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CHARACTERIZATION OF PECTINASE AND ANTIBACTERIAL ACTIVITY BY USING *Nocardiopsis dasnonivelli* S10 ISOLATED FROM MARINE SAMPLES

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ABSTRACT:

Enzymes are highly efficient and specific biocatalysts which may be intracellular or extra cellular. Actinomycetes are Gram-positive filamentous bacteria with high guanine and cytosine contents. They are unquestionably the most important in the synthesis of antibiotics and other bioactive compounds. Six marine sediment samples from various locations and depths were collected in Visakhapatnam, India, with the aim of isolating actinomycetes from the marine environment. Among all the 120 isolates were screened for antibacterial activity by using cross streak method, around 10 isolates showed antibacterial activity against all the bacteria, 25 isolates showed antibacterial activity only against some bacteria and 85 isolates did not posses any antibacterial activity. isolates S10 showed excellent antibacterial activity. Again of the 120 isolates, eight bacterial isolates showed activity on pectinase indicator plate. However maximum activity was observed for isolate S10 after three days. Zone formation for S10 isolate was observed. The selected strain was identified as a *Nocardiopsis* species based on biochemical tests. Based on molecular characterization, the strain has been closely related to *Nocardiopsis dassonvillei* strain (100% similarity) and designated as *Nocardiopsis dassonvillei* S10

Keywords: Enzymes, Actinomycetes, pectinase and antibacterial activity, biochemical and molecular characteristics, 16s rDNA sequencing, UPGMA tree

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I INTRODUCTION:

Life depends upon a set of organized and regulated chemical reactions that occur rapidly through the mediation of natural catalysts called enzymes. Biocatalysts found in living matter are enzymes. In a cell, almost every reaction involves the presence of a particular enzyme. The Enzyme is defined as a protein that accelerates a biochemical reaction but is not consumed in the process (or) simply a biological catalyst (Smith et al., 1997)[1].

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Enzymes are highly efficient and specific biocatalysts which may be intracellular or extra cellular. Enzymes have found their way into several industrial processes with the advances in protein engineering techniques. In 1960, the first complete enzymatic industrial process was established (Illanes et al.,2008)[2]. Following then, scientific study has led to a rise in the use of enzymes in industry. Moreover, increasing public awareness about the adverse consequences of pollution and laws of environment protection has been forcing industries to replace traditional chemical processes with green chemistry processes involving microorganisms and enzymes, wherever possible (simran jot kaur et al .,2017)[3]. In the above context, increased usage of enzymes is justified because they are natural and biodegradable. The compatibility of enzymes with the environment as well as their ability to increase process efficiency and product specificity has led to expansion of the global enzyme market. The industrially important enzymes include pectinase, xylanase, lipase, laccase, cellulase, phytase, mannanase, β-galactosidase and so on. Pectinases have 25% share in the worldwide sale of the food enzymes (Jayani et al., 2005)[4].The enzymes application in industries has developed as a huge 'Global Enzyme Industry'. In 2019, the worldwide enzymes market was valued at $8,636.8 million, and is expected to reach $14,507.6 million in the year 2027 at a compound annual growth rate (CAGR) of 6.5% from 2020 to 2027 (https://www.alliedmarketresearch.com/enzymes-market)[5].

Henri Braconnot (1825) was the first to isolate and describe pectin. Pectin is a jelly-like matrix cellular polysaccharide found in the main cell walls and middle lamellae of fruits and vegetables (Torres etal, 2006)[6]. Homogalacturonan, Rhamnogalacturonan I, and Rhamnogalacturonan II are three main groups of pectic polysaccharide that all contain D-galacturonic acid to varying degrees. Pectic substances appear to form gel shape when segments of homogalacturonan are cross linked, forming a three-dimensional crystalline network in which water and solutes are trapped. These polysaccharides have been used in the food industry as a detoxifying agent and as a bioactive ingredient (Kusuma et al., 2014)[7]. A broad range of sources including plants and microorganisms such as bacteria including actinomycetes (Beg *et al.,* 2000)[8], yeast (Reid and Ricard, 2000)[9] and fungi (Rasheedha *et al.,* 2010)[10] are used for pectinases production. However, most of the commercially available enzymes are produced by bacteria and fungi (Rasheedha *et al.,* 2010)[10].

Actinomycetes are Gram-positive filamentous bacteria with high guanine and cytosine contents. They are unquestionably the most important in the synthesis of antibiotics and other bioactive compounds. They can produce a number of extracellular hydrolytic enzymes that aid in the degradation and recycling of natural biopolymers (Olano et al. 2009)[11]. Several actinomycetes, including *Nocardiopsis* species, have been isolated from various sites and have a wide range of enzymatic abilities. Kumar et al. (2012)[12] isolated actinomycetes from earthworm castings and screened them for antimicrobial activity and industrial enzymes on another occasion. It was possible to obtain *Streptomyces, Streptosporangium, Saccharopolyspora, Nocardia, Micromonospora, Actinomadura, Microbispora, Planobispora, and Nocardiopsis.*

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II MATERIALS AND METHODS:

II.1. Isolation and screening of actinomycetes for their pectinase activity and determination of their antibacterial activity

II.1.1.Source of sample and sampling site:

Six sediment samples from the Bay of Bengal (Visakhapatnam, India) were obtained by grab sampler and kept in sterile containers for actinomycetes screening. Each sample weighed around 50g and was gathered from several locations (at different distances and different depths).

II.1.2.Screening and isolation of actinomycetes:

Marine sediment samples were maintained at 4^{o}C until isolation. Actinomycetes are isolated by plating suitable dilutions of samples. Actinomycetes colonies are easily identified from fungus and pure bacteria on the plate. They are usually compact, leathery, conical, and have a dry surface. 1 g of each of the following samples was placed in a 250 mL conical flask containing 100 mL of sterile water and agitated on a rotary shaker for 15 minutes. The suspension was serially diluted up to a concentration of 10^{-6} . Isolation was done on starch casein agar (SCA medium one containing distilled water and another sea water)- soluble 10.0g starch; 0.3g vitamin free casein; 2.0g KNO_3; 2.0g NaCl; 2.0g K_2HPO_4; 0.05g $MgSO_4.7H_2O$; 0.02g $CaCO_3$; 0.01g $FeSO_4.7H_2O$; 20.0g agar; 1.0 lt sterilized natural aged sea water; pH, 7.2 and 1.0 lt of distilled water of the same composition enriched with rifampicin 2.5g/ml and cycloheximide 75g/ml to suppress bacterial and fungal contamination, respectively) plates were seeded with 1.0 ml sediment sample suspensions and incubated at 28^{o}C for 14 days (Ramesh and Narayanasamy, 2009)[13]. Actinomycete colonies were carefully separated from various plates after 14 days to avoid bacterial or fungal contamination. The actinomycete colonies that were visually distinct from one another (surface texture) were transferred and incubated at 28^{o}C for 2 weeks before being subcultured in a test tube with the appropriate media. The isolates were combined, and cultures that appeared similar to the naked eye in terms of aerial mycelium colour, reverse colour, soluble pigment, and colony texture were removed.

II.1.3.Antibacterial activity studies:

The test organisms (*Bacillus cereus, Bacillus subtilis ,Staphylococcus aureus, Pseudomonas auroginosa, Klebsiella pneumonia ,Escherichia coli)* for this research were obtained from the National Collection of Industrial Microorganisms (NCIM) at the National Chemical Laboratory (NCL) in Pune, India, and the Microbial Type Culture Collection (MTCC) at the Institute of Microbial Technology (IMTECH) in Chandigarh, India

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II.1.4. Primary cross-streak method:

The cross streak technique was used to test the marine actinomycete isolates for antibacterial activity (Venkata et al., 2011)[14] on agar plates containing equal amounts starch casein agar (SCA) and nutrient agar. Each plate was streaked with a single isolate through the centre and incubated at 28°C for 5 days. After 5 days, test organisms were streaked perpendicular to the growth of the actinomycete culture. After 24 hours of incubation, the intensity of inhibition produced by each isolate against the test bacteria was measured. As a control, a plate with the same medium and without actinomycete streaking but with test organism streaking was maintained.

II.1.5.Selection of promising isolates using well diffusion method:

The antibacterial activity of the test bacteria was measured using the well diffusion method on nutrient agar medium. For the well diffusion method, nutrient agar plates were used. Molten sterile nutrient agar was cooled to approximately 45°C before being inoculated with test bacteria, thoroughly mixed, poured into sterile petriplates, and allowed to settle. A sterile cork borer was used to make wells in the solidified agar plates. Using a micropipette, the clear supernatant from the fermentation broth (antibacterial metabolite produced by the actinomycetes test isolates) was added to each well (50µl). The plates were allowed to cool for about 2 hours to allow antibiotic diffusion before being incubated at 37ºC. The inhibition zones were recorded after 24 hours. Experiments were carried out in triplicate, with the average of the three default trails used to calculate the results. The method was carried out for each of the potential active isolates. identified during primary screening (Venkata et al., 2011)[14].

II.1.6.Submerged fermentation:

Actinomycetes isolates with well-sporulated slants (7 days old) were used for production for antibacterial metabolite studies. Five millilitres of sterile distilled water were transferred into each slant aseptically, and the isolate which is present on surface of the medium was scraped with a sterile inoculating loop and transferred into 45 millilitres of production medium and incubated at 28°C on a oribital shaker at 180 rpm for seven days. After seven days the samples taken out from the oribital shaker, were then placed in sterile falcon tubes and centrifuged at 10000 rpm for 20 minutes at 4°C to separate the clear culture filtrate. Using the well diffusion method, the clear supernatant was used for antibacterial assay (Venkata et al., 2011)[14].

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II.1.7.Screening of isolates for Pectinase Activity:

Actinomycetes were streaked on pectin-enriched agar plates containing (g/l) pectin – 5, yeast extract – 5, and agar – 16. (pH 7.0). Plates were incubated at 28°C for three days. Clear zones were observed after 3 days using a 1% cetrimide solution (Beg et al., 2000)[8].

II.2.Identification of the isolate:

Identification of isolate was carried out by studying their morphological, physiological, biochemical and molecular characteristics by standard method. Bacterial isolate was identified by using Bergey‘s manual of determinative bacteriology (Holt *et al*., 1994)[15].16s rDNA sequencing was done for the final evaluation of isolated bacteria and the sequence was amplified by using Universal primers forward and reverse primers, 704F (5'GTAGCGGTGAAATGCGTAGA3') and 907RC (5' CCGTCAATTCCTTTGAGTTT3'). The obtained sequence was then compared with the other NCBI databases. Further to this, multiple sequence alignment was carried out by MEGA 6.0 software to construct a UPGMA tree (Tamura et al., 2013)[16].

III .RESULTS AND DISCUSSION:

III.1.Isolation and screening of actinomycetes for their pectinase activity and determination of their antibacterial activity:

Six marine sediment samples from various locations and depths were collected in Visakhapatnam, India, with the aim of isolating actinomycetes from the marine environment. On the plate, colonies of actinomycetes are easily appears from fungi and true bacteria. They are oftenly leathery, compact, conical in appearance, and also have a dry surface. The isolates were combined, and cultures that appeared identical to the naked eye in terms of aerial mycelium colour, soluble pigment, colony texture, and reverse colour were eliminated. From the six marine sediment samples, altogether 120 isolates belongs to actinomycetes were isolated and were designated as A batch (A1–A25), B batch (B1–B13), C batch (C1-C21), D batch (D1-D14), E batch (E1-E15), S batch (F1-F32) (Table 3.2, Fig. 4.1).

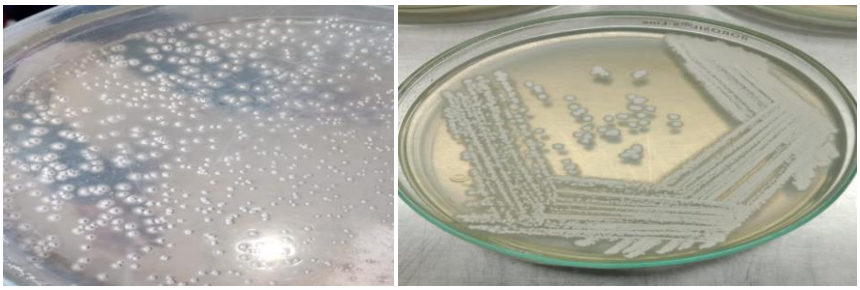

Fig. 1. Actinomycetes isolation from marine sediment samples using SCA medium (shows the distinct features of actinomycetes having compact, leathery, with a dry surface and conical appearance)

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III.1.2. Determination of antibacterial activity of isolated marine actinomycetes

III.1.2. 1 Cross-streak method

Among all the 120 isolates were screened for antibacterial activity by using cross streak method, 8 isolates (A3, B11, C8, D13, E5, E11, S3, **S10)** were screened for antibacterial activity by using cross streak method, around 4 isolates showed antibacterial activity against all the bacteria, 4 isolates showed antibacterial activity only against some bacteria. For the preliminary screening, all observations were made with the naked eye and different intensities of inhibition were observed and noted as following: **"N", "G" and "E" represents Normal, Good and Excellent / Significant Inhibition zone, respectively.** The following test organisms were used for the antibacterial activities by cross streak method: *Bacillus subtilis (B. sub), Staphylococcus aureus (S. aur), Klebsiella pneumonia (K. pne), Bacillus cereus (B. cer), Pseudomonas auroginosa (P. aur)* and *Escherichia coli (E. coli).*

Table 1: Antibacterial activities of series isolates by cross streak method

Series	***S. aur***	***B. sub***	***K. pne***	***B. cer***	***P. aur***	***E. coli***
	Inhibition Zone					
A3	N	G	N	G	N	G
B11	G	E	N	G	N	G
C8	G	-	G	N	-	-
D13	-	N	G	-	-	N
E5	G	N	G	G	N	E
E11	N	-	N	G	-	N
S3	-	N	-	G	N	-
S10	E	N	G	G	E	N
N , G and E represents Normal, Good and Excellent inhibition zone, respectively						

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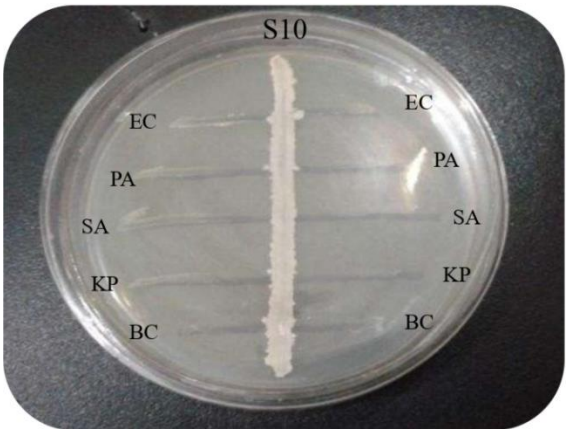


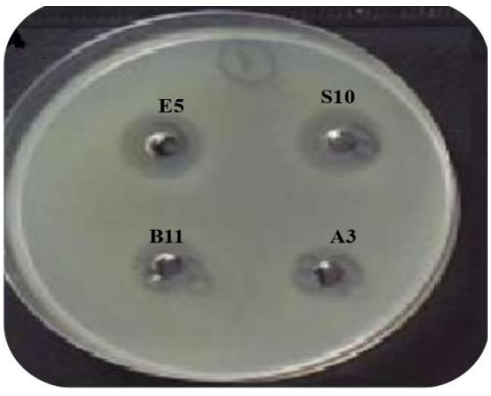
Fig. 2. Isolate S10 showing significantintensity of inhibition

III.1.3..Selection of promising isolates using cup-plate method

Even though 4 isolates showed very good antibacterial activity out of 10 marine isolates studied; isolates S10 showed excellent antibacterial activity (Table 2, Fig. 4.3); and thus selected as the best isolates and used for further studies.

Table 2. Cup-plate method was used to test the antibacterial activity of selected marine sediment isolates (inhibition zone in mm)

Series	***S. aur***	***B. sub***	***K. pne***	***B. cer***	***P. aur***	***E. coli***
	Inhibition Zone Diameter (in mm)					
A3	25	23	17	14	-	18
B11	14	18	-	15	12	17
E5	26	17	15	25	18	14
S10	**20**	**16**	**32**	**29**	**14**	**17**



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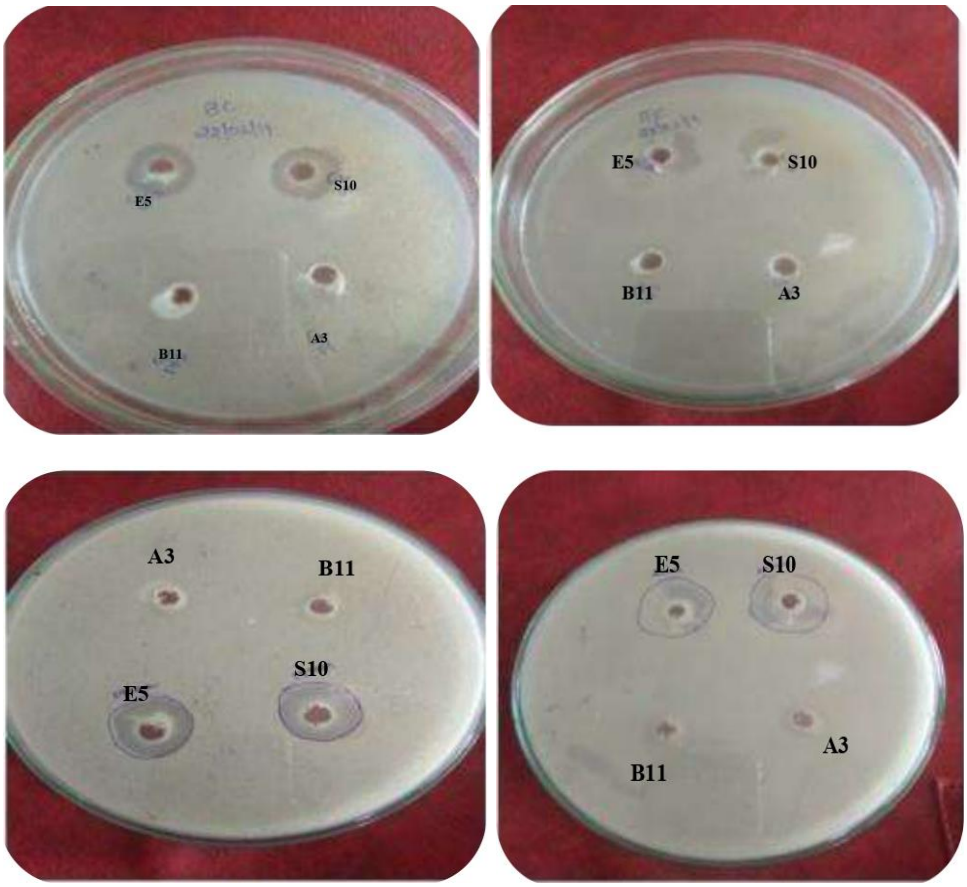

Fig. 3. Antibacterial activities of the selected marine isolate A3, B11, E5, S10 by cup- plate method against test bacterial organisms. Zones shows the inhibition area against the tested bacteria by the isolates

III.1.4..Screening of isolates for Pectinase production:

Of the 120 isolates, eight bacterial isolates showed activity on pectinase indicator plate. However maximum activity was observed for isolate S10 after three days. Zone formation for S10 isolate is as shown in figure.

Table 3 Zone formation for potential Pectinase isolates

Isolates	**Zone size(mm)**
A3	20
B11	15
C8	13
D13	8

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E5	23
E11	17
S3	11
S10	**26**

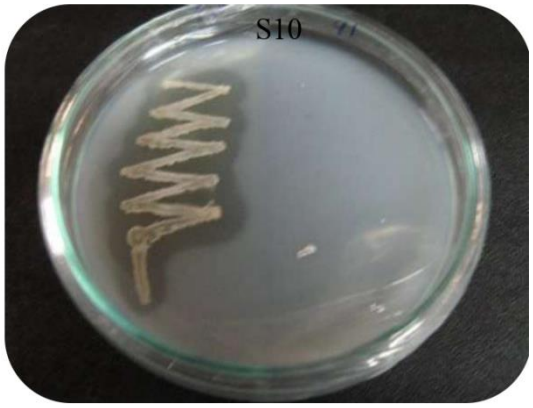

Fig.4 Zone formation for S10 isolate

III.2.Taxonomic and biochemical identification of isolate S10

The S10 isolate colony morphology was observed on starch casein agar plate on 14th day. The isolate S10 exhibits growth on agar plate as small, spherical, convex and cream colonies. Microscopic observation shows that cells are gram positive, filamentous shaped and non motile (Fig. 4.5).The isolate S10 was tested for phenotypic and biochemical activities. The biochemical tests showed that the strain is gram positive, catalase positive and also positive for MR and nitrate reduction tests. It could not utilize citrate as sole carbon source (Table 4.3).

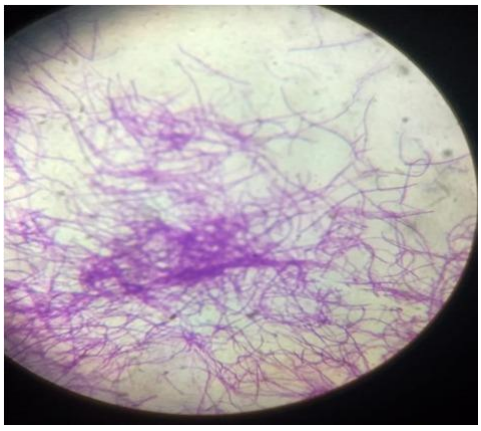

Fig. 5 Isolate S10 cells observed under microscope at 100x magnification.

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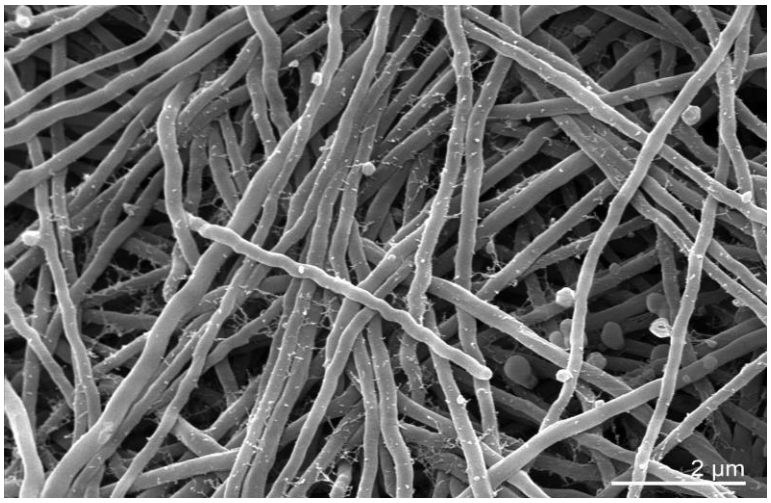

Fig. 4.6 Isolate S10 cells observed under Scanning Electron microscope at 7000x magnification

Table 4 A summary of the isolate S10 morphological characteristics

Sl.No	Morphological Test	S10 Isolate
	Colony morphology	
1.	Configuration	Irregular
2.	Margin	Uneven
3.	Elevation	Slight rinse
4.	Surface	Rough
5.	Texture	Mucoid
6.	Pigment	Off White
7.	Opacity	Opaque
8.	Gram's reaction	+
9.	Motility	-
10.	Spore(s)	+
11.	Cell shape	filamentous shaped

Table 5 S10 isolate biochemical characterization summary

Sl.No	Biochemical Test	Isolate S10
1.	Casein	+
2.	Voges Proskauer test	_
3.	Nitrate	+
4.	Indole	_
5.	Methyl red test hydrolysis	+
6	Citrate	_
7	Ornithin	_
8	Lysine	_
9	Arginine	_

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10	Oxidase test	+
11	Catalase test	+
12	Esculin hydrolysis	+
13	Starch hydrolysis	+
14	Gelatin hydrolysis	+

+: Positive ; – : Negative

When the above data were compared to Bergey's handbook of determinative bacteriology, it was determined that isolate S10 belonged to the *Nocardiopsis species*.

III.2.1.16 s r DNA Sequencing analysis (1200bp)

The 16s rRNA gene sequence similarity between the isolate S10 and its nearest neighbour in the *Nocardiopsis* genus, *Nocardiopsis dassonvillei* strain S10 was 100%. Based on the 16s rRNA gene the phylogenetic tree of isolate S10 and its closest representatives of the family *Nocardiopsaceae* and related taxa obtained by NCBI BLAST search using BLASTN program by UPGMA tree method was constructed.Based on the physiological and morphological characteristics of the isolate S10,sequence analysis of its gene encoding 16s rRNA confirmed that the isolate belonged to *Nocardiopsis* genus and according to BLAST evidence, it was *Nocardiopsis dassonvillei* (Fig.4.7). The amplified PCR product size was ~1200 bp. Therefore from biochemical and morphological characteristics and 16s rRNA sequence comparison, isolated S10 was designated as *Nocardiopsis dassonvillei*. The nucleotide sequence of the16srRNA of *Nocardiopsis dassonvillei* was submitted to GenBank (accession number ON183151).

>S10
TCAGGACGAACGCTGGCGGCGTGTATAACACATGCAAGTCGAGCGGTAAGGCCCTT
CGGGGTACACGAGCGGCGAACGGGTGAGTAACACGTGAGCAACCTGCCCCTGACTC
TGGGATAAGCGGTGGAAACGCCGTCTAATACCGGATACGACCCTCCACCTCATGGT
GGAGGGTGGAAAGTTTTTCGGTCAGGGATGGGCTCGCGGCCTATCAGCTTGTTGGTG
GGGTAACGGCCTACCAAGGCGATTACGGGTAGCCGGCCTGAGAGGGCGACCGGCCA
CACTGGGACTGAGACACGGCCCAGACTCCTGCGGGAGGCAGCAGTGGGGAATATTG
CGCAATGGGCGAAAGCCTGACGCAGCGACGCCGCGTGGGGGATGACGGCCTTCGGG
TTGTAAACCTCTTTTACCACCAACGCAGGCTTCCAGTTCTCTGGAGGTTGACGGTAG
GTGGGGAATAAGGACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTC
CGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCGTGTCGCGTCTG
CTGTGAAAGACCGGGGCTTAACTCCGGTTCTGCAGTGGATACGGGCATGCTAGAGG
TAGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAG
GAACACCGGTGGCGAAGGCGGGTCTCTGGGCCTTACCTGACGCTGAGGAGCGAAAG
CATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCG

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CTAGGTGTGGGGACTTTCCACGGTTTCCGCGCCGTAGCTAACGCATTAAGCGCCCCG
CCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACA
AGCGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGTTTG
ACATCACCCGTGGACTCGCAGAGATGTGAGGTCATTTAGTTGGCGGGTGACAGGTG
GTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGC
GCAACCCTTGTTCCATGTTGCCAGCACGTAATGGTGGGGACTCATGGGAGACTGCCG
GGGTCAACTCGGAGGAAGGTGGGGATGACGTCAATCATGCCCCTTATGTCTTGGGCT
GCAAACATGCTACAATGGCCGGTACAATGGGCGTGCGATACCGTAAGGTGGAGCGA
ATCCCTAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGGT
GGAGTCGCTAGTAATCGCGGATCAGCAACGCCGCGGTGAATACGTTCCCGGGCCTT
GTACACACCGCCCGTCACGTCATGAAAGTCGGCAACACCCGAAACTTGCGGCCTAA
CCCCTTGTGGGAGGGAGTGAGTGAAGGTGGGGCTGGCGATTGGGACGAAGTCGTAA
CAAGGTAGCCGTACCGGAAGGTGCGG

Fig.6: The DNA sequence for 16s rRNA gene of *Nocardiopsis dassonvillei* S10

Table 6.16s rRNA gene sequence similarity values between *Nocardiopsis dassonvillei* and the representatives of the genus *Nocardiopsis*.

Description	**Scientific Name**	**Max Score**	**Total Score**	**Query Cover**	**E value**	**Per. Ident**	**Acc.Len**	**Accession**
Nocardiopsis dassonvillei strain NOCA502F chromosome, complete genome	*Nocardiopsis dassonvillei*	2723	22854	100%	0	99.6	6585441	CP017965.1
Nocardiopsis dassonvillei strain VTT E-062968 16S ribosomal RNA gene, partial sequence	*Nocardiopsis dassonvillei subsp. dassonvillei*	2723	4581	100%	0	99.6	1512	EU430534.1

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Nocardiopsis sp. strain 10K510 16S ribosomal RNA gene, partial sequence	*Nocardiopsis sp.*	2717	4570	100%	0	99.53	1496	MG770819.1
Nocardiopsis sp. strain 5K521 16S ribosomal RNA gene, partial sequence	*Nocardiopsis sp.*	2717	4570	100%	0	99.53	1497	MG770745.1
Streptomyces sp. Ahbb4 16S ribosomal RNA gene, partial sequence	*Streptomyces sp.* Ahbb4	2684	4504	100%	0	99.13	1523	KM214828.1
Actinomycetales bacterium XJ-1 16S ribosomal RNA gene, partial sequence	*Actinomycetales* bacterium XJ-1	2682	4535	99%	0	99.53	1500	EU882851.1
Actinomycetales bacterium XJ-6 16S ribosomal RNA gene, partial sequence	*Actinomycetales* bacterium XJ-6	2676	4524	99%	0	99.46	1500	EU882854.1
Nocardiopsis synnemataformans DSM 44143 strain NBRC 102581	*Nocardiopsis synnemataformans* DSM 44143	2654	4454	99%	0	98.92	1487	NR_112742.1

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16S ribosomal RNA, partial sequence								
Nocardiopsis synnemataformans strain AlS2 16S ribosomal RNA gene, partial sequence	*Nocardiopsis synnemataformans*	2645	4460	99%	0	98.79	1488	MF321793.1

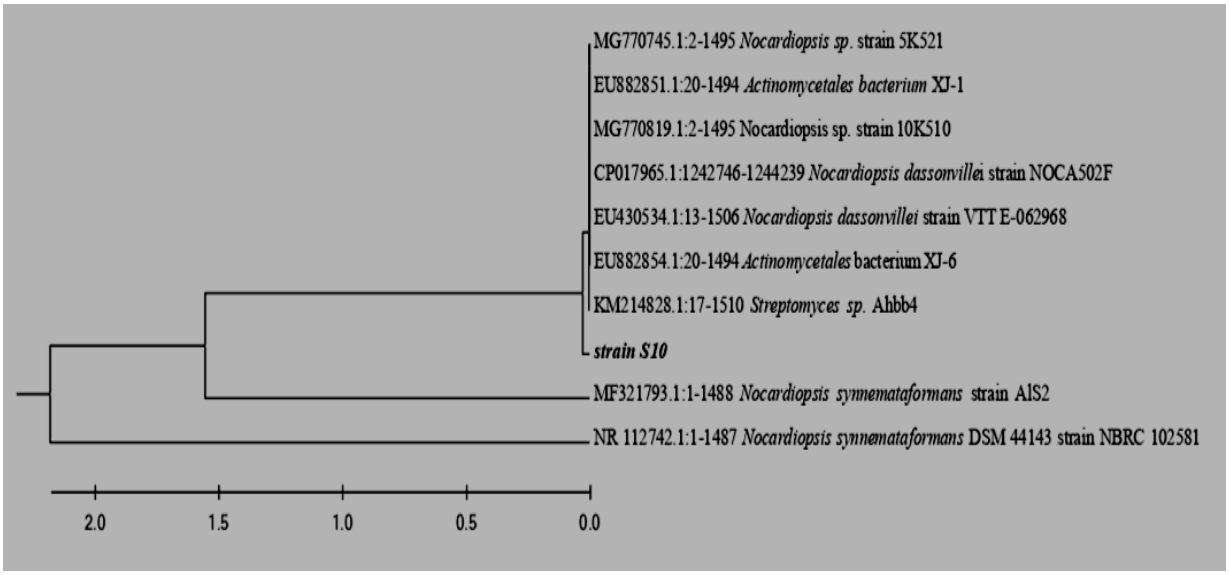

Fig. 7: UPGMA tree based on nearly complete 16S rRNA gene sequence shows similarities between isolate S10 and members of the *Nocardiopsaceae* family and allied taxa.

IV.CONCLUSION:

Altogether 120 actinomycetes were isolated from the six marine sublimate samples collected from Visakhapatnam, India. After preliminary screening programme by cross streak method, 10 isolates with good inhibition were chosen for extracellular antibacterial activity assessments using the cup-plate method of submerged fermentation. Among the four active isolates, S10 showed good antibacterial activity against Gram positive and Gram negative bacteria. All the 120 isolates were streaked on to the pectin agar media pertri plates and observed for growth. After observation of growth, clear zone was visualized using 1% cetrimide solution. From out of 120 isolates, 8 isolates showed pectinase activity and in that isolate S10 showed maximum

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activity. The isolate S10 was best pectinase producer comparatively hence, for further studies it was selected. The selected S10 isolate was identified as a *Nocardiopsis* sp. by performing of biochemical parameters and *Nocardiopsis dassonvillei* strain with the help of 16s rRNA gene sequencing.

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CONFLICTS OF INTEREST:

The authors declare that they have no conflict of interest.

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