ijlpr 2020; doi 10.22376/ijpbs/lpr.2020.10.3.P52-59

International Journal of Lifescience and Pharma Research

Research Article

Marine Biotechnology for better medical care

Isolation and Characterization of Novel Marine Bacterium from the Marine Soil Sample and its Antimicrobial Efficacy of the Secondary Metabolites

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Abstract: The marine bacterium was widely recognized as a natural manufacturer of novel bioactive secondary metabolites during the last decades, making the marine ecosystems a huge treasure of novel isolates. Marine coastal areas of the southern part of India have huge varieties of ecosystems that produce many marine microbes and organisms which are considered as a perennial source of many potential drug leads. This study focuses on the isolation of marine soil samples collected from the shores of the Bay of Bengal at Kanyakumari, Tuticorin, and Mandapam and followed by the characterization of the novel marine bacterium from that soil sample for the secondary metabolites production with potential pharmacological importance. The isolated pure culture from colonies was cultured and taxonomically characterized by gram staining and other supported biochemical tests. Further, from the genetic analysis results through 16s rDNA sequencing and by comparing the nucleotide homology and phylogenetic analysis of the organisms it was confirmed as *"Paenibacillus dendritiformis* and *Paenibacillus thiaminolyticus"*. Moreover, it was found that the bacterial isolates produced the inhibitory activity to other bacterial strains including both gram-positive and gram-negative bacteria at a concentration of 32 µl/ml. Thus, the bacterial isolates were found to produce secondary metabolites that comprise antimicrobial components and enzymes of commercial importance. Henceforth, this preliminary study looks promising and may lead to the discovery of potential antibiotics and other bioactive compounds.

Keywords: Marine soil bacteria, secondary metabolites, *Paenibacillus sp*., antibacterial activity, 16s rDNA sequencing, phylogenetic analysis

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Recieved On 18 December 2019
Revised On 10 March 2020
Accepted On 13 March 2020
Published On 02 July 2020

Funding This research did not receive any specific grant from any funding agencies in the public, commercial or not for profit sectors.

Citation Subbiah Latha, Palanisamy Selvamani and Thangavelu Prabha , Isolation and Characterization of Novel Marine Bacterium from the Marine Soil Sample and its Antimicrobial Efficacy of the Secondary Metabolites.(2020).Int. J. Life Sci. Pharma Res.10(3), P52-59 http://dx.doi.org/10.22376/ijpbs/lpr.2020.10.3.P52-59

1. INTRODUCTION

Marine biotechnology is a rapidly developing field in the branch of bioscience, in which huge research is underway to explore the marine organisms and resources either a part or as a whole. Due to the advancement of molecular and biotechniques, scientists have been able to elucidate novel approaches in both aquatic and terrestrial microorganisms. Oceans are found to be a gorgeous arena and great efforts have been accomplished worldwide aiming the isolation of novel molecules and biotherapeutic products from marine organisms. Though, oceans cover more than 70% of the earth's surface, very little is known about the organism and microbial diversity of marine ecosystems and sediments. The oceanic atmosphere of the Indian peninsula is ironic in microbial diversity. However, the prosperity of marine micro-flora has not been entirely explored to date.[1] Marine microorganisms seemed to have adapted brilliantly to the wide range of environmental stresses that occurs in the aquatic atmosphere, thus have added distinct attention because of their assorted metabolic abilities. The marine environment is a prolific resource for the isolation of many scientifically unexplored microorganisms. In recent years, marine microorganisms have become a potential natural source for producing the novel microbial products[2] exhibiting antimicrobial, antiviral, antitumor as well as anticoagulant and cardioactive properties. These bioactive compounds may serve as model systems in the discovery of new drugs.[3-5]When compared with terrestrial soils, marine sediments contain limited amounts of readily available organic matter, with most sources of carbon being present in complex forms (i.e., cellulose and chitin). Hence, the marine environment provides a spectrum of molecules and metabolites that possess both chemical and biological diversity. However, culture-independent methods have demonstrated that marine sediments contain a wide range of unique microorganisms not present in the terrestrial environment.[6,7] The present study was aimed to isolate, identify and to optimize the nutritional requirements, and taxonomic characterization of the isolated organisms obtained from the marine soil samples. Here, we describe a new selective enrichment procedure that has resulted in the discovery of two novel marine bacteria from marine soil collected from the shores of the Bay of Bengal at Kanyakumari, Tuticorin and Mandapam. A polyphasic approach including morphological and chemotaxonomic analyses as well as phylogenetic analysis based on 16S rDNA gene sequences has led to the identification of two novel marine bacteria within the family *Paenibacillus sp*. Evidence is also provided that these new marine-derived bacteria produced a variety of bioactive secondary metabolites.

2. MATERIALS AND METHODS

2.1 Sample collection

Marine soil samples were collected from the shores of the Bay of Bengal at Kanyakumari and Tuticorin in the first phase in May 2005 and from Mandapam in the second phase in August 2005. These samples were collected in sterile containers and were stored in a refrigerator for further use. The isolation of the marine bacteria from these soil samples was carried out by the spread plate technique.[8]

2.2 Growth optimization

The isolated organism was arbitrarily optimized for its growth requirements by altering pH, temperature, and sodium chloride content based on experiments that were done to verify the actual requirements using the Broth culture-Turbidity Method.[9]

2.3 Characterization of the test organism

2.3.1 Gram staining

Gram staining was performed for the isolated colony according to the standard procedure. A smear of bacterial cells was prepared on a clean glass slide by a gentle heat fixation. The heat-fixed smear was flooded with a crystal violet solution for one minute. Smear was washed with water followed by adding mordant Gram's iodine. The smear was decolorized with 95% ethyl alcohol and washed with water. Finally, safranin was used to counterstain the culture for 60-80 sec and washed with water. Stained cells were then examined under a microscope. Simple and acid-fast staining also performed for the smooth identification of bacterial isolates.[10]

2.3.2 Identification and Characterization of Bacteria

The shape, size and arrangement of the isolate and biochemically characterized by various tests like Indole, MR, VP, Citrate, TSI, Nitrate Reduction, Catalase, Urease, Oxidase, Starch Hydrolysis, Gelatin Utilization, Decarboxylase and Phenylalanine - Deaminase Test including carbohydrate fermentation.[11]

2.3.3 DNA Sequencing and PCR amplification

Genomic DNA was isolated from the pure culture pellet provided. Using consensus primers, ~1.5 kb 16S rDNA fragment was amplified using high-fidelity PCR Polymerase. The PCR product was cloned and plasmid DNA was bi-directionally sequenced using the forward and reverse primer. Sequence data were aligned and analyzed for finding the closest homologs for the microbe. Furthermore, using the BLAST tool, the determined sequence was compared with the sequences deposited in NCBI GenBank and the accession number was obtained.[12]

2.3.4 Phylogenetic analysis

The 16S rDNA nucleotide sequence obtained from the PCR product. A BLAST of an acquired series was performed with that of available EMBL database using the site http://www.ncbi.nlm.nih.gov/genebank and the 16S rDNA nucleotide sequence was subjected to phylogenetic analysis using bioinformatics tool available in online using software Mega 3.1.[13]

2.3.5 Fermentation Process

A 250 ml conical flask was used as the fermentation vessel. The nutrient medium was autoclaved at 121°C for 15 minutes and a loop of inoculum was added to the media for the growth of an organism. The culture vessels were placed inside the air bath shaker, at the speed of 60 rpm and allowed it to ferment for 3 days under the room

temperature of 37°C and maintained in a shaker. After 3 days of fermentation, dilute HCl is added to the culture broth.

2.3.6 *Isolation and Purification of secondary metabolites*

The selection of the solvent is crucial for solvent extraction. The isolation of secondary metabolites was achieved by the solvent extraction method.[14] The organic solvent, ethyl acetate and an equal volume of the sample were shaken vigorously followed by the addition of HCl. The precipitates were formed and the cell debris was removed by centrifugation method. Due to the cell wall breakage, the contents of the cell weredissolved in the solvent which was then extracted. The supernatant fluid was evaporated by keeping it in a water bath to obtain the crystals. By using column chromatography, the obtained crystalline microbial metabolites were purified.

2.3.7 Evaluation of Antimicrobial Activity

Antimicrobial activity of the marine microbial metabolites was tested on the pathogenic microorganism by well diffusion method. The wells were filled with different amounts of the secondary metabolite solution in a concentration of 4 µl, 8 µl, 16 µl and 32 µl. The plates were incubated at 37°C for 24 h. Antimicrobial activity of each sample against the test bacteria was indicated by growth free "Zone of inhibition" near the respective wells.[15,16]

3. RESULTS AND DISCUSSION

3.1 Characterization of the test organism

The diversity of terrestrial bacterium has been of extraordinary significance in several areas of science and medicine. The decreasing rate of discovery of novel drugs from established terrestrial sources has motivated the evaluation of new sources of chemically diverse bioactive compounds.[17] The oceans represent an underexplored environment for microbial discovery and although new methods are under development,[18] relatively few have been applied to reveal the microbial diversity of the ocean environment. Microorganisms can live in the environment along with humans and in extreme conditions such as hot springs, miles deep in the ocean, inside rocks and extremely cold temperatures.[19] Although the nature and number of microorganisms vary in different places, it has been indicated that the mass of carbon from these microorganisms could be trillions of tons.[20-22] Most of the bacteria in the soil remain unidentified. Both academic and industrial scientists realized that soil bacteria are a potential source to find unique biologically active metabolites and novel commercially important products. Bacteria have been found as a source of producing many valuable chemicals including ethanol, acetone, enzymes, perfumes and antibiotics. In the last few decades, thousands of antibiotics have been discovered.[23-25] In our study, the morphology of the isolated bacterium had played a major role in distinguishing bacteria and in the characterization of bacterial species. The morphological characteristics of the various organisms were determined by the method described by Shirling and Gottlieb.[26] The isolated organism was found to be rod-shaped; Gram-negative bacteria and it was optimized for its favourable growth conditions. The optimized growth parameters were of the temperature between 40 to 50°C (since it showed maximum growth at this temperature), the optimized pH was at 7 and the growth media concentration was 2% Sodium Chloride. However, the microscopic analysis of some bacteria was found to be round in shape and some were rod-shaped. The results obtained in this study were consistent with the previous studies reported elsewhere.[27,28] These soil microorganisms may be an important source of producing chemicals having biochemical and pharmacological importance. Although many microbial metabolites have been discovered as bioactive molecules, there might be many more products yet to be discovered from the soil as well as marine microorganisms.[29] After performing various biochemical tests for the characterization of selected and isolated marine microorganism it was found that the organism was able to produce catalase enzyme as it showed positive results by giving vigorous bubbling on the addition of hydrogen peroxide solution in the catalase test. Moreover, the organism also produced urease enzyme which was indicated by pink colour in the medium on the addition of phenol red. The organism used citrate as the carbon source, ammonium salts as the nitrate source and hence the media turned deep blue upon evaluation in the citrate utilization test. In Voges-Proskauer test glucose was converted into acetoin and turned the medium to red colour. The organism was able to produce decarboxylase enzyme which turned the media to purple colour on the addition of Bromocresol purple. The results were tabulated in Table.1. Based on the biochemical characterization and fermentation of sugars the isolate was identified as *Paenibacillus* sp.

Table 1. Consolidated results of Biochemical Tests of the isolated marine bacteria

S.No	Biochemical Tests			Results	
1.	Simple Stain			Slightly Violet	
2.	Gram Stain			Gram-Negative	
3.	Acid Fast Stain			Negative	
4.	Catalase Activity			Positive	
5.	Urease Activity			Positive	
6.	Nitrate Reduction			Negative	
7.	Starch Hydrolysis			Negative	
8.	Gelatin Hydrolysis			Slightly Positive	
9.	Citrate Utilisation			Positive	
10.	Indole Production			Negative	
11.	Oxidase Test			Negative	
12.	Methyl Red Test			Negative	
13.	Voges - Proskauer Test			Positive	
14.	Decarboxylase Test			With Lysine	Without Lysine
				Positive	Positive
15.	Carbohydrate Fermentation			Acid	Gas
	Lactose			Positive	Positive
	Dextrose			Positive	Negative
	Sucrose			Positive	Positive
	Glucose			Positive	Positive
16.	Hydrogen Sulphide Production			Negative	
17.	Phenylalanine Deaminase Test			Negative	
18.	TSI Test	Alkaline Slant	Acid Butt	H^2S	Gas
		Negative	Positive	Negative	Positive

Further, the isolated marine microorganism was subjected to Genetic analysis by 16s rDNA sequencing followed by phylogenetic analysis. The results were identified based on nucleotide homology and phylogenetic analysis the microbe was detected to be *Paenibacillus dendritiformis* (GenBank Accession Number: AJ320490) (Table 2). The nearest homolog species was found to be *Paenibacillus thiaminolyticus* (GenBank Accession Number: EU330645). The aligned sequence data were presented here.

3.2 Aligned Sequence Data: (1457 bp)

TGCCTAATACATGCAAGTCGAGCGGAGTGGATGTAGTGCTTGCACTCCTGATGCTTAGC
GGCACACGGGTGTGTAACGCGTAGGTAACGTGCCCTTAACTGCGGGATAACTCGGGG
ACACGTGCTCTTATACCGGATAGGCGATTTCCTCGCATGTGGAAATCGGGAAAGGCCG
AGCAATCTGCCTTTTGTGGATGGCCCTACGGCGCATTATCTAGTTGGTGGTATAACCTC
TCCCCGGGGCGACGATGCATAGCCGACCTGAGAGGGAGATCGGCCACACTGGCACTG
ACACACGCCCCACACTCCTACGAGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGCA
AGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTT
GCCAGGGAAGAACGCTATGGAGAGTAACTGTTCCATAGGTGACGGTACCTGAGAAGAA
AGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTC
CGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCATGTAAGTCTGGTGTTTAAACCC
GGGGCTCAACTCCGGGTCGCATCGGAAACTGTGTGACTTGAGTGCAGAAGAGGAAAGT
GGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAG
GCGACTTTCTGGGCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGA
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGCTAGGTGTTAGGGGTTTCGAT
ACCCTTGGTGCCGAAGTTAACACATTAAAGCATTCCGCCTGGGGAGTACGGTCGCAAG
ACTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCAGTGGAGTATGTGGTTTAATT
CGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCTCTGACCGTCCTAGAGATA
GGGCTTCCCTTCGGGGCAGAGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTC
GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAACTTTAGTTGCCAGCATT
GAGTTGGGCACTCTAGAGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACG
TCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTACTACAATGGCTGGTACAAC
GGGAAGCGAAGCCGCGAGGTGGAGCGAATCCTAAAAAGCCAGTCTCAGTTCGGATTG
CAGGCTGCAACTCGCCTGCATGAAGTCGGAATTGCTAGTAATCGCGGATCAGCATGCC
GCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTTTACA
ACACCCGAAGTCGGTGGGGTAACCGCAAGGAGCCAGCCGCCGAAGGTGGGGTAGATG
ATTGGGGTG

Table 2. DNA sequencing Alignment View using a combination of NCBI GenBank and RDP database

Alignment View	ID	Alignment results	Sequence description
	TC1	0.84	Studied sample
	AY359885	0.95	Paenibacillusdendritiformis
	EU330645	0.95	Paenibacillusthiaminolyticus strain 8118
	AJ320490	0.93	Paenibacillusthiaminolyticusst. DSM 7262T
	AB073198	0.98	Paenibacilluspopilliae
	AB073199	0.99	Paenibacilluslentimorbus
	AB073201	0.86	Paenibacillusapiarius
	EF190502	0.94	Paenibacilluslentimorbus strain DNG 15
	DQ232773	0.96	Paenibacilluscampinasensis strain BL11
	EF190495	0.99	Paenibacilluspopilliae strain BPHD
	AB045092	0.91	Paenibacillusdendritiformis

On identifying the isolates at the species level, the 16S rDNA sequence of strain *Paenibacillus sp.* was determined and compared to those deposited in GenBank using the BLAST and BIBI, a Bioinformatics Bacterial Identification Tool (Fig. 1) shows a phylogenetic tree derived from these sequences. It was observed that the sequence was similar to those sequences of more than one species (Table 3). Species identification was performed based on a maximum score, identity and coverage values. Although 16S rDNA sequencing has been used increasingly for bacterial identification in clinical microbiology laboratories there are some limitations of this technique like the ever-expanding sequence databases, taxonomic complexity and the inaccuracies in some databases.[30,31] The use of 16S rDNA sequencing for bacterial identification depends on significant interspecies differences and small interspecies differences in 16S rDNA sequences. Therefore, one of the major limitations is that when two different bacterial species share almost the same 16S rDNA sequence, this technique would not be useful for distinguishing between them.[32] However, there are 'blind spots' within some major genera, in which 16S rDNA sequences are not sufficiently discriminative for the identification of certain species.[33,34] The 16S rDNA gene sequencing is highly useful in regards to bacterial classification; it has low phylogenetic power at the species level and poor discriminatory power for some genera.[35,36] Considering these limitations, to increase the accuracy of 16S rDNA sequencing for the identification of bacteria, it would be necessary to interpret the results of 16S rDNA sequencing with preliminary morphological, cultural, biochemical and physiological tests results. The results obtained on analyzing various characteristics and phylogenetic studies based on partial sequences of the 16S rDNA region encoding gene demonstrate that though the strain *Paenibacillus dendritiformis* (AJ320490) and *Paenibacillus thiaminolyticus* (EU330645) is most closely related to the organism *Paenibacillus sp.* in phylogenetic development.

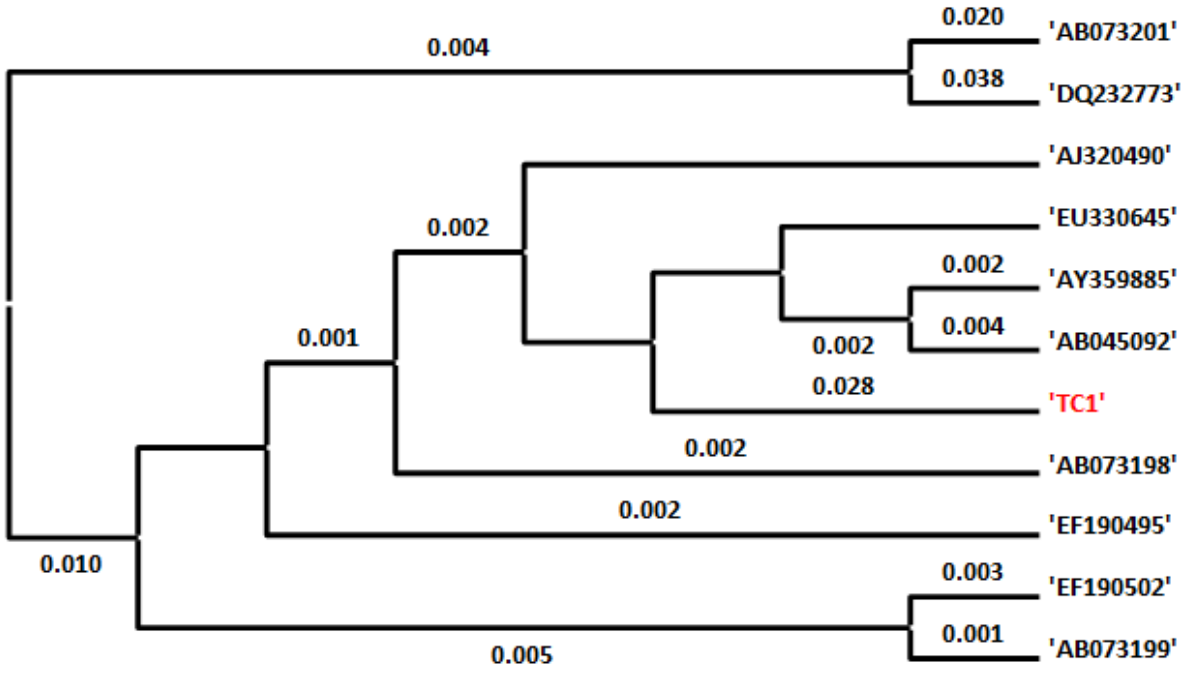

Fig 1. Phylogenetic Tree using Neighbour Joining method

Table 3. ***Distance Matrix based on Nucleotide Sequence Homology (Using Kimura-2 Parameter)***

Distance Matrix											
		1	2	3	4	5	6	7	8	9	10	11
AJ320490	1	---	0.964	0.999	0.988	0.996	0.944	0.996	0.994	0.994	0.990	0.971
AB073201	2	0.036	---	0.963	0.957	0.963	0.942	0.960	0.961	0.957	0.958	0.938
EU330645	3	0.001	0.037	---	0.988	0.995	0.943	0.997	0.995	0.995	0.991	0.972
EF190502	4	0.012	0.043	0.012	---	0.989	0.942	0.985	0.989	0.983	0.996	0.960
AB073198	5	0.004	0.037	0.005	0.011	---	0.946	0.992	0.995	0.990	0.990	0.967
DQ232773	6	0.056	0.058	0.057	0.058	0.054	---	0.940	0.948	0.937	0.943	0.919
AY359885	7	0.004	0.040	0.003	0.015	0.008	0.060	---	0.992	0.995	0.988	0.968
EF190495	8	0.006	0.040	0.005	0.011	0.005	0.052	0.009	---	0.989	0.992	0.966
AB045092	9	0.006	0.043	0.005	0.017	0.010	0.063	0.005	0.011	---	0.985	0.966
AB073199	10	0.010	0.042	0.009	0.004	0.010	0.057	0.012	0.009	0.015	---	0.962
TC1	11	0.029	0.062	0.028	0.041	0.033	0.081	0.032	0.034	0.034	0.038	---

Table 3 indicates that the nucleotide similarity (above diagonal) and distance (below diagonal) identities between the studied sample 'Sample TC1' and ten other closest homologs microbe.The secondary metabolites were produced and its antimicrobial activity was evaluated by a zone of inhibition method against *Aeromonassp, B- haemolytic streptococcus, Bacillus polymyzia, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella typhimurium, Salmonella paratyphi, Streptococcus faecalis*and *Vibrio cholerae* (Table. 4). Table 4 showed that there was no activity found at 4 µl and 8 µl whereas in 16 µl concentration of secondary metabolites there was an activity against *Aeromonas* sp. However, at a concentration of 32 µl, the promising activity was found for all the tested pathogenic microorganisms. In spite, the maximum activity was found for *Aeromonas* sp. The isolate was tested for antibacterial activity against both gram-positive and gram-negative bacteria. The bacterial isolate from marine soil showed better bactericidal activity against pathogenic bacteria. This result indicates that the isolates were produced bactericidal/inhibitory activity for the other bacterial organism. Further studies are in progress to isolate and characterize the antibacterial principle produced by our bacterial isolates from the marine environment. Thus, further studies need to be carried out in respect of the DNA relatedness studies, DNA-DNA hybridization, small subunit (SSU) sequences, cell wall composition, and other characteristics to confirm the taxonomic position of the strain *Paenibacillus dendritiformis* (AJ320490) and *Paenibacillus thiaminolyticus* (EU330645).

Table 4. Antimicrobial activity of the isolated secondary metabolites (Zone of inhibition)

Organism	Zone of inhibition (mm)			
	4 µl	8 µl	16 µl	32 µl
Acinetobacter sp	-	-	-	-
Aeromonas sp	-	-	9	11
β-streptococcus	-	-	-	9
Bacillus polymyzia	-	-	-	7
Citrobacter sp	-	-	-	-
E. coli	-	-	-	-
Proteus vulgaris	-	-	-	8
P.aeruginosa	-	-	-	8
S. typhimurium	-	-	-	8
S. paratyphi	-	-	-	8
S. faecalis	-	-	-	9
Shigella flexneri	-	-	-	-
S. aureus	-	-	-	-
Vibrio cholerae	-	-	-	7

4. CONCLUSIONS

Hereby we conclude thatthe microorganism isolated was probably a new bacterium that was isolated from a marine soil source and it is reported for the first time. Through a series of biochemical assays, the marine bacteria's characteristics were determined. From the genetic analysis, 16s rDNA sequencing and by comparing nucleotide homology and phylogenetic analysis the organisms were confirmed as *Paenibacillus dendritiformis* and *Paenibacillus thiaminolyticus*. From the secondary metabolites produced from these organisms, the antimicrobial activity against the pathogens like *Aeromonas* sp, *B-haemolytic streptococcus, Bacillus polymyzia, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella typhimurium, Salmonella paratyphi, Streptococcus faecalis* and *Vibrio cholerae* were found to be significant at 32 µl/ml concentration. From the optimization results, the marine organism (*Paenibacillus dendritiformis* and *Paenibacillus thiaminolyticus*) was found to have maximum growth at 40-50° C temperature at pH 7 at 2% NaCl concentration. Thus, the marine bacterial isolate was found to produce secondary metabolites comprise of antibacterial principles and enzymes with potential to be developed as novel antibiotic and of commercial importance. This preliminary study may lead to the discovery of antibiotics and other bioactive compounds henceforth with more detailed studies in near future. Hence, further studies are needed to elucidate the mechanism of

action for its potential antibacterial activity and to identify the presence of any other bioactive compounds present in the secondary metabolites produced by the identified and prospective bacterial isolates from the marine soil samples.

5. AUTHORS CONTRIBUTION STATEMENT

Subbiah Latha and Palanisamy Selvamani contributed to the design and implementation of the research and to the analysis of the results. Thangavelu Prabha contributed in drafting the manuscript in consultation with Subbiah Latha and Palanisamy Selvamani.

6. CONFLICT OF INTEREST

Conflict of interest declared none.

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