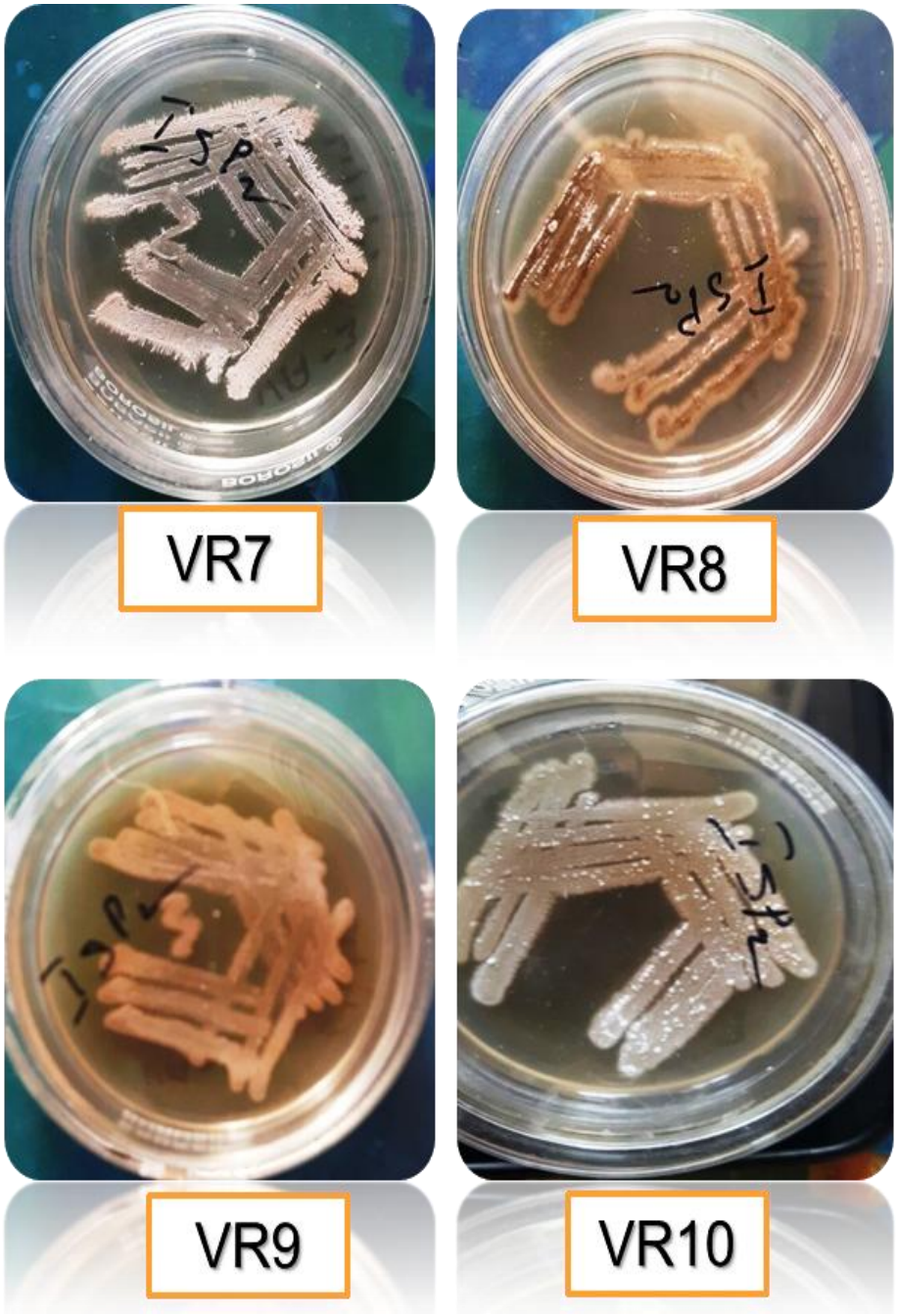

Fig. 3. Agar plates shows the morphologically distinct actinobacterial strains

Cellulase enzyme production

Screening for cellulase enzyme production was done for the ten isolated actinobacterial strains, using the Carboxy methyl cellulose agar medium. Higher cellulase enzyme activity, was found in two strainsVR2 and VR7(Fig. 4) with 18 mm of clear halo (Fig. 5).

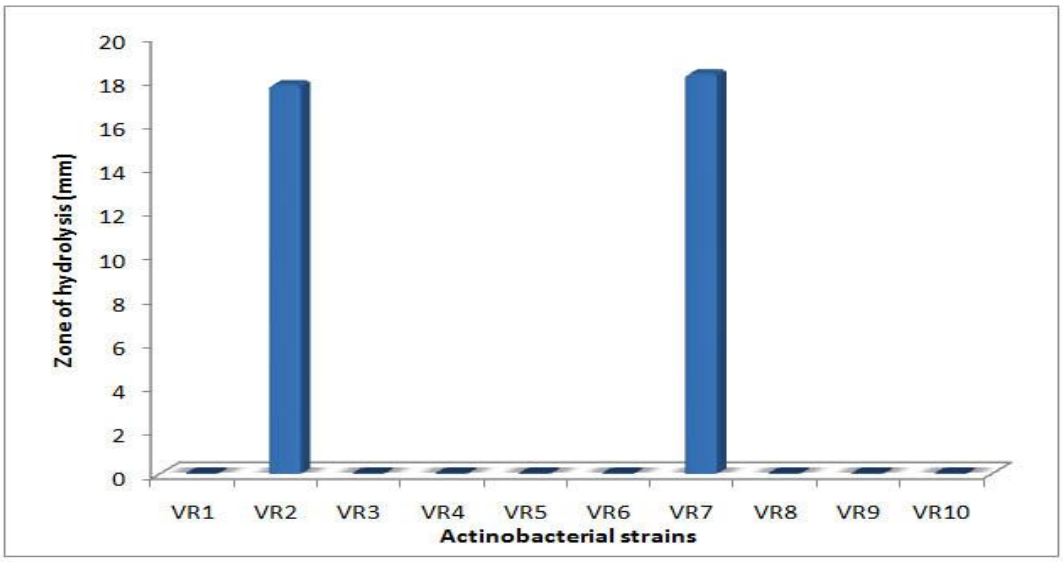

Figure 4. Cellulase enzyme activity in Carboxy methyl cellulose agar medium.

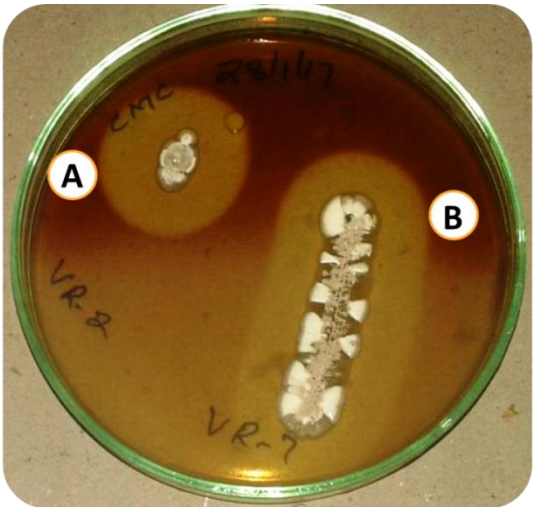

Figure 5. Cellulase screening results in Carboxy methyl cellulose agar medium [Positive strains, A-VR2, B-VR7)**.**

Enzyme activity

Based on the performance of the actinobacterial strains for cellulase production in the Carboxy methyl cellulose agar, the higher cellulose degrading actinobacterial strains *viz*. VR2 and VR7 were selected as potential cellulase enzyme producers.

Further, cellulase enzyme produced by these strains (VR2 and VR7),was subjected to enzyme activity usingthe DNS method and glucose was used as the standard. The standard solutions (glucose) as well as samples (crude enzyme cellulase) were estimated colorimetrically using a UV-Vis spectrophotometer (UV-2450, Shimadzu, Japan) at 575 nm. The glucose standard curve and its results are shown in Table 1.

By comparing the glucose standard curve, cellulase enzyme production of the selected actinobacterial strains was measured. Higher amount of cellulase enzyme production was 14.379 U/ml in VR7 and followed by VR2 (14.213 U/ml(Table 2).

Table: 1

Spectrophotometericvalues at different glucose concentrations.

S. No.	Glucose concentration	Absorbance value
1	0	0.061
2	25	0.243
3	50	0.574
4	100	1.508
5	200	3.181
6	500	5.045

Table: 2

Spectrophotometericvalues and cellulase enzyme concentration of the two actinobacterial strains.

S. No	Strain name	Absorbance value (575 nm)	Concentration (U/ml)
1	VR2	0.123	14.213
2	VR7	0.126	14.379

Actinobacteria, one of the known cellulase produces, has attracted considerable research interest due to the potential application (Jang and Chen, 2003; Arunachalam*et al.*, 2010). *Streptomyces* are the largest and well-studied group of actinobacteria. A wide variety of bacteria are known for their production of hydrolytic enzymes with *Streptomyces* being the best (Chellapandi and Jani, 2008). Considering these, present study examines cellulase activity screening in ten actinobacterial isolates and among them, the strain AUBN-9 exhibited higher cellulase activity and identified as *Streptomyces* genus.

Murugan*et al.* (2007) isolated 35 actinobacterial strains from Vellar estuary, India and examined cellulase production. Among them, Starin CL-30 (*S. actuosus*) showed maximum cellulase activity. Sirisha*et al.*, (2013) reported bioactive compounds from marine actinobacteria isolated from the sediments of Bay of Bengal, and 24 % strains exhibited cellulase activity. Meena*et al.* (2013) reported marine sediments actinobacteria from Andaman and Nicobar islans and isolated 26 actinobacteria strains, among them two *Streptomyces* species (NIOT-VKKMA02 and NIOT-VKKMA26) have showed excellent activity of cellulase. Recently, Gobalakrishnan (2013) reported cellulase produced actinobacterial stains from Havelock island, the Andamans.

Amylase enzyme production

Screening for amylase enzyme production was done for the ten isolated actinobacterial strains, using the starch agar medium. Strongamylase enzyme activity was found in the strains VR2 (14 mm) and trace activity was found in the strain VR6.(Fig. 6 &7)

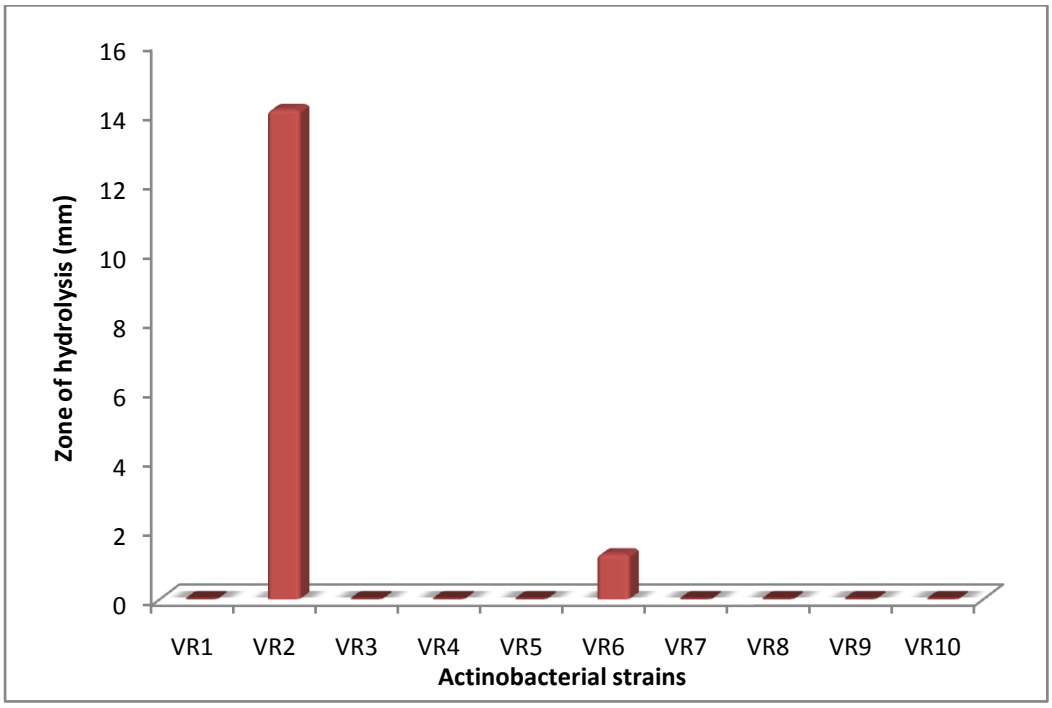

Figure 6. Amylase enzyme activity in starchagar medium.

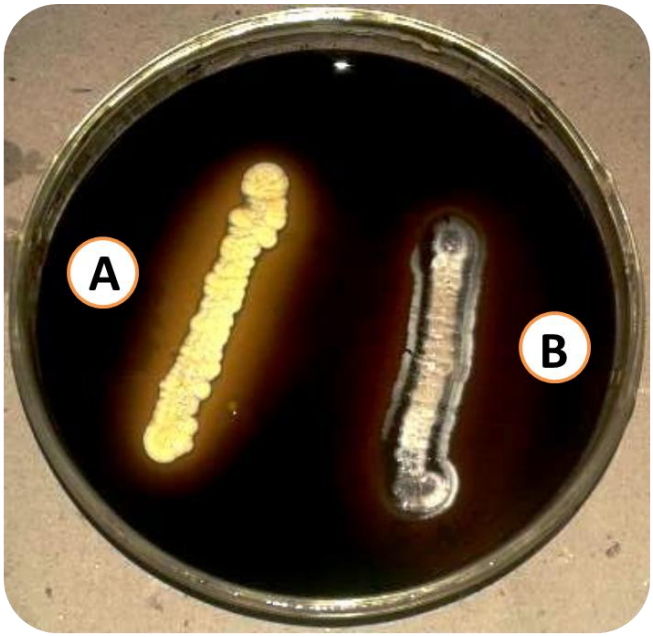

Figure 7. Amylase screening results in Starchagar medium [A- Positive strain (VR2), B- Trace activity (VR6)]**.**

Enzyme activity

Based on the performance of the actinobacterial strains for amylase production in the starchagar, the higher starch degrading actinobacterial strains VR2 was selected as potential cellulase enzyme producer.

Further, theamylase enzyme produced by the strain VR2,was subjected to enzyme activity using the DNS method and glucose was used as the standard. The standard solutions (glucose) as well as samples (crude enzyme amylase) were estimated colorimetrically using a UV-Vis spectrophotometer (UV-2450, Shimadzu, Japan) at 540 nm. The glucose standard curve and its results are shown in Table 3.

By comparing the glucose standard curve, amylase enzyme production of the selected actinobacterial strains was measured. Higher amount of amylase enzyme production was 67.13 U/ml was observed in the strains VR2 (Table 4).

Table: 3

Spectrophotometericvalues at different glucose concentrations.

S. No.	**Glucose concentration**	**Absorbance value**
1	5	0.215
2	20	0.476
3	35	0.682
4	50	0.883
5	65	1.040
6	80	1.320
7	95	1.390
8	100	1.680

Table: 4

Spectrophotometericvalues and amylase enzyme concentration of the actinobacterial strains VR2

S. No	**Strain name**	**Absorbance value (540 nm)**	**Concentration (U/ml)**
1	VR2	0.656	67.13

Amylase is one of the most important enzymes in various point of view especially in industries, that catalyses the breakdown of starch into sugar. These amylase enzymes account for about 30 % of the world's enzyme production. The world market for enzymes remains inexcess of $4500 million (Sivakumar*et al*.,2012) presently about US$ 2.7 Billion and increase by 4% annually (Deb *et al*., 2013). Bacteria which can produce the amylase are widely present in nature can easily be screened and tested for the production of amylase (Pokhrel*et al*., 2013).

Liu and Xu (2008) isolate a novel raw starch digesting a-amylase bacteria *Bacillus* sp.YX-1: they worked on the purification and characterization of amylase enzyme. Maximum amylaseactivity(53U/mL^{-1}) was obtained at 45 °C after 44 h of incubation. The enzyme waspurified using ammonium sulfate precipitation, ion exchange and gel filtrationchromatography, and showed a molecular weight of 56 kDa by SDS-PAGE. This enzyme activity was maximum at pH 5.0, and optimum active temperature was at 40-50°C. deSouzaandMagalhães (2010) studied about the application of microbial a-amylase in different types of industries.

Alkando and Ibrahim (2011) isolate a new potential microorganism *Bacillus licheniform is* for amylase production and compared the enzymatic activity in two different method and found maximum amylase activity of 0.7947 U/mg/ml at pH of 8 in iodine method and (0.024 U/mg/ml) in 3.5.dinitrosalicylic acid (DNS) method. Amutha and Priya(2011) analyzed the effect of pH, Temperature and Metal Ions on amylase activity from *Bacillus subtilis*KCX 006. They found maximum enzymatic activity in stationary phase with optimum temperature of 37°C. Residual activity was found maximumupto117.5%.

Sani*et al*., (2014) isolate *Bacillus subtilis* for the production, purification and

characterization of amylase. There result showed that the partially purified enzyme hasspecific activity of 0.144±0.019 U/mg, these was increase of 33.5 times than the raw enzymeextract. The optimum pH of the purified enzyme was 6.0, but the enzyme can work in the pHrange of 5.0 – 9.0. The optimum temperature of the enzyme was 60°C. SundarramandMurthy (2014) has studied on the different technique, methods and process parameters foramylase production. They studied the different enzymatic activity determination methods and there applications.

Also some studies were done in relation to the industrial enzyme production for examples. Sharma and Pant (2001) isolated marine actinobacteria in Bengal gulf and their results showed that the marine actinobacteria could be a source for production of bioactive compounds and industrial enzymes. Ward and Bora (2006), introduced the marineactinobacteria as an important source to discover new secondary metabolites.

Leon *et al*. (2007) succeeded to isolate actinobacteria with multi-enzymatic activities from the central coast in Peru. Ramesh and Mathivanan (2009) reported that Bay of Bengal is the potential source with rang of marineactinobacteria that have ability to produce industrial enzymes such as lipase, amylase,cellulase, caseinase and gelatinase. Peela*et al*. (2005) isolated the marine actinobacteria from Andaman Islands coast that belonged to *Streptomyces* genus and had ability to produce industrial enzymes. Also, Selvin*et al*. (2009) isolated three types of action bacteria from the southwest coast of India which had ability to produce industrial enzymes such as amylase, cellulase and lipase.

The acquired results were consistent with other different studies throughout the world which indicated *Streptomyces* from mangrove sediments is appropriate to produce industrial enzymes.

Identification

Strain VR2

Strain VR2 is a mesophilicactinobacterium, which forms an extensively branched substrate mycelium and aerial hyphae that differentiate into short, spiral spore chains (Fig. 6). Gray-brown coloured aerial spores were formed in ISP2 agar. Reverse side pigment was produced. Soluble and melanoid pigments were not produced. The culture grew well when it was supplemented with the carbon sources *viz.* xylose, mannitol and fructose. The strain VR2 showed the presence of LL-DAP along with glycine of the peptidoglycan layer with no characteristic sugar pattern, indicating that this strain belongs to the cell wall chemo type I (Table 3).

The 16S rRNA gene sequence (1396 bp) of the strain VR2 was analysed for sequence similarity. Phylogenetic relationship of the strain VR2 showed close relationship with the members of the genus *Streptomyces.* Comparison of the 16S rRNA gene sequence of the strain VR2 with the previously obtained sequences from the GenBank (NCBI) was made and the phylogenetic tree was constructed (Fig. 7). This indicated that the strain VR2 forms a clade with *Streptomyces costaricanus*with 99.5% similarity. *Bacillus subtilis*served as an out group.

Results of the cultural, morphological, physiological, chemotaxonomical and molecular characters were compared between the strain VR2 and its closest phylogenic member. The strain VR2 showed variation in some characters when compared to those of the reference species *S. costaricanus* i.e. variation in carbon source utilization. Except these, all the other characters were similar to those of *S. costaricanus*(Table 3). Hence, the strain VR2 was identified as a species close to *S. costaricanus*.

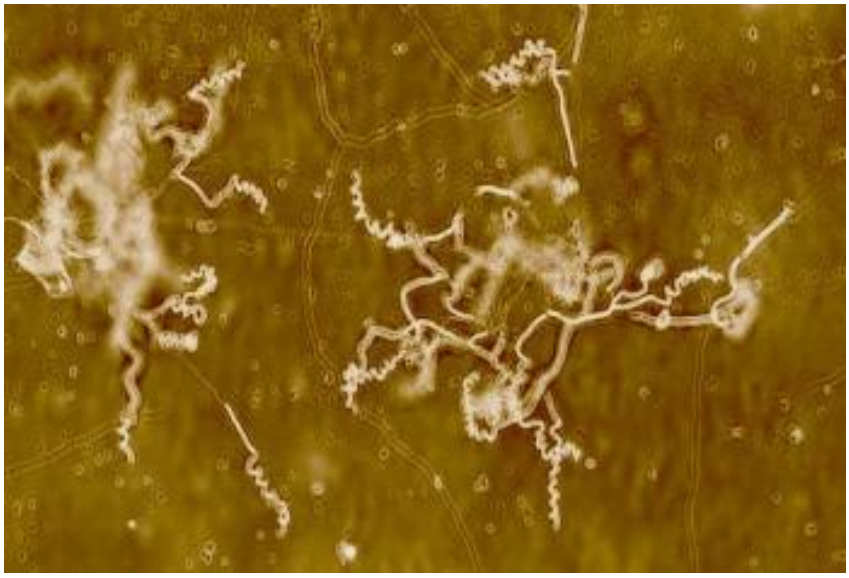

Fig. 6. Shows the spirale spore chain of the strain VR2

Table. 3. General characteristics of the strain VR2 and the closely related *Streptomyces* species.

Characters studied	**Strain VR2**	***Streptomyces costaricanus***
Morphological and cultural characteristics		
Colour of aerial mycelium	Gray-Brown	Gray-Brown
Melanoid pigment	-	-
Reverse side pigment	+	+
Soluble pigment	-	-
Spore chain	Spirale	Spirale
Carbon source assimilation		
Arabinose	-	-
Xylose	+	+
Inositol	±	+
Mannitol	+	+
Fructose	+	+
Rhamnose	±	-
Sucrose	-	-
Raffinose	-	-
Cell wall amino acids		
LL-DAP	+	+
Meso- DAP	-	-
Glycine	+	+
II. Cell wall chemotype	**I**	**I**

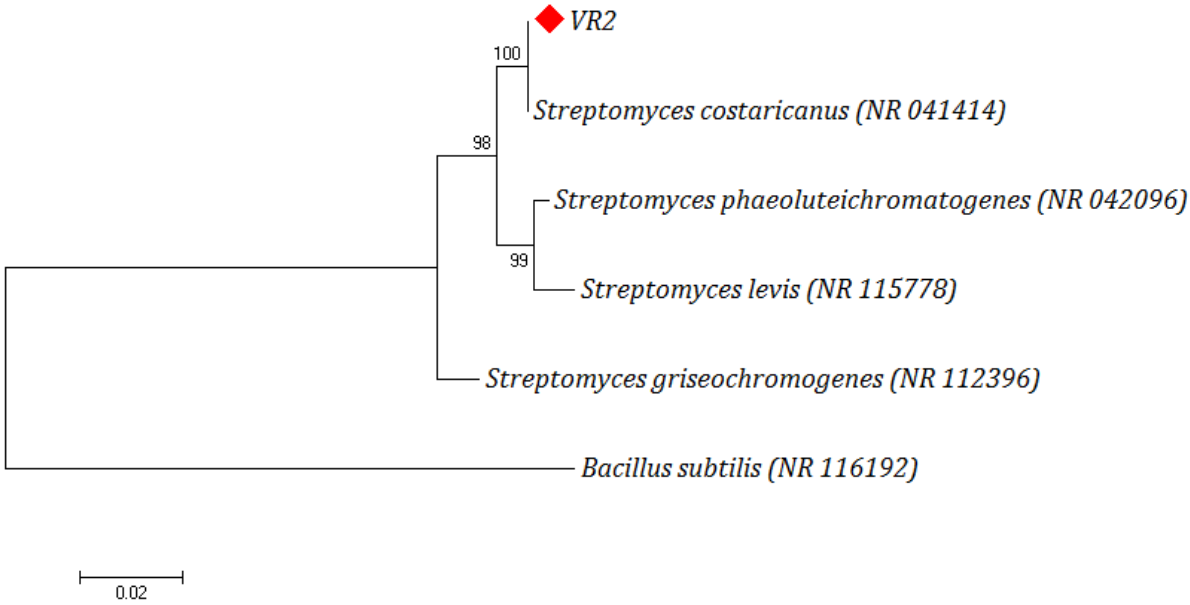

Fig. 7. Phylogenetic tree constructed based on the 16S rRNA gene sequences showing the position of the strain PS3 and related strains.

Strain VR7

Strain VR7 is a mesophilicactinobacterium, which forms an extensively branched substrate mycelium and aerial hyphae that differentiate into rectiflexibile spore chains (Fig. 8). White coloured aerial spores were formed in ISP2 agar. Reverse side, soluble and melanoid pigments were not produced. The culture grew well when it was supplemented only with the carbon source mannitol and not grown on other carbon sources. The strain VR7 showed the presence of LL-DAP along with glycine of the peptidoglycan layer with no characteristic sugar pattern, indicating that this strain belongs to the cell wall chemo type I (Table 4).

The 16S rRNA gene sequence (1134 bp) of the strain VR7 was analysed for sequence similarity. Phylogenetic relationship of the strain VR7 showed close relationship with the members of the genus *Streptomyces.* Comparison of the 16S rRNA gene sequence of the strain VR7 with the previously obtained sequences from the GenBank (NCBI) was made and the phylogenetic tree was constructed (Fig. 9). This indicated that the strain VR7 forms a clade with *Streptomyces rubidus*with 99.6% similarity. *Bacillus subtilis*served as an outgroup.

Results of the cultural, morphological, physiological, chemotaxonomical and molecular characters were compared between the strain VR7 and its closest phylogenic member. The strain VR7 showed variation in some characters when compared to those of the reference species *S. rubidus* i.e. carbon source utilization. Except these, all the other characters were similar to those of *S. rubidus* (Table 4). Hence, the strain VR7 was identified as a species close to*S. rubidus*.

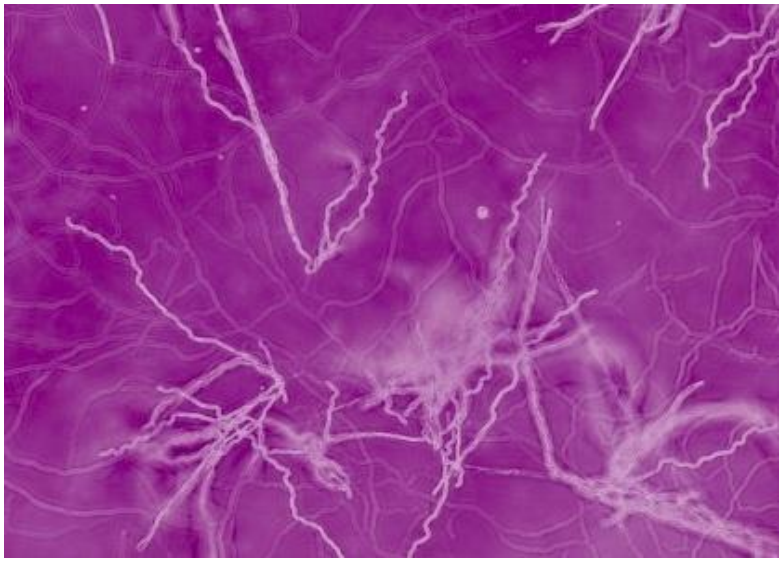

Fig. 8. Shows the spirale spore chain of the strain VR7

Table. 4. General characteristics of the strain VR7 and the closely related *Streptomyces* species.

Characters studied	**Strain VR7**	***Streptomyces rubidus***
Morphological and cultural characteristics		
Colour of aerial mycelium	White	White
Melanoid pigment	-	-
Reverse side pigment	+	+
Soluble pigment	-	-
Spore chain	Rectiflexibles	Rectiflexibles
Carbon source assimilation		
Arabinose	-	-
Xylose	-	-
Inositol	-	-
Mannitol	+	+
Fructose	-	-
Rhamnose	-	-
Sucrose	-	-
Raffinose	-	-

Cell wall amino acids		
LL-DAP	+	+
Meso- DAP	-	-
Glycine	+	+
II. Cell wall chemotype	**I**	**I**

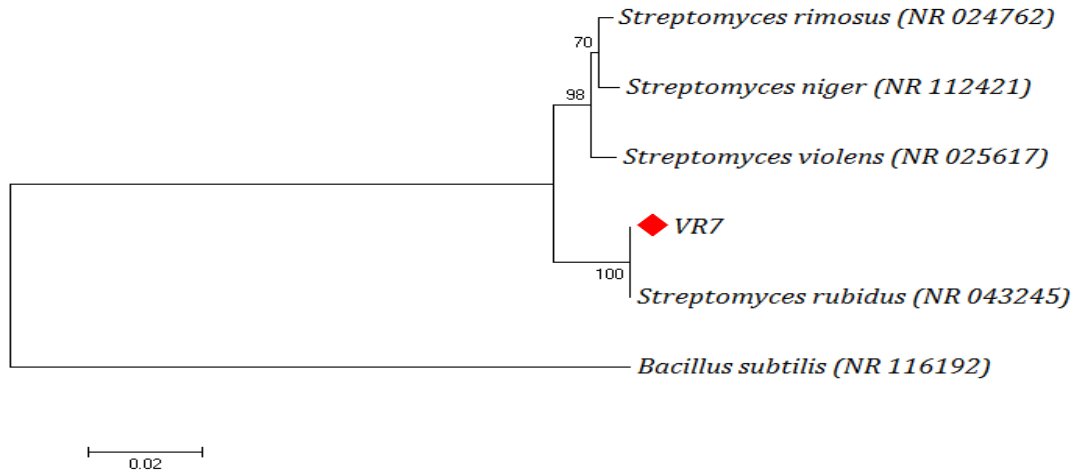

Fig. 9. Phylogenetic tree constructed based on the 16S rRNA gene sequences showing the position of the strain VR7 and related strains.

Various criteria have been used for taxonomical classification of microorganisms. Cell wall composition determinations have demonstrated important differences, such procedures of cell wall anaylsis, in particular, have clear defined members of the genus *Actinobacteria* (Boone and Pine, 1968) and rapid chromatographic procedure developed for routine use in the laboratory for the identification of species of *Actinobacteria*. Chemical characters are being used increasingly in the classification and identification of microbes and they have been responsible for some of the improvements in the taxonomy of actinobacteria (Lechevaliear and Lechevaliear, 1970) and examination of whole cell hydrolysates will give sufficient data for accurate identification.

Hence, presently isolated 10 actinobacterial strains were subjected to the analyzing of whole cell wall sugars and amino acids to identify them. In the present study, all the strains showed the presence of LL-DAP along with glycine of the peptidoglycan layer with no characteristic sugar pattern, indicating that these strains belong to the cell wall chemo type I.

Spore morphology is considered as one of the important characteristics in the identification of actinobacteria and it greatly varies among the genera and also within the species (Tresner*et al.*, 1961; Sivakumar*et al.*, 2005). In the present study, it was found that the strain VR2 found to have spirale spore chain and the strain VR7 found to have rectiflexibles spore chain. The aerial mycelium color systems, which seem most practically appealing and effective to specialists on actinobacteria are those embracing a limited number

of color names and groups. The broad groupings allow placement of isolates into reasonably well-defined categories based on color of aerial mycelium (Pridham, 1965). In addition, morphological features of the aerial mycelium are regarded as more significant for characterization and they include the mode of branching, configuration of the spore chains and surface of the spores. Surface of the conidial wall often has convoluted projection which, together with the shape and arrangement of the spore bearing structures, is the characteristics of each genus of the actinobacteria and used for the separation of genera (Anderson and Wellington, 2001) and there has been increasing evidence that certain morphological properties are finally and properly being accorded major status as criteria for species differentiation (Tresner*et al.*, 1961).

Genus level identification of microorganisms by 16S rRNA gene sequencing has emerged as a more objective, accurate and reliable method for bacterial identification, with the added capability of defining taxonomical relationships among bacteria (Clarridge, 2004) and a modern approach including physiological and chemotaxonomic analyses as well as phylogenetic analysis based on 16S rRNA gene sequences will lead to the identification of actinobacterial strains (Magarvey*et al.*, 2004).

Application of both traditional techniques, such as micromorphological, chemosystematic, and numerical taxonomy of phenotypic measurements, and the modern molecular systematic techniques are instrumental in clarifying the relationships within the actinobacteria (Stackebrandt*et al.*, 1997; Atalan*et al.*, 2000), In addition, current taxonomical approach replacing minimal numbers of characteristics by large numbers of features and characterizing different aspects of bacterial cells has resulted in more stable classification (Gillis *et al.*, 2001). Further, it is widely recognized that multi-characteristic taxonomic approaches are better, since they take into account all available phenotypic and genotypic data and integrate them in a consensus type of classification (Vandamme*et al.*, 1996). Present study pursued the modern taxonomical approach *viz.* cultural, cell wall chemotypical, micro-morphological, physiological and molecular characteristics for the identification of the twoactinobacterial strains (VR2 and VR7) isolated from the mangrove rhizosphere sediment of the Vellar Estuary, Bay of Bengal.

SUMMARY AND CONCLUSION

Present study was on the "Isolation, screening and identification of cellulolytic marine actinobacteria from the mangrove sediments of Vellar Estuary, Bay of Bengal".

- The actinobacterial colonies isolated from the mangrove sediment samples of the station of the Vellar estuary using SCNA medium.
- A total of ten morphologically distinct actinobacterial strains were selected. These strains eight were designated as VR1, VR2, VR3, VR4, VR5, VR6, VR7, VR8, VR9 and VR10 were subjected to preliminary screening for cellulase enzymes activity.
- Screening for cellulase enzyme production was made using the Carboxy methyl cellulose agar medium. Strong cellulase enzyme activity was found in the strain VR7(18.3 mm) followed by VR2 (17.7 mm).Further, these strains were showed higher amount of cellulase enzyme production viz. 14.379 1U/ml in VR7 and VR2 (14.213 1U/ml.

- Screening for amylase enzyme production was made using the starch agar medium. Strong amylase enzyme activity was found in the strain VR2 (14 mm). Further, the strain was showed higher amount of amylase enzyme production 67.13 U/ml.
- Based on the performance of enzyme production, the potential actinobacterial strainsVR2 and VR7were selected for further identification using conventional and molecular techniques.
- The cultural and morphological characters were analyzed to identify the cellulase positive strainsVR2 and VR7 and compared with the *Streptomyces* species given in the key of Nonomura (1974) and those described in the Bergy's manual of determinative bacteriology.
- Based on the results, the cellulase positive strain VR2 was tentatively identified as *S. costaricanus*and the strain VR7 was tentatively identified as *S. rubidus*.

The present investigation concludes that the mangrove sediment samples of the Vellar Estuary, Bay of Bengal contain a good diversity of culturableactinobacterial strains of *Streptomyces*. These strains are able to produce cellulase and amylase enzymes. These strains can be further evaluated for the commercial scale production of enzymes and possess vast potential in varied biotechnological and industrial applications.

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