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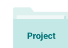

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MASS SPECTROMETRIC IDENTIFICATION OF SECONDARY METABOLITES FROM MARINE SEAGRASS - *CYMODOCAEA SERRULATA* (R. BROWN) ASCHERSON & MAGNUS AND ITS ASSOCIATED BACTERIA *BACILLUS CEREUS*

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Keywords:

Associated microbes, Enzyme activity, Mass spectrometry, Phenolics and Seagrass

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ABSTRACT: A marine sea-grass *Cymodocaea serrulata* was collected, dried and extracted with a different solvent. The presences of phytochemical constituents were confirmed and the ethyl acetate extraction was considered to be a suitable solvent. In addition, the crude seagrass was serially diluted and spread onto the Zobell Marine Agar plates. Among the eight colonies obtained, the red-pigmented bacterium (CS8) which was identified as *Bacillus cereus* (MG890216.1) through 16S rRNA was selected for further studies. Subsequently, the secondary metabolites from seagrass and its bacterial pellet of ethyl acetate extract were detected through Mass Spectroscopic analysis. On comparison of MS spectrum of the seagrass phenolic derivatives such as p-Coumaric acid (163Da), syringic acid (197Da), 3, 4, 5-trimethoxy benzoic acid (213Da), Oleuropine derivative (334Da) and 1-acetoxy pinoresinol (415Da) were found to be major compound. GC-MS revealed the major unknown compound as Androst-5-En-3.Beta.-Ol, 4; 4-Dimethyl-and Acetate which is a steroidal unsaturated carboxylate had not been reported from any sources so far and along with seven other known compounds in the bacterial pellet. In addition to this, hydrolysis of casein and starch shows that the bacterial strain has the ability to produce protease and amylase enzyme. In this conclusion, as a new trial, we have targeted secondary metabolites from both seagrasses as host and from its microbiome.

INTRODUCTION: In recent years, sizeable importance is attached to the discovery of new biodynamic agents from marine sources so as to unearth new sources of drugs from the sea [1].

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Excellent natural products are available in the marine environment which can serve as medicines, ornaments, nutrients, *etc.*

Sea grasses play a vital role in defining the habitat complexity, species diversity and abundance of associated invertebrates; thereby shaping the structure of marine communities. There are about 340 animals which directly feed upon sea grasses [2]. Although sea grasses comprise only a few species, their importance to coastal marine environments and pharmaceuticals is remarkable.

Generally, sea grasses are the abundant source of secondary metabolites which aid in their defense mechanisms. These metabolites -act as anti-hyperlipidemic and hypoglycemic agents, reducing blood pressure and regulating blood cholesterol levels [3]. The possibility of collecting seagrasses directly from the ocean opened a new gate to a largely untapped resource with a wide range of unique and novel compounds. Seagrasses are well documented for the presence of potent diverse secondary metabolites [4]. Associated microorganism continues to be the subject demanding the chemical investigation. Marine microorganisms and their isolates remain as a major source of unexplored specific potential. As a result, natural products isolated from these microorganisms occupant environments other than soils act as an attractive research tool. These products not only benefit the microbiologists and biochemists but also the pharmacologists and clinicians [5]. Marine bacteria still constitute as an emerging source of novel natural products. Among them, Bacillus species are expert in producing extracellular proteases.

Many of the commercial alkaline proteases was isolated from Bacillus species [6] and constitutes one of the most important groups of hydrolytic enzymes which act upon native proteins to break up them into small peptides and amino acids [7]. Proteases are the key industrial enzymes accounting for about 50% of the total industrial enzymes [8] Many marine origin bioactive natural products from invertebrates show similarities to metabolites of their associated microorganisms including bacteria [9, 10]. Likewise, the data on the diversity of bacterial symbionts in the seagrass is also still very limited. Therefore, the current study simultaneously focuses on the secondary metabolites produced from the seagrass and its associated symbionts and chemical characterization of the same.

Methododology:

Extraction of Seagrass and Isolation of Bacterial Symbionts from Sea grass: Fresh seagrass *Cymodecea serrulata* was collected in low tide region from Gulf of Mannar Coast, Mandapam (09º17.417′N; 079º08.558′E), Rameshwaram, India, during the period of March 2018. The collected specimens were brought to the laboratory and washed thoroughly with autoclaved seawater to remove the epiphytes and other extraneous materials. Finally, the sample material was air-dried under the shade for 2 weeks. The dried leaves were milled to a fine powder which was then packed in air-tight bags and stored in a refrigerator for further use. *C. serrulata* powder (20 g/100 mL was extracted with ethyl acetate by subjecting it to sonication for 10 min later evaporated and lyophilized.

Simultaneously, a small amount of the same seagrass powder (100 mg/1mL) was spread onto the surface of the Zobell marine agar plate. The Petri dish was then incubated at 30 °C for two days. After incubation, the plate was observed for the growth of several bacterial colonies along with pigmented colonies. They were isolated and streaked onto the media plates and maintained as a subculture. After incubation, the pigmented red colony was further mass cultured on Zobell Marine broth, which was later centrifuged for obtaining the bacterial pellet. A total of 2 grams of the pellet was taken and then extracted with ethyl acetate with the aid of sonicator and rotary shaker11and the same was sent for microbial identification by 16S rRNA sequencingat RGCB, sirkazhi.

Genomic DNA Isolation, PCR Amplification, and Sequencing: Genomic DNA was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel) following the manufacturer's instructions. The quality of the DNA isolated was checked using 1% agarose gel electrophoresis. The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). The sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) following the manufacturer's protocol. Purified PCR products 16srRNA gene was used for Cycle sequencing using the Big Dye® Terminator 3.1 sequence kit (Applied Biosystems, Foster City, California, USA). After cycle sequencing, the products were purified using Ethanol-EDTA purification protocol to remove the un-incorporated dNTP's, ddNTPs and primer dimer. The purified products were dissolved in 12 µl Hi-Di formamide and the samples were subjected for denaturation at 95 °C for 5 min. Denatured products were used for sequencing in forward and reverse directions using

Genetic Analyzer 3500 (Life Technologies Corporation, Applied Biosystems®, California 94404, USA) as per manufacture's instruction. Sequences were aligned and edited using Mega software version 6 [12].

GC-MS Analysis: The Clarus 680 GC was used in the analysis employed a fused silica column, packed with Elite-5MS (5% biphenyl 95% dimethylpolysiloxane, 30 m × 0.25 mm ID × 250 μm df) and the components were separated using Helium as carrier gas at a constant flow of 1 mL/min.

The injector temperature was set at 260 °C during the chromatographic run. 1 μL of bacterial pellet extract (CS-8) was injected into the instrument and the oven temperature was adjusted at frequent time intervals: 60 °C (2 min); followed by 300 °C at the rate of 10 °C min^{-1} and 300 °C, where it was held for 6 min.

The mass detector conditions were transferred line temperature set at 240 °C; ion source temperature at 240 °C; and ionization mode electron impact at 70 eV, scan time 0.2 sec and scan interval of 0.1 sec. The fragment ranges from 40 to 600 Da. The spectrums of the components were compared with the database of the spectrum of known components stored in the GC-MS NIST (2008) library.

UHPLC-ESI/MS Analysis of Seagrass Extract: UHPLC–ESI/MS analyses were performed at 254 nm and 280 nm and 30 °C using an RP C18 column (150 × 4.6) × 1.8 μm possessing a Shimadzu apparatus equipped by a binary pump coupled to a UV detector and a Triple Quad (ESI) mass spectrometer (Shimadzu Japan). The injected sample volume was 10 μL along with that a blank was run simultaneously for eliminating contaminants. The mobile phase (0.4 mL/min) consisted of solvent A (water +0.1% Formic acid) and solvent B (Methanol). The six-step gradient was applied, for a total run time of 20 min, as follows: Starting from 95% solvent A and 5% solvent B increasing to 100% solvent B over 12 min, then hold for 2 min, decreased to 70% solvent B over 2 min, to 30% over 2 min and to 5% solvent B over 1 min, and finally stopped at 20 min. ESI ionization conditions were spray voltage 4 kV, capillary 350 °C, 15 V. Pure nitrogen was the sheath gas and pure (99.9%) argon was the collision gas. The full scan mass data m/z was obtained in both positive and negative mode ranged from 100 to 2000 Da. Spectrum obtained was matched with metlin database library.

Enzyme Activity:

Screening for Amylase Activity (Starch Iodine Test): Isolated colonies were picked up from each plate containing pure culture and streaked in straight lines on the starch agar plates with starch as the only carbon source [13].

After incubation at 37 ºC for 24-48 h, the individual plates were flooded with Gram's iodine solution (Gram's iodine- 250 mg iodine crystals added to 2.5 gm potassium iodide solution, and 125 ml of water, stored at room temperature) to produce a deep blue colored starch-iodine complex.

Screening for Protease Activity: In order to screen the protease activity, the isolated bacteria were spread onto skimmed milk agar plates containing beef extract (3 g/L), peptone (10 g/L), NaCl (10 g/L), skimmed milk powder (10 g/L), and 18 g/L agar, with pH adjusted to 8.5. Plates were incubated for 24 h at 37 ºC. Later was screened for the production of protease by observation of the clean zone around the bacterial colony [14].

TABLE 1: SHOWS THE PHYTOCHEMICAL ANALYSIS OF SEAGRASS FROM DIFFERENT SOLVENT EXTRACTION

Phyto	M	EA	A	H	DCM	CHCL	PE	BEN	Aq
Protein	+	-	+	-	+	+	+	+	+
Glycosides	-	-	-	+	-	+	+	+	-
Reducing Sugars	-	-	-	-	+	-	-	-	+
Flavanoids	-	-	+	-	+	+	+	+	+
Terpenoids	+	+	+	-	+	+	+	+	+
Phenol	-	+	+	-	-	-	-	+	-
Carbohydrates	+	-	-	-	+	-	-	-	+
Steroids	-	-	-	-	-	-	-	-	-

Where M-Methanol; EA-Ethyl acetate; A-acetone; H-Hexane; DCM-Dichloromethane; CHCL-Chloroform;PE-Petroleum ether; BEN-Benzene; Aq- Aqueous

RESULTS:

Phytochemical Analysis: The phytochemical analysis revealed the presence of several biomolecules such as protein, glycosides, reducing sugars, flavonoids, terpenoids, phenols, carbohydrates. Steroidal compounds were absent shown in **Table 1**.

For detecting the compounds more specifically we have used ethyl acetate throughout the process as it showed positive results towards phenols and terpenoids only.

Isolation of Characterization of Associated Bacterial Symbiont: Bacteria(CS-8) and its genomic DNA was isolated from the strain and its quality was checked by loading in 1% agarose gel with the DNA marker which showed the intact DNA shown in **Fig. 1**, **Fig. 2A** and **2B**.

16S rDNA of the strain SU12 was amplified through PCR which shows the molecular weight of 1.472 kb corresponding to that of the DNA ladder in 1% agarose gel.

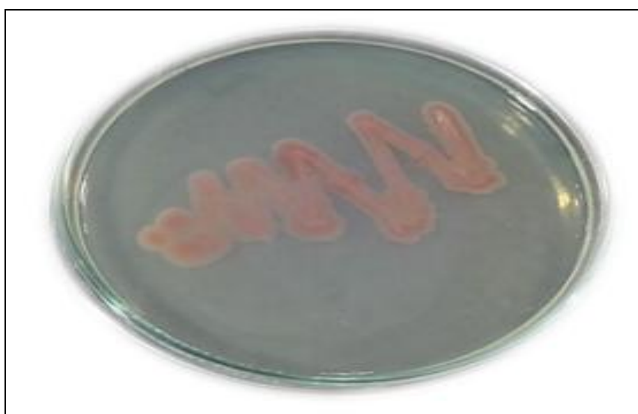


FIG. 1: THE RED PIGMENTED BACTERIA (*B. CEREUS*) ISOLATED FROM SEA GRASS (*C. SERRULATA*)

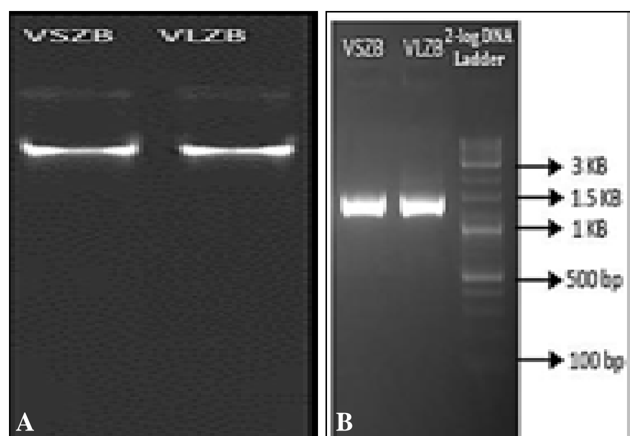


FIG. 2A & 2B ISOLATED AND PCR AMPLIFIED DNA

The amplified product was further sequenced to identify the genome. The obtained 16S rRNA gene sequence (1472 bp) of the strain CS-8 was preliminary compared with previously available sequences of Bacillus sp. deposited in GenBank (NCBI) and it indicated that this organism is phylogenetically related to the members of the genus Bacillus.

The phylogenetic tree of 16S rRNA sequences was constructed by using the three valid representative species of the genus Bacillus to know the relationship of the strain CS8 and the BLAST result showed that they appeared close match in the neighbor-joining tree. The sequence of *B. cereus* (MG890216.1) served as the reference of the operational taxonomic unit. The species *B. cereus* (MG890216.1) and Bacillus sp. (MG814015.1) has the closest sequence similarity of 99%. The sequence base pairs were given below

CS8-16SF: TGAGTTAGCGGCGGACGGGTGA
GTAACACGTGGGTAACCTGCCCATAAGACT
GGGATAACTCCGGGAAACCGGGGCTAATCC
GGATAACATTTTGAACCGCATGGTTCGAAT
TGAAAGGCGGCTTCGGCTGTCACTTATGGA
TGGACCCGCGTCGCATTAGCTAGTTGGTGA
GGTAACGGCTCACCAAGGCAACGATGCGTA
GCCGACCTGAGAGGGTGATCGGCCACACTG
GGACTGAGACACGGCCCAGACTCCTACGGG
AGGCAGCAGTAGGGAATCTTCCGCAATGGA
CGAAAGTCTGACGGAGCAACGCCGCGTGA
GTGATGAAGGCTTTCGGGTCGTAAAACTCT
GTTGTTAGGGAAGAACAAGTGCTAGTTGAA
TAAGCTGGCACCTTGACGGTACCTAACCAG
AAAGCCACGGCTAACTACGTGCCAGCAGCC
GCGGTAATACGTAGGTGGCAAGCGTTATCC
GGAATTATTGGGCGTAAAGCGCGCGCAGGT
GGTTTCTTAAGTCTGATGTGAAAGCCCACG
GCTCAACCGTGGAGGGTCATTGGAAACTGG
GAGACTTGAGTGCAGAAGAGGAAAGTGGA
ATTCCATGTGTAGCGGTGAAATGCGTAGAG
ATATGGAGGAACACCAGTGGCGAAGGCGA
CTTTCTGGTCTGTAACTGACACTGAGGCGC
GAAAGCGTGGGGAGCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGATGAG
TGCTAAGTGTTAGAGGGTTTCCGCCCTTTGT
GCTGAAGTTAACGCATTAAGCACTCCGCCT
GGGGAGTACGGCCGCAAGGCTGAAACTCA
AAGGAATTGACGGGGGCCCGCACAAGCGG
TGGAGCATGTGGTTTAATTCGAAGCAACGC

GAAGAACCTTACCAGGTCTTGACATCCTCT
GAAAACCCTAGAGATAGGGCTTCTCCTTCG
GGAGCAGAGTGACAGGTGGTGCATGGTTGT
CGTCAGCTCGTGTCGTGAGATGTTGGGTTA
AGTCCCGCAACGAGCGCAACCCTTGATCTT
AGTTGCCATCATTAAGTTGGGCACTCTAAG
GTGACTGCCGGTGACAAACCGGAGGAAGG
TGGGGATGACGTCAAATCATCATGCCCCTT
ATGACCTGGGCTACACACGTGCTACAATGG
ACGGTACAAAGAGCTGCAAGACCGCGAGG
TGGAGCTAATCTCATAAAACCGTTCTCAGT
TCGGATTGTAGGCTGCAACTCGCTACATGA
AGCTGGAATCGCTAGTAA

CS8-16SR: GTTAGCGGCGGACGGGTGAGTA
ACACGTGGGTAACCTGCCTGTAAGACTGGG
ATAACTCCGGGAAACCGGGGCTAATACCGG
ATGGTTGTTTGAACCGCATGGTTCAAACAT
AAAAGGTGGCTTCGGCTACCACTTACAGAT
GGACCCGCGGCGCATTAGCTAGTTGGTGAG
GTAACGGCTCACCAAGGCAACGATGCGTAG
CCGACCTGAGAGGGTGATCGGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGA
GGCAGCAGTAGGGAATCTTCCGCAATGGAC
GAAAGTCTGACGGAGCAACGCCGCGTGAG
TGATGAAGGTTTTCGGATCGTAAAGCTCTG
TTGTTAGGGAAGAACAAGTACCGTTCGAAT
AGGGCGGTACCTTGACGGTACCTAACCAGA
AAGCCACGGCTAACTACGTGCCAGCAGCCG
CGGTAATACGTAGGTGGCAAGCGTTGTCCG
GAATTATTGGGCGTAAAGGGCTCGCAGGCG
GTTTCTTAAGTCTGATGTGAAAGCCCCCGG
CTCAACCGGGGAGGGTCATTGGAAACTGGG
GAACTTGAGTGCAGAAGAGGAGAGTGGAA
TTCCACGTGTAGCGGTGAAATGCGTAGAGA
TGTGGAGGAACACCAGTGGCGAAGGCGAC
TCTCTGGTCTGTAACTGACGCTGAGGAGCG
AAAGCGTGGGGAGCGAACAGGATTAGATA
CCCTGGTAGTCCACGCCGTAAACGATGAGT
GCTAAGTGTTAGGGGGTTTCCGCCCCTTAG
TGCTGCAGCTAACGCATTAAGCACTCCGCC
TGGGGAGTACGGTCGCAAGACTGAAACTCA
AAGGAATTGACGGGGGCCCGCACAAGCGG
TGGAGCATGTGGTTTAATTCGAAGCAACGC
GAAGAACCTTACCAGGTCTTGACATCCTCT
GACAATCCTAGAGATAGGACGTCCCCTTCG
GGGGCAGAGTGACAGGTGGTGCATGGTTGT
CGTCAGCTCGTGTCGTGAGATGTTGGGTTA
AGTCCCGCAACGAGCGCAACCCTTGATCTT
AGTTGCCAGCATTCAGTTGGGCACTCTAAG
GTGACTGCCGGTGACAAACCGGAGGAAGG
TGGGGATGACGTCAAATCATCATGCCCCTT
ATGACCTGGGCTACACACGTGCTACAATGG
ACAGAACAAAGGGCAGCGAAACCGCGAGG
TTAAGCCAATCCCACAAATCTGTTCTCAGTT
CGGATCGCAGTCTGCAACTCGACTGCGTGA
AGCTGGAATCGCTAGTAATCGCG

GC-MS Analysis: The GC-MS analysis was only carried out for the detection of secondary metabolites in ethyl acetate extracted *B. cereus* pellet. The ethyl acetate extract of *B. cereus* showed 8 peaks. Among these, a major peak holding 50 % of the area which was found at Rt-26.253. This peak represented Androst-5-En-3.Beta.-Ol, 4, 4-Dimethyl-, Acetate, a steroidal unsaturated carboxylate compound along with 7 other minor peaks of known reported compounds. The detected compounds were shown in chromatogram and spectrum **Fig. 3** & **4** and listed in **Table 2**.

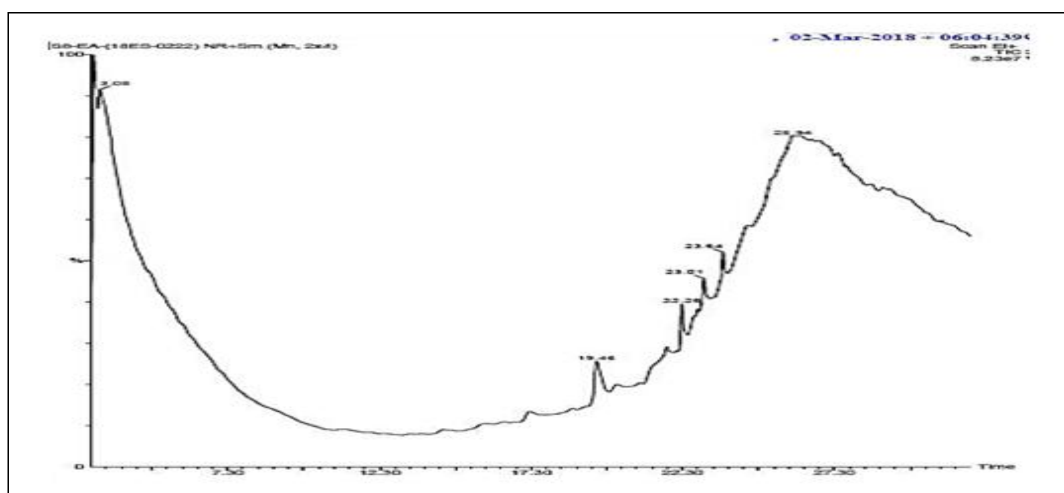


FIG. 3: THE CHROMATOGRAM PROFILE FOR ETHYL ACETATE EXTRACTED *B. CEREUS* (CS-8)

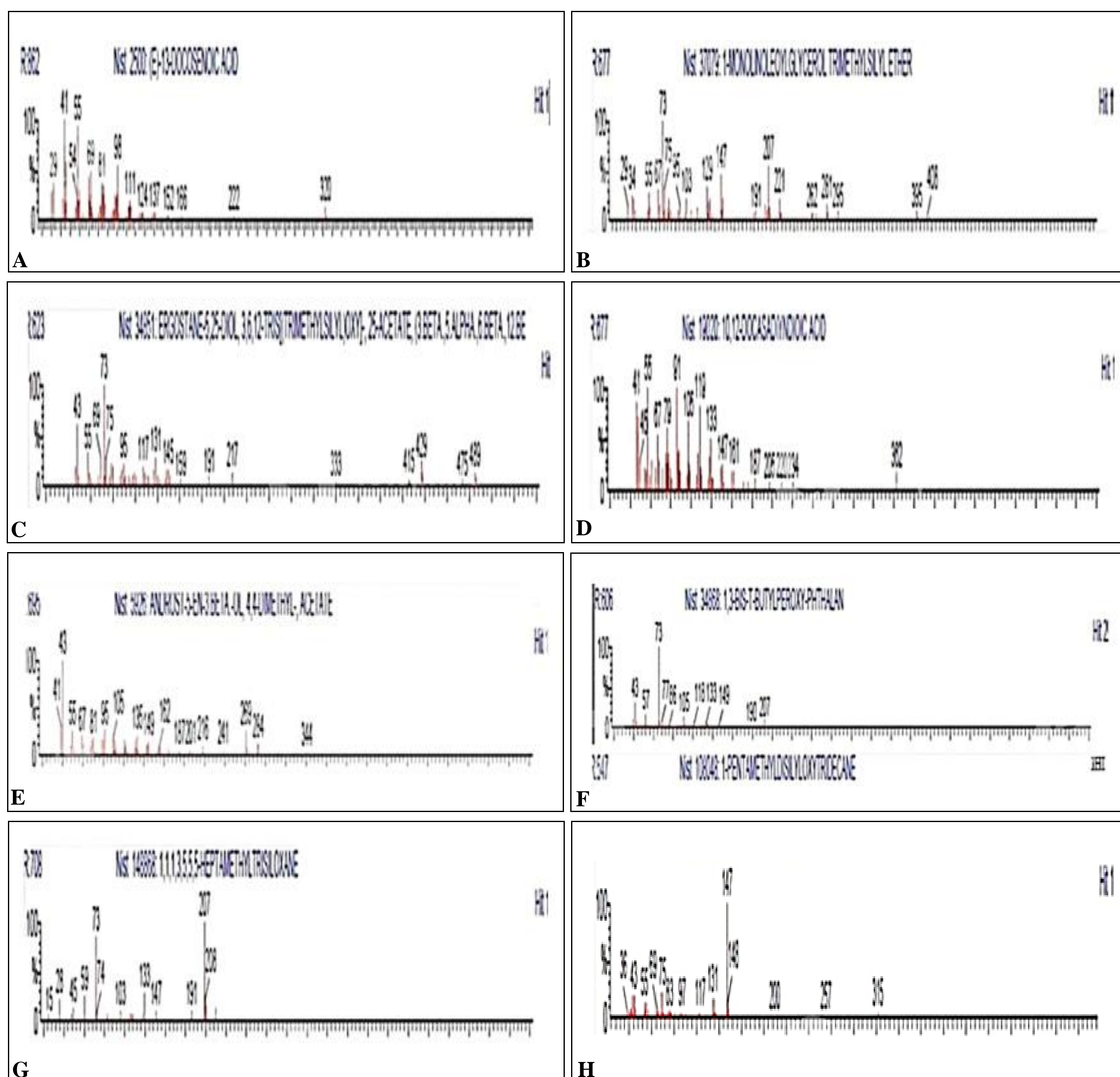


FIG. 4: THE GC-MS SPECTRUM OF ETHYL ACETATE EXTRACTED B.CEREUS (CS-8)

TABLE 2: SHOWS THE GC-MS DETECTION OF SECONDARY METABOLITES FROM ETHYL ACETATE EXTRACTED B.CEREUS (CS-8)

S. no.	RT	Name of the Compound	Mol. Wt	Formula	Nature of Compound
1	19.45	(E)-13-Docosanoic Acid	338	$C_{22}H_{42}O_2$	Unsaturated fatty acids
2	25.19	L-Alanine, N-Propoxycarbonyl-, Undec-10-Enyl Ester	327	$C_{18}H_{33}O_4N$	Unsaturated alanine dicarboxylate
3	25.32	9,12,15-Octadecatrienoic Acid, 2-[(Trimethylsilyl)Oxy]-1-[[(Trimethylsilyl)Oxy]Methyl]Ethyl Ester	496	$C_{27}H_{52}O_4Si_2$	Unsaturated fatty acid ester
4	25.45	1-Ethyl-3-[2-(Octadecylthio)Ethyl]Thiourea	416	$C_{23}H_{48}N_2S_2$	Saturated lon chain thioether thiorea
5	26.253	Androst-5-En-3.Beta.-Ol, 4,4-Dimethyl-, Acetate	334	$C_{23}H_{36}O_2$	Steroidal methyl carboxylate
6	26.64	1,3-Bis-T-Butylperoxy-Phthalan	498	$C_{27}H_{54}O_4Si_2$	Oxy compound
7	26.75	1,1,1,3,5,5,5-Heptamethyltrisiloxane	222	$C_7H_22O_2Si_3$	Silyl methyl ether
8	28.119	1-Pentamethyldisilyloxytridecane	330	$C_{18}H_{42}OSi_2$	

UHPLC-ESI/MS Analysis of Seagrass: For identification of the presence of secondary metabolites from ethyl acetate extract of seagrass UHPLC-ESI/MS, an analysis was carried. Chromatograms and spectrum were recorded at 254 nm and it revealed the presence of five types of known phenolic derivatives in both the seagrass extract **Fig. 5 A** & **B** & **6 A-E**. The phenolic compounds were identified by comparing their m/z value with similar compounds that have been previously obtained under similar conditions. Among the phenolics identified in the seagrass *C. serrulata*, the major compounds were found to p-coumaric acid (165Da- [M+H] +), dihydroferulic acid (197Da – [M+H] +), urolithin B (213Da - [M+H]+), oleuropin derivative (334Da - M+H+) and 1-acetoxy pinoreserpinol (448Da – [M+CH_3OH+H]+) were found to be major phenolic derivatives considered as secondary metabolites. In this all phenolic acid derivatives were detected with a positive ionization.

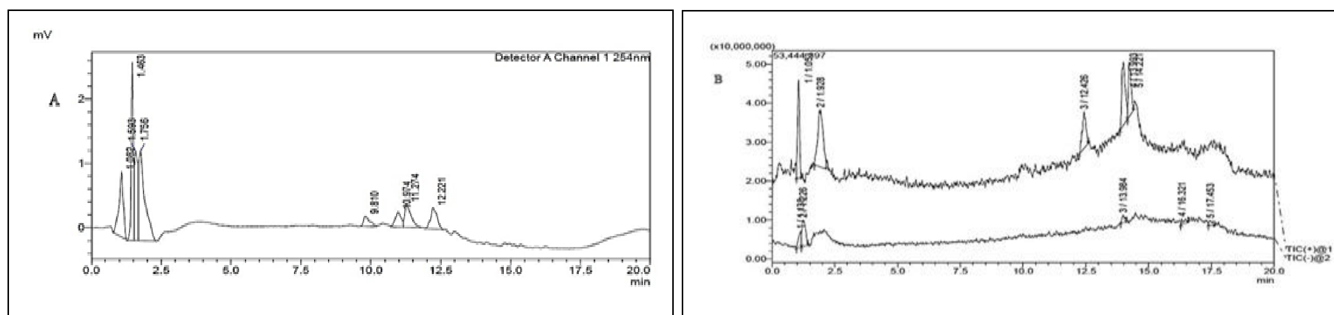
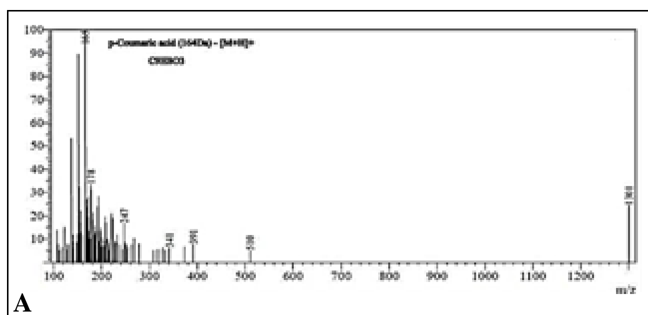


FIG. 5: SHOWING THE CHROMATOGRAM AND TIC FOR THE ETHYL ACETATE EXTRACT



B

C

D

E

FIG. 6: SHOWING THE SPECTRUM OF IDENTIFIED PHENOLIC ACID DERIVATIVES FROM SEAGRASS *C. SERRULATA*

Enzyme Activity Test:

Screening for Amylase Activity (Starch Iodine Test): CS-8 bacteria were streak inoculated on to the starch agar medium and incubated at 37 °C for 24 h. After incubation, iodine solution was added and the plate was observed for the presence of the zone of clearance around the bacteria. Our results revealed that the *B. cereus* (CS8) bacteria can produce amylase and therefore is capable of utilizing the starch as the carbon source **Fig. 7**.

Screening for Protease Activity (Casein Hydrolysis Test): CS-8 (*B. cereus*) organism was streaked onto skim milk agar plate and incubated for 24 h. After incubation, it was observed that CS8 colonies could produce a zone of clearance, which confirmed the hydrolysis of casein **Fig. 8**.

FIG. 7: SHOWS AMYLASE ACTIVITY OF *B. CEREUS*

FIG. 8: EXHIBITION OF PROTEASE ACTIVITY BY *B. CEREUS*

DISCUSSION: The present study focuses on isolation of the sea grass and its associated microorganisms for the production of secondary metabolites and industrially important enzymes. Microorganism plays a considerable role in seagrass biology as they are associated with either extracellular, intracellular or both. We thus focused on the phenolic derivatives from seagrass and its associated microbe. In this current seagrass study, the wide range of phytochemicals was identified in the sample especially phenolic substances. From the phytochemical study, we have studied that all the used solvents could extract as much as phytochemicals but ethyl acetate showed favorable towards the extraction of phenolics and terpenoids only. Since, we targeted phenolic derivatives from both seagrass and seagrass associated micro-organisms, therefore we chose ethyl acetate as the better solvent system for extracting phenolic compounds.

The previous study from [15] has shown the presence of phenols, flavonoids, terpenoids, and tannins in ethylacetate extract of *Halophila ovalis*. In this study, we have extracted phenolic compounds and its derivatives and confirmed it through HPLC analysis. Presence of caffeic acid in different seagrasses and ρ-Coumaric acid in *H. pinifolia* and *C. rotundata* has been illustrated in earlier reports. Other than these two predominant compounds, [16] reported the presence of compounds such as gallic acid, gentisic acid, p-Coumaric acid, along with other phenolics derivatives moderate to trace levels. From another study quantitative analysis of phytochemicals revealed that phenols are rich in seagrass and found to be rich in *H. ovalis* followed by saponins, flavonoids and other phytochemicals were found to be less [17]. Phenolic content was higher in *C. serrulata* seagrasses of Gulf of Mannar, in contrast to which *S. isoetifolium* showed higher phenol compounds that, were obtained from both the Gulf of Mannar and Palk Bay in the previous study. The fluctuations in phenolic acids concentrations amongst the seagrasses could be due to the abiotic andbiotic factors including depth, environmental parameters, interactions of herbivores, competition between species, *etc.* [18] Another aspect of our research showed the presence of fatty acids derivatives besides phenolics derivatives, which was detected from GC-MS chromatogram. In a study of [19] has led to the isolation of fatty acid esters, chlorophyll in about 1:1 ratio from the dichloromethane extract of *C. rotundata*. Fatty acid esters obtained from the extract of *C. rotundata* exhibited antibacterial activity and partly showed antimicrobial activity *B.*

cereus was isolated and further characterized by GC-MS. In the present study, GC-MS analysis was carried out to detect the presence of secondary metabolites from ethyl acetate extract of B. cereus strain. The results showed that several compounds have been detected in *B. cereus* bacteria isolated from *C. serrulata*. Our results were compared with the compounds obtained from several sources such as terrestrial plants, aquatic plants and micro-organisms. From our results, we found the compound Androst-5-En-3.Beta.-Ol, 4, 4-Di-methyl-, Acetate, a steroidal methyl carboxylate compound which has never been reported so far. This study from [20] reported the presence of 9, 12,1 5-Octadecatrienoic Acid, 2-[(Trimethylsilyl) Oxy]-1- [[(Trimethylsilyl) Oxy] Methyl]Ethyl Ester, an unsaturated fatty acid ester obtained from ethanolic extract of Nelumba Nucifera and methanol extract of Enicostemma auxiliary [21]. Likewise, compound 1, 3-Bis-T-Butylperoxy-Phthalan an oxy compound was previously reported from *Andrographis paniculata* [22]. A study carried out by [23] found the compound Z, Z-2, 5-Pentadecadien-1-Ol from *Adhatoda vasica*. All these fatty molecules were obtained using solvent extraction method hence, the type of solvent used plays a significant role in the detection of metabolites. Protease enzyme has wide application in detergent, pharmaceutical, photography, leather, food and agricultural industries. In our study, marine bacteria were isolated and then screened for protease and amylase production *via* casein hydrolysis and starch hydrolysis.

Until now no previous study has been reported on *Bacillus cereus* bacteria isolated from seagrass that produces proteases enzyme. Apparently, another protease producing bacterium *B. cereus* sp. was isolated from oyster *Saccostrea cucullata* and this was screened and optimized for the production of protease enzyme [14]. Also, an enterotoxigenic *B. cereus* was isolated from a variety of tropical marine fishes [24]. Another strain of *B. cereus* was isolated from marine waters of Vishakhapatnam which showed the ability to degrade oil as well as grease. Hence, *B. cereus* isolated could be used as biodegrading agent [25]. In other study *Bacillus cereus* species isolated from Asian Sea Bass (*Lates calcarifer*) has displayed the ability to produce exopolysaccharides [26]. Likewise, 11 reported that *Bacillus licheniform* is from *T. hemprichiiis* capable of producing carotenoid pigment such as diodinoxanthin. An epiphytic cyano-bacteria was isolated from the leaves of *C. rotundata* which show some nitrogenase activity has been studied [27]. An anthraquinone rich compound from Streptomyces sp RAUACT-1 has been isolated from seagrass *S. isoetifoliuem* and has been investigated to have antagonistic property [28].

Several Bacillus sp. isolated from Odisha coast was studied to produce extracellular enzymes [29]. Green pigment-producing bacteria strain *B. cereus* isolated and characterized for 9-methyl-1 4 5 8-tetra-azaphenanthrene responsible for the pigmentation and antibacterial activity [30]. Apart from this, phosphate solubilizing bacteria have been isolated from the rhizosphere of two seagrasses (*H. ovalis* and *H. pinifolia*) namely *B. circulans* and other two Bacillus sp [31]. This study from [32] reported isolates *B. cereus*, *B. sphaericus* along with *B. pumilus* showed high protease and lipase activity. Likewise, *Bacillus cereus* isolated from Sponge *H. cribriform* is tested for its bioactive potential and antibacterial activity [33].

The spectra generated from the ESI/MS were the identification of secondary metabolites such as phenolic derivatives from ethyl acetate extract of seagrass. The spectrum reveals about the major phenolic compounds found in the seagrass extract such as p-coumaric acid (165Da - [M+H] +) which has m/z value of 164Da with the adduct formation of hydrogen molecules which can possess the anti-inflammatory that was previously reported by [34], also in food P-coumaric acid can be found in a wide variety of edible plants like peanuts, navy beans, tomatoes, carrots and garlic.

It is found in wine and vinegar [35]. Dihydroferulic acid, also known as 3-(4-hydroxy-3- methoxy-phenyl) propionic acid or dihydroconiferylate, is classified as a member of the phenylpropanoid acids. Phenylpropanoid acids are compounds with a structure containing a benzol conjugated to propionic acid. It is considered to be slightly soluble (in water) and acidic. It has a role as a human xenobiotic metabolite, a plant metabolite, a mouse metabolite and an antioxidant. Another phenolic derivative such as Oleuropein derivative (334Da- [M+H] +) with a molecular mass of 333Da having a adduct formation hydrogen molecule

which possess anti-oxidant activity [36] and 1-acetoxy pinoresinol (448Da- [M+CH_3OH+H]) of having molecular mass of 415Da with an adduct formation of CH_3OH+H which is capable of inhibiting bacterial growth and acts as anti-oxidants, anticancer and hepato-protective activities [37]. Urolithin B (3-hydroxy-6H-dibenzo[b, d]pyran-6-one) is considered to be one of the gut microbial metabolites of ellagitannins that are found in diverse plant foods, including pomegranates, berries, walnuts, tropical fruits and medicinal herbs [38, 39].

Urolithin B is the final product catabolized among the urolithin derivatives. Its most well-known effect is that of anti-cancer activity. Urolithin B inhibits the proliferation of colon, prostate, and bladder cancer [39].

In this current study, urolithin B was reported as one of the major phenolic metabolites from the ethanolic extracts of seagrass *C. serrulata* which was reported previously by [40] possessing antioxidant and anti-inflammatory activity. Also, these compounds have been extracted and identified previously from Moroccan olive mill wastewater [41].

CONCLUSION: The present study reported here is about metabolites from sea-grass and its associated bacteria *B. cereus* from the seagrass *C. serrulata*. The obtained results revealed that both seagrass and its associated microbe could produce secondary metabolites such as phenolic derivatives, fatty acid derivatives, lignans type compounds respectively. Further target extraction and purification of them would fetch many potent compounds for treating multiple illnesses. Further optimizing enzyme-producing conditions could offer valuable enzymes for a wide range of applications.

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REFERENCES:

1. Aswathi EM, Bharathi V and amp Jamila P: Antibacterial activity and preliminary phytochemical analysis of sea grass *Cymodocea rotundata*. International Journal of Microbiological Research 2012; 3 2: 99-03.
2. Kannan L and Thangaradjou T: Seagrass ecosystem Importance and need for conservation. national symposium on marine plants, their chemistry, and utilization. Souvenir seaweed research utilization association and suganthi devadason marine research institute. Tuticorin 2005; 76-78.
3. Krishnamurthy V, Kaliaperumal N, Patterson Edward JK, Edwin Joseph V, Murugan V, Chidambaram P and amp Ramalingam JR: National symposium on marine plants, their chemistry and utilization. Souvenir Seaweed Research Utilization Association and Suganthi Devadason Marine Research Institute, Tuticorin 2005; 1-4.
4. Puglisi MP, Engel S, Jensen PR and Fenical W: Antimicrobial activities of extracts from Indo-Pacific marine plants against marine pathogens and saprophytes. Mar Biol 2007; 150: 531-540.
5. Soliev AB, Kakushi H, and amp, Keiichi E: Bioactive Pigments from Marine Bacteria Applications and Physiological Roles. Evidence-Based Complementary and Alternative Medicine 2011.
6. Priest FG: Extracellular enzyme synthesis in the genus Bacillus Bacterial. Rev 1977; 41: 711-53.
7. Nascimento W and Martins M: Production and properties of an extra cellular protease from thermophilic Bacillus sp. Braz J Microbial 2004; 35: 91-96.
8. Rao MB, Tankasale AM, Ghatge MS and Deshpande VV: Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev 1998; 62: 597-34.
9. Radjasa OK, Salasia SIO, Sabdono A, Weise J Imhoff, JF, Lammler C and Risk MJ: Antibacterial activity of marine bacterium Pseudomonas sp. associated with soft coral *Sinularia polydactyla* against Streptococcus equi subsp. zooepidemicus. Int J Pharmacol 2007; 32: 170-74.
10. Proksch P, Edrada PA and Ebel R: Drugs from the seas-current status and microbiological implications. Appl Microbiol Biotechnol 2002; 59: 125-34.
11. Nugraheni SA, Khoeri MM, Lia K, Yustin W and Radjasa OK: Characterization of carotenoid pigments from bacterial symbionts of seagrass *Thalassia hemprichii*. Journal of Coastal Development 2010; 141: 51-60.
12. Koichiro T, Peterson SG, Daniel, Alan F and Sumitxl K: MEGA6 Molecular Evolutionary Genetics Analysis Version 6.0. Molecular biology and evolution 2013; 30. 10.1093 molbevmst 197.
13. Shaw JF, Fu Pang L, Su Chiu C and Hsing C: Purification and properties of an extracellular a-amylase from Thermus sp. Bot Bull Acad Sin 1995; 36: 195-00.
14. Umayaparvathi S, Meenakshi S, Arumugam M and Balasubramanian T: Purification and characterization of protease from *Bacillus cereus* SU12 isolated from oyster *S. cucullata*. African J of Biotech 2009; 12(40): 5897-08.
15. Sangeetha J and Asokan S: Isolation and characterization of stigmasterol from the sea grass *Halophila ovalis*. EJBPS 2018; 55: 500-05.
16. Jeyapragash D, Subhashini P, Raja S, Abirami K and Thangaradjou T: Evaluation of *in-vitro* antioxidant activity

of seagrasses, signals for potential alternate source. Free Radicals and Antioxidants 2016; 6: 1.

17. Yuvaraj N, Kanmani P, Satishkumar R, Paari A, Pattukumar V and Arul V: Seagrass as a potential source of natural antioxidant and anti-inflammatory agents, Pharmaceutical Biology. 2012; 50(4): 458-67.
18. Athiperumalsami T, Kumar V and Jesudass L: Survey and phytochemical analysis of seagrasses in the Gulf of Mannar, South east coast of India. Botanica Marina 2008; 51: 269-77.
19. Perez J, Shen CC, Ragasa CY and Schweinf: J. Chemical constituents of *Cymodocea rotundata*. J Pharmacog 2018; 10(4): 620-1.
20. Moustafa SA, Elena IK and Natalya AL: GC-MS analysis of phytocomponents in the ethanolic extract of Nelumbo nucifera seeds from Russia. Journal of Applied Pharmaceutical Science 2015; 5(04): 115- 18.
21. MullaiNila K and Karthikeyan J: Phytochemical profiling, antibacterial potential and gc-ms analysis of methanol leaf extract of *Enicostemma axillare*. Int Journal of Current Microbiology and Applied Sciences 2017; 6(7): 2688-95.
22. Sithara NV, Komathi S and Rajalakshmi G: Identification of bioactive compounds using different solvents through FTIR studies and GCMS analysis. Journal of Medicinal Plants Studies 2017; 5(2): 192-94.
23. Rachna Verma, Ashwani T and Sunil Puri: Phytochemical profiling and GCMS study of *Adhatoda vasica* Nees. an Ethnomedicinal Plant of North Western Himalaya. Biological Forum an Int Journal 2016; 8(2): 268-73.
24. Prasad MP: Molecular characterization of Enterotoxigenic *Bacillus cereus* species isolated from tropical marine fishes using RAPD markers. Int J Pure App Biosci 2014; 2(4): 189-95.
25. Kiranmai MR and Anima SD: Isolation of marine bacteria from Visakhapatnam Coast for Degradation of Oil. J Bioremediat Biodegrad 2017; 8: 392.
26. Mohamed Orsod, Mugambwa J and Fahrul H: Characterization of Exopolysaccharides produced by *Bacillus cereus* and *Brachy bacterium* sp. Isolated from Asian Sea Bass (*Lates calcarifer*) Malaysian Journal of Microbiology 2012; 8 3, 170-174.
27. Mariam H, Beatriz D, Thomas L, Karolina I and Birgitta B: Epiphytic cyanobacteria of the seagrass *Cymodocea rotundata* diversity, diel nifH expression and nitrogenase activity. Enviro Microbiology Reports 2013; 5(3): 367-76.
28. Ravikumar S, Gnanadesigan A, Saravanan A, Monisha N, Brindha, V and Muthumari S: Antagonistic properties of seagrass associated Streptomyces sp. RAUACT-1 A source for anthraquinone rich compound. Asian Pacific Journal of Tropical Medicine 2012; 887-90.
29. Bal S, Mishra RR, Rath B, Sahu HK and Thatoi HN: Characterization and extracellular enzyme activity of predominant marine Bacillus spp. isolated from sea water of Orissa Coast, India. Malaysian Journal of Microbiology 2009; 5(2): 87-93.
30. Debopam BC, Sandipan UC, Banerjee KG and Arun Lalitagauri R: Green Pigment from *B. cereus* M1 16 (MTCC 5521). Production Parameters and Antibacterial Activity. Appl Biochem Biotechnol 2011; 164: 767-79.
31. Upasana G, Subhashini P, Dilipan E, Raja S, Thangaradjou T and Kannan L: Isolation and characterization of phosphate-solubilizing bacteria from seagrass rhizosphere Soil J Ocean Univ China (Oceanic and Coastal Sea Research). 2012; 11(1): 86-92.
32. Parvathi A, Kiran K, Jiya J, Neetha J and Santha Nair: Biochemical and molecular characterization of bacillus pumilus isolated from coastal environment in Cochin, India. Brazilian J of Microbiology 2009; 40, 269-275.
33. Brammavidhya S and Usharani G: Bioactive potential of sponge associated *Bacillus cereus* isolated From *Hyattela cribriformis*. International Journal of Research in Environmental Science and Technology 2013; 3(2): 61-64.
34. Hao Z, Qing HL, gui X, Xiong, Wang Y, hui Z, Mei Z, Lu JSX and Dan Wu: Anti-inflammatory effects of p-coumaric acid, a natural compound of *Oldenlandia diffusa*, on Arthritis Model Rats. Evidence-Based Complementary and Alternative Medicine 2018; 9.
35. Alvez MCG, Barroso CG and Perez Bustamante JA: Analysis of polyphenolic compounds of different vinegar samples. Zeitschrift fur Lebensmittel-Untersuchung and Forschung 1994; 199(1): 29-31.
36. Raffaella B, Francesco LC, Maria PT, Ferdinando F and Roberto N: Antioxidant activity of the main bioactive derivatives from oleuropein hydrolysis by Hyper-thermophilic alpha-Glycosidase. J Agric Food Chem 2001; 49: 3198-03.
37. Chaitali YM and Pinoresinol: A potential Biological warrior in edible foods. IOSR Journal of Environmental Science, Toxic and Food Technology 2015; 1(1): 44-47.
38. Okuda T, Yoshida T and Hatano T: Ellagitannins as active constituents of medicinal plants. Planta Med 1989; 55: 117-22.
39. Barberan TFA, Sarrias GA, Villalba GR, Sanchez NMA Selma MV, Conesa GMT and Espin JC: Urolithins, the rescue of old metabolites to understand a new concept Metabotypes as a nexus among phenolic metabolism, microbiota dysbiosis, and host health status. Mol Nutr Food Res 2017; 61: 1500901.
40. Gyeongjin L, Jin Sun P, Eun Jung L, Jung HyuckAhn and Hee Sun Kim: Anti-inflammatory and antioxidant mechanisms of urolithin B in activated microglia, Phytomedicine. 2018; doi10.1016j.phymed.2018.06.032.
41. Inass L, Abdelmajid Z, Ali A, Moulay Ali O, Hassan Ait M and Mbarki M: Identification and characterisation of phenolic compounds extracted from Moroccan olive mill wastewater. Food Sci Technol Camp 2014; 34(2): 249-57.

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